The plasma membrane in the tolerance of yeast to stress.

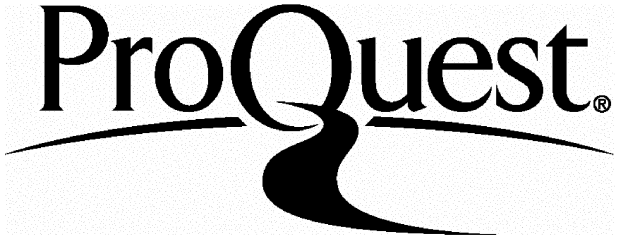
by

Barry Panaretou

Thesis submitted for the degree of doctor of philosophy.

University college (University of London)

January 1993



Abstract.

In most organisms a mild sub-lethal stress increases the capacity for survival during a subsequent lethal stress. The mechanisms that implement this acquisition of tolerance have been the subject of numerous studies. Best characterised is the induction of heat shock proteins (hsps) at elevated temperatures. Very little is known about heat shock-associated events occurring at the plasma membrane. This is surprising bearing in mind that the first component of the cell to encounter environmental stress is the cell envelope.

Work described in this thesis established action of the plasma-membrane-associated H^+-translocating ATPase (H^+-ATPase) of *S. cerevisiae* as an important determinant of tolerance to several types of stress including heat shock, exposure to ethanol and high salt concentrations. Activity of this enzyme also influences the capacity of cells to synthesize hsps. The importance of this enzyme in thermotolerance is probably due to its role in limiting the intracellular acidification that is one of the consequences of heat shock.

It is conceivable that cells target proteins with a damage limitation function to the plasma membrane as part of an inducible stress response. Two prominent changes to the protein composition of the *S. cerevisiae* plasma membrane were seen on heat shock: (I) a marked (55%) decrease in levels of the H^+-ATPase; and (II) the acquisition of a previously uncharacterised hsp of M_r 30,000 (hsp30). The ATPase decline must have an important influence over the ability of cells to maintain intracellular pH during extended periods of stress. *In vitro* assays of ATPase activity revealed that enzyme from heat shocked cells is almost twice as active as that from unstressed cells, indicating the existence of a system that activates ATPase during stress. Hsp30 is an integral membrane protein exclusively located at the plasma membrane which can be phosphorylated *in vitro*. The strong evolutionary conservation of hsp induction makes it probable that structural (and possibly functional) homologs of hsp30 will be found in organisms other than yeast. Both of these events were also observed when cells entered stationary phase; in addition, hsp30 is induced when cells are starved for nitrogen.

Covalent modification of proteins is the most recurrent form of biological control. Any notable differences between phosphoprotein profiles from unstressed and stressed cell fractions may shed light on how events associated with stress are regulated. The *in vitro* kinase/autophosphorylation activities associated with cytosolic (S100) fractions of unstressed and heat shocked yeast were examined. One polypeptide of M_r 66kDa was heavily phosphorylated in heat shock extracts only. This protein is not an hsp, but its phosphorylation is hsp dependent. The phosphoproteins detected by these experiments are particularly interesting since a large proportion of them involve the poorly-understood (rare) acid-labile, N-linked phosphoryl groups. This suggests the existence of a signal transduction system in yeast, based on N-linked phosphorylation, much like the one mediating chemotaxis in bacteria.

TABLE OF CONTENTS.

page no.

Chapter 3 Figures

Appendix I. Publications arising from work presented in this thesis.
(see pocket inside back cover)

Panaretou, B. and Piper, P.W. (1990) Plasma-membrane ATPase action affects several stress tolerances of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as well as the extent and duration of the heat shock response. *J. Gen. Microbiol.* 136, 1763-1770.

Panaretou, B. and Piper, P.W. (1992) The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and entry into stationary phase. *Eur. J. Biochem.* 206, 635-640.

Acknowledgements.

I would like to thank Peter Piper for the opportunity of working in his lab and for supervising this project over the last three years. Also, I thank Niall Kirk, Lili Cheng, Yaping Chen, Greg Rossi, Richard Watt and Emmanuela Guerra for making the lab a good humoured place in which to work.

In addition I am indebted to Darryl Papin and Dina Raman for their expert help with N-terminal sequencing. Moreover, certain problems to do with protein sequencing were solved with relative ease due to the many helpful discussions with Brian Coles, who gave me a great deal of his time.

The last few years have been made considerably easier thanks to the ceaseless support of my family, who also coped very well with the occasional grumpiness that invariably accompanies anybody that attempts research. For these reasons this thesis is dedicated to Andriani and Zacharias Panaretou, and Christina Panaretou.

Abbreviations.

ATP	adenosine 5'-triphosphate
bp	base pairs
bis-acrylamide	N,N'-methylene bisacrylamide
BSA	bovine serum albumin
Ca^{2+}_i	intracellular calcium level
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
Ci (μCi)	curies (microcuries)
cpm	counts per minute
DCCD	N,N'-dicyclohexylcarbodiimide
Des	diethylstilbestrol
DNAase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fig.	Figure
HPLC	high-performance (pressure) liquid chromatography
HSE	heat-shock element
HSF	heat-shock transcription factor
hsp	heat shock protein
IgG	immunoglobulin G
kDa	kilodaltons
KHZ	kilohertz
Mes	2-(N-morpholino)ethanesulphonic acid
M_r	apparent molecular mass
NMR	nuclear magnetic resonance
pH_i	intracellular pH
PMSF	phenylmethylsulphonyl fluoride
PVDF	polyvinylidene difluoride
RNAase	ribonuclease
s	seconds
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide-gel electrophoresis
$t_{1/2}$	half life
TCA	trichloroacetic acid.
TEMED	N,N,N',N,-tetramethylethylenediamine
TLCK	tosyl-lysine chloromethyl ketone
TPCK	tosylamido-2-phenylethyl chloromethyl ketone

Tris	tris(hydroxymethyl)aminomethane
xg	acceleration due to gravity
4CN	4-chloro-1-napthol

Single letter codes for amino acids:

A, alanine	**C**, cysteine
D, aspartic acid	**E**, glutamic acid
F, phenylalanine	**G**, glycine
H, histidine	**I**, isoleucine
K, lysine	**L**, leucine
M, methionine	**N**, asparigine
P, proline	**Q**, glutamine
R, arginine	**S**, serine
T, threonine	**V**, valine
W, tryptophan	**Y**, tyrosine

X, (in Fig. 3.9, denotes unknown amino acid)

Abbreviations used for strain names:

Escherichia coli	*E. coli*
Saccharomyces cerevisiae	*S. cerevisiae*
Schizosaccharomycs pombe	*Sch. pombe*
Tetrahymena pyriformis	*T. pyriformis*

homologs analogue — American spellings (homolog, analog) used throughout.

Sphaeroplas- — Equivalent to spheroplast.

Any other abbreviations are explained where appropriate in the text.

Chapter 1 Introduction.

1.1 The response to stress.

Major environmental changes cause stress, damage, or death. Even minor changes can hamper the physiological capacities of cells. For example, changes in temperature of the order of 1^{0}C can cause the rates of biological reactions to change by nearly 10% (Cossins and Bowler, 1987). Not surprisingly, organisms have evolved a variety of adjustments that help buffer the physiological impact of environmental change. These adjustments have been studied from diverse perspectives ranging from the gross changes in protein synthesis to the morphological and physiological alterations elicited by stress. Studying stress-inducible phenomena has also yielded a great deal of information concerning processes essential to the lifestyle of the normal unstressed cell.

Living organisms respond at the cellular level to unfavourable conditions such as heat shock by a rapid repression in the synthesis of most proteins and the concomitant but transient acceleration in the rate of expression of a small number of specific proteins (heat shock proteins or hsps). This heat shock response is universal having been observed in archaebacteria, eubacteria, yeasts, plants, invertebrates and vertebrates, including humans. Although there are differences among the various organisms, for instance in the precise molecular weights and number of the induced hsps, it is remarkable how similar the response has remained throughout evolution. Comparison of the sequences of the equivalent heat shock genes from organisms as far apart on the evolutionary scale as *E. coli* and man indicate that hsps are among the most highly conserved proteins in nature (for reviews see Lindquist, 1986; and Lindquist and Craig, 1988).

The temperature at which hsp induction occurs varies considerably with the organism, but is usually a few degrees above the optimal growth temperature. For instance, *S. cerevisiae* grows optimally at ca. 36^{0}C and induces hsp synthesis maximally over the 38-42^{0}C range. However, it has become increasingly clear that many hsp genes are activated in the apparent absence of stress, with some hsps appearing at specific stages of development, in specific tissues and even during the normal cell-growth cycle (Morimoto *et al.*, 1990). This suggests that these proteins play a role in normal growth and development. Figure 1.1 gives an overview of conditions other than stress that induce hsp gene expression.

Besides heat shock the induction of hsps can be achieved by many other types of environmental stress such as exposure to heavy metals, ethanol, amino acid analogs, and release from anoxia (see Table 1.1 for a more comprehensive list). For this reason one should refer to these proteins as stress proteins rather than hsps. Many reviews of the response to stress (e.g Lindquist and Craig, 1988) give the mistaken impression that most types of stress induce the same changes in gene expression and are regulated by a similar

Table 1.1 Inducers of the heat shock response. [1]

Inducing[2] agent or treatment	Proposed effect
Heat shock Ethanol Amino acid analogs Various heavy metals Iodoacetamide	Increasing levels of denatured protein by causing synthesis of aberrant protein or by binding to sulphydryl groups, for example.
Return from anoxia Hydrogen peroxide Superoxide ions Other free radicals	Oxygen toxicity, free radical fragmentation of proteins.
Antimycin Rotenone Oligomycin Azide Dinitrophenol Ionophores	Disturbing the processes involved ir energy metabolism by inhibiting oxidative phosphorylation, or dissipating the ionic gradients that exist across membranes.

1
Compiled from data reviewed in Ashburner and Bonner, 1979; Ananthan *et al.*, 1986; Burdon, 1986 and Lindquist, 1986.

2
By definition, heat shock is the one universal stimulus that induces synthesis of hsps. Many other stimuli induce the synthesis of all hsps or a subset of them and may even induce stress proteins not seen during heat shock.

Many of these inducers are effective across a broad range of species. Ethanol induces hsps in mammalian cells (Li, 1983), yeast (see Fig. 6.5.2), and *E. coli* (Neidhardt *et al.*, 1984). However there are many species-specific differences. For example, ethanol and cadmium do not induce hsps in *Dictyostelium* (Lindquist, 1986).

mechanism. This is a common misconception; ethanol stress in yeast for example induces some (not all) of the heat inducible proteins (see Figure 6.5.2). Furthermore, the induction of transcription of these genes occurs via a mechanism which is distinct from that controlling heat-shock induced transcription (Kirk and Piper, 1991).

Figure 1.1 An overview of the conditions that induce hsps.

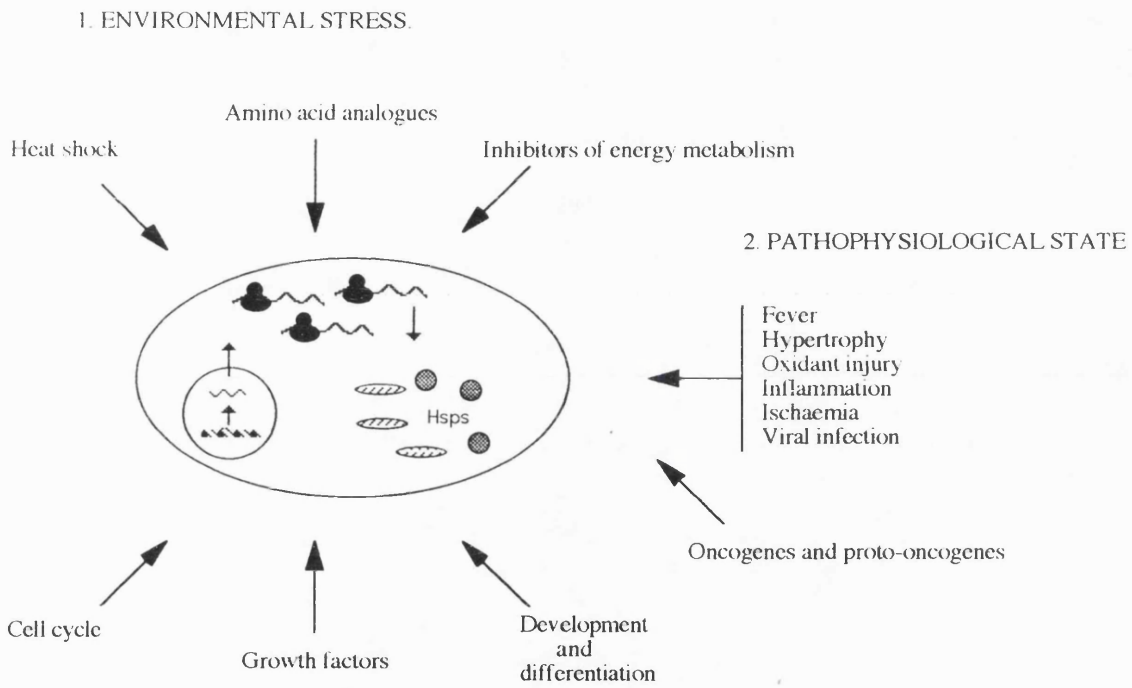


Figure from Morimoto *et al.*, 1990.

For many years the search for the functions of the hsps seemed fruitless, and interesting hypotheses were difficult to test. This situation has now changed; many hsps are involved in subtle interactions with other proteins to perform particular tasks (these developments are discussed in sections (1.21-1.2.4). In addition to this, it seemed obvious that hsps would play a role in tolerance to thermal stress (thermotolerance). Many studies showed that a pre-shock treatment, that induced hsp synthesis, rendered an organism more resistant to subsequent stress, an effect that was transient. Furthermore, positive correlations existed between the amount of hsps present and the amount of thermotolerance. At best this can only be regarded as indirect proof of hsp involvement in thermotolerance since heat shock elicits other changes in cells, as well as hsp induction. The situation became more confusing when thermotolerance could be induced in the absence of hsps e.g by glucocorticoids in mammalian cells (Hahn and Li, 1990) and by incubating yeast cells in the presence of cycloheximide during the adaptive heat shock (Watson et al, 1984; Widelitz et al, 1986). However, applying the powerful techniques of

molecular genetics (involving deletion or overexpression of hsp genes) has indicated that hsps do play a role in stress tolerance, albeit a minor one. All available data now leads to the conclusion that the molecular basis of adaptive thermotolerance involves both hsp synthesis and other poorly-understood responses to environmental stress (see section 7.2).

1.1.1 The complex nature of the heat shock response.

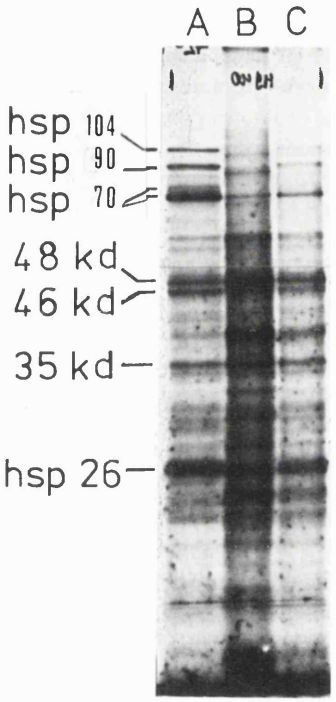
The response to heat shock can be divided into: i) the changes in gene expression, involving induction of hsp gene transcription, repression in transcription of other genes and effects on translation (see sections 1.2-1.3.2); and ii) changes in cell morphology, physiology, and metabolism (see sections 1.8-1.8.5). Most of the work pertaining to stress biology concentrates on the changes in gene expression, particularly cloning and characterisation of hsp genes. Indeed, many reviews use the term "heat shock response" to refer to these changes only, and fail to discuss any other type of stress response. It is important to point out that changes in morphology and physiology are no less important, particularly from the aspect of tolerance to stress. The rest of this introduction will summarise recent progress in both of these areas; moreover, work presented in this thesis is a preliminary attempt to deal with both aspects of the heat shock response as applied to the plasma membrane of *S. cerevisiae* .

1.2 The heat shock proteins.

The induction of heat shock proteins was first observed in *Drosophila busckii* by Ritossa, who reported that when larvae raised at 25^{0}C were exposed to 32^{0}C several new puffs appeared on the giant salivary gland chromosomes, representing regions of high transcriptional activity. At the same time puffs active prior to heat shock treatment regressed (Ritossa,1962). The heat shock genes from *Drosophila* were among the first eukaryotic genes to be cloned (Livak *et al.*, 1978; Craig, *et al.*, 1979; Voellmy *et al.*, 1981). For many years hsp research was done solely on *Drosophila*. At the end of the 1970s, however, a typical heat shock response was reported in chicken fibroblasts (Kelley and Schlesinger, 1978), in *E. coli* (Lemaux *et al.*, 1978) in yeast (McAlister and Finkelstein, 1980) in plants (Barnett *et al.*, 1980) and in many other organisms.The major hsps can be classified into five families:

1. hsps of ca. 100-110 kDa.
2. hsp90 (ranging from 83-90 kDa).
3. The hsp70 family (ranging from 66-78 kDa). Hsp70 is often the most prominent product of protein synthesis in stressed cells and is by far the most well characterised of all hsp families. In many organisms the hsp70 family

Figure 1.2 Heat shock proteins of *S. cerevisiae.*



Protein synthesis in yeast (*Saccharomyces cerevisiae*) cells following mild and severe heat-shocks. The centre track (B) of this one-dimensional 12.5% polyacrylamide gel shows the proteins made during a 20 min 25°C pulse-labelling of unstressed cells. The right-hand track (C) shows proteins synthesized 10–30 min after a 25–38°C temperature shift. 38°C is about 1°C below the maximum growth temperature of this yeast strain (MD40-4c), yet hsps are made transiently for about 1 h even though the cells will continue to divide. Production of hsps and cessation of the synthesis of most other proteins is much more dramatic following a severe 25–42°C heat-shock (track A). At 42°C the cells cease all proteins synthesis within 1 h, although they are not killed rapidly. The bands of 48, 46 and 35 kilodaltons (Kd) are not hsps, but glycolytic enzymes. Their synthesis both before and after heat-shock has so far only been noted in yeast. Hsps are generally denoted by their mass in Kd, e.g. hsp70, the hsp of 70 Kd.

Figure from Piper, 1987.

comprises many proteins - there are nine members in *S. cerevisiae*, for example (see Table 1.2).

4. hsp60. Found in bacteria, chloroplasts and mitochondria.
5. The small hsps. A diverse group ranging from 15 to 30 kDa with two forms in yeast and approximately 30 forms in higher plants.

A substantial amount of work has been carried out on the heat shock response of yeast (*S. cerevisiae* in particular). This is mainly due to the powerful techniques of yeast molecular genetics which not only allow dramatic alteration of hsp gene expression but also enable us to delete hsp genes (the latter is only achievable at low frequencies in mammalian cells). Figure 1.2 shows the hsps produced by *S. cerevisiae* during exposure to elevated temperatures. The following section reviews the roles of each hsp family. The existence of a previously undiscovered hsp (hsp30) is reported in chapter 4 of this thesis. This 'new' hsp may share some of the properties of the hsps described below.

1.2.1 The hsp70/DnaK family.

This family of proteins has been universally conserved, its members from *E. coli*, yeast and humans, for instance, being at least 50% identical to each other at the level of the amino acid sequence (Lindquist and Craig, 1988). In *E. coli* it is represented by the single copy *dnaK* gene. The *HSP70* genes, the eukaryotic homologs, belong to a multigene family whose members respond to a variety of physiological conditions as well as to temperature (Lindquist and Craig, 1988). Genetic analyses in *S. cerevisiae* show that many of these genes are essential for growth at all temperatures (Werner-Washburne *et al.*, 1989; Craig *et al.*, 1989). Also, deleting the *E. coli dnaK* gene results in poor growth within a narrow temperature range (30-37°C) (Georgopoulos, 1977). It is likely that such strains would be completely inviable but for the extragenic suppressors that these cells rapidly acquire (Bukau *et al.*, 1990). These effects on cell viability indicate a crucial role in normal cellular physiology for these proteins.

DnaK/hsp70 is often found in association with other proteins (Rothman, 1989; Liberek *et al.*, 1991). In addition to this, the known biochemical properties of DnaK/hsp70 are:

1. (Prokaryote homolog only) a nonspecific 5'-nucleotidase activity inhibited by the alarmone AppppA which accumulates on temperature upshift (Bochner *et al.*, 1986).

2. The prokaryote and eukaryote homologs can both catalyse their own autophosphorylation (Zylicz *et al.*, 1983 and Leustek *et al.*, 1989, respectively).

3. The prokaryote and eukaryote homologs bind ATP, the mammalian homolog binding with high affinity (Pelham, 1990). However rates of ATP hydrolysis are low, ranging from 0.15 (DnaK; McCarty and Walker, 1991) to 1 (BiP; a eukaryotic homolog; Pelham, 1990) hydrolysis eventsper molecule of protein per minute.

4. A clathrin-uncoating ATPase activity. Clathrin, the major structural component of coated vesicles, is found in a three legged structure called a triskelion. The cages these triskelions form can be disassembled by one of the mammalian hsp70 homologs in an ATP-dependent manner (Chappell *et al.*, 1986).

The significance of the first two activities remains unknown. However, the ATPase activity, though weak, is thought to be of importance in the protective response of this protein (see below).

DnaK, (along with two other bacterial hsps DnaJ and GrpE) was originally discovered because mutations in any of the proteins blocked phage replication *in vivo* (Friedman *et al.*, 1984). Subsequent studies established that mutations in these genes exerted global pleiotropic effects on host metabolism including effects in DNA and RNA synthesis, proteolysis, cell division and overproduction of hsps. These proteins disassemble a very stable *ori* λ DNA- λO-λP-DnaB complex, liberating the DnaB helicase thus enabling it to perform in λ DNA replication. This is accomplished via protein-protein interactions and with the aid of ATP hydrolysis. Such a role can be easily extended to normal *E. coli* physiology. In the unstressed cell DnaK/hsp70 binds to the exposed hydrophobic regions of cellular proteins, inhibiting their aggregation by limiting the concentration of hydrophobic surfaces. At intervals hydrolysis of ATP leads to dissociation of hsp70 from its substrate which then has a chance to fold before hsp70 reverts to its binding conformation (Pelham, 1986). Under conditions of heat shock the concentration of denatured protein in the cell increases (Lepock *et al.*, 1988, 1990); the primary roles of hsp70 in this case may be to prevent mass aggregation of aberrant protein. During recovery from heat shock hsp70 will then promote refolding and reassembly of these proteins. The literature contains extensive experimental evidence for such a role, including preferential binding of DnaK to the unfolded form of bovine trypsin inhibitor as opposed to its native form (Georgopoulos *et al.*, 1990), protection of enzymes from thermal inactivation by DnaK (Skowyra *et al.*, 1990) and enhanced restoration of activity of heat denatured λ repressor by DnaK (Gaitanaris *et al.*, 1990). Though very weak, the ATPase activity is crucial. There is a perfect correlation between ATP hydrolysis and release of bound polypeptide (Liberek *et al.*, 1991). Rapid and quantitative release of hsp70 from the complexes it forms with other proteins is only achievable in the presence of ATP, non hydrolysable analogs being ineffective (Pelham, 1990).

Two other *E. coli* hsps, DnaJ and GrpE, stimulate the ATPase activity of DnaK at

Table 1.2 Regulation and function of the HSP70 multigene family of *S. cerevisiae.*

HSP70 cognate gene [1]	Levels of mRNA at: 23°C	39°C	Approach to stationary phase:	Location	Function
SSA1 [2] *SSA2*	Moderate	↑ x10	↑ x2-3 prior to diauxic shift then ↓ x10 thereafter.	Predominantly cytoplasmic	Essential for growth at all temperatures. Stimulates translocation of precursor polypeptides into the ER, and mitochondria.
SSA3 *SSA4*	Very low	↑ x10-100	↑ x100 after diauxic shift. / As *SSA1/SSA2*.		
SSB1 [2] *SSB2*	Moderate	↓ x80%	↓ x10 as cells reach diaxic shift.	Unknown	Needed for optimal growth at low tempratures.
SSC1	Moderate	↑ x10	As *SSB1/SSB2*.	Mitochondrial matrix	Essential for growth. Needed for import of protein into mitochondria
SSD1	Moderate	↑ x10	As *SSB1/SSB2*.	Unknown	Unknown. Deletion strains have no apparent phenotype.
KAR2	Moderate	↑ x10	Unknown	Exclusive to the ER	Essential for growth. Mutants are defective in nuclear fusion.

SSA : Stress Seventy sub-group A.
KAR : Refers to defect in nuclear fusion (karyogamy).

[1] The homology these genes exhibit towards each other ranges from 50-97%, at the level of the nucleotide sequence (Lindquist and Craig, 1988).

[2] Levels of mRNA were detected by hybridization studies. In this type of analysis *SSA1* and *SSA2* transcripts are indistinguishable, as are *SSB1* and *SSB2* transcripts, since the DNA sequences are 97% and 94% identical, respectively (Werner-Washburne *et al.*, 1989).

References: Data for the above table, concerning the SS genes, are compiled from the numerous articles published by E.A Craig. The most recently discovered member was *KAR2* (Rose *et al.*, 1989 [a]). Regulation, function and location of all nine members was recently reviewed (see Craig, 1990 and references therein).

least five fold. DnaJ accelerates hydrolysis *per se*, whilst GrpE stimulates nucleotide release. In some organisms the three genes coding for DnaK, DnaJ and GrpE form an operon (Wetzstein and Schumann, 1990). It is likely that the DnaK/DnaJ/GrpE system has been highly conserved throughout evolution since there have been recent publications revealing the existence of at least three genes in yeast with partial or total homol to DnaJ (Blumberg and Silver 1991; Caplan and Douglas 1991; Luke *et al.*, 1991).

S. cerevisiae contains at least nine *HSP70* related genes, five of which are heat shock inducible. They are divided into five sub-groups on the basis of structural and functional criteria (see Table 1.2). These genes are regulated differentially during exponential growth, heat shock and entry into stationary phase. The extensive amount of data concerning differential expression of these genes, as well as function and location of the corresponding proteins, are summarised in Table 1.2.

Genetic studies have shown that hsp70 proteins must be present in the cytoplasm, endoplasmic reticulum (ER) and mitochondria for yeast cell viability (Craig *et al.*, 1989). It seems likely, due to the homology between them, that these proteins perform biochemically similar functions but have access to a different set of substrates. As evolution has progressed the role of hsp70 has been extended in tandem with the increased complexity of the cell. There is considerable data (from both *in vivo* and *in vitro* studies) to show that the proteins of the yeast *SSA* sub group (see Table 2) and its analogs in higher eukaryotes facilitate the post-translational import of precursor polypeptides into the lumen of the ER (Chirico *et al.*, 1988), the mitochondrion (Deshaies *et al.*, 1988), the lysosome (Chiang *et al.*, 1989) and the chloroplast (Waegemann *et al.*, 1990). The most likely mechanism involved here is the maintenance of precursors in an unfolded state, a prime requirement for translocation of proteins across membranes (Eilers and Schatz, 1988). Furthermore, one of the heat inducible members of the yeast hsp70 family (the product of the *SSC1* gene) is an essential protein located in mitochondria. It is thought to initiate the events leading to refolding of imported precursors once they have reached the mitochondrial matrix (Ostermann *et al.*, 1990; Scherer *et al.*, 1990).

It has become apparent that this entire system is highly conserved in eukaryotes. The human genome contains at least 6 hsp70 related genes (Morimoto and Milarski, 1990). Of these there are members that exhibit constitutive (hsp73) and heat inducible regulation (hsp72), and there are members that are located exclusively in mitochondria (grp75) and the lumen of the ER (grp78/BiP) (Welch, 1990).

1.2.2 hsp60/GroEL.

Like the DnaK/hsp70 family the hsp60/GroEL proteins are not only heat inducible but are essential for survival during non-stressful situations. Members of this family have been found in bacteria (Georgopoulos and Hohn, 1978), plants (Hemmingsen *et al.*,

1988), and mammals (Mizzen *et al.*, 1989).

The *groEL* and *groES* genes of *E. coli* were originally discovered because mutations in them resulted in blockage of bacteriophage growth by inhibition of phage assembly. There is a great deal of genetic (Tilly and Georgeopoulos, 1982) and biochemical (Chandrasekhar *et al.*, 1986) evidence demonstrating that GroEL interacts with another bacterial hsp, GroES. The two genes are completely indispensable for *E. coli* growth at all temperatures (Ang *et al.*, 1991). Figure 1.3 presents the current model proposed by Georgopoulos, that incorporates all known structural and biochemical properties of both proteins (Ang *et al.*, 1991).

FIGURE 1.3 Hypothetical scheme depicting mode of action of GroEL (hsp60) and GroES heat shock proteins.

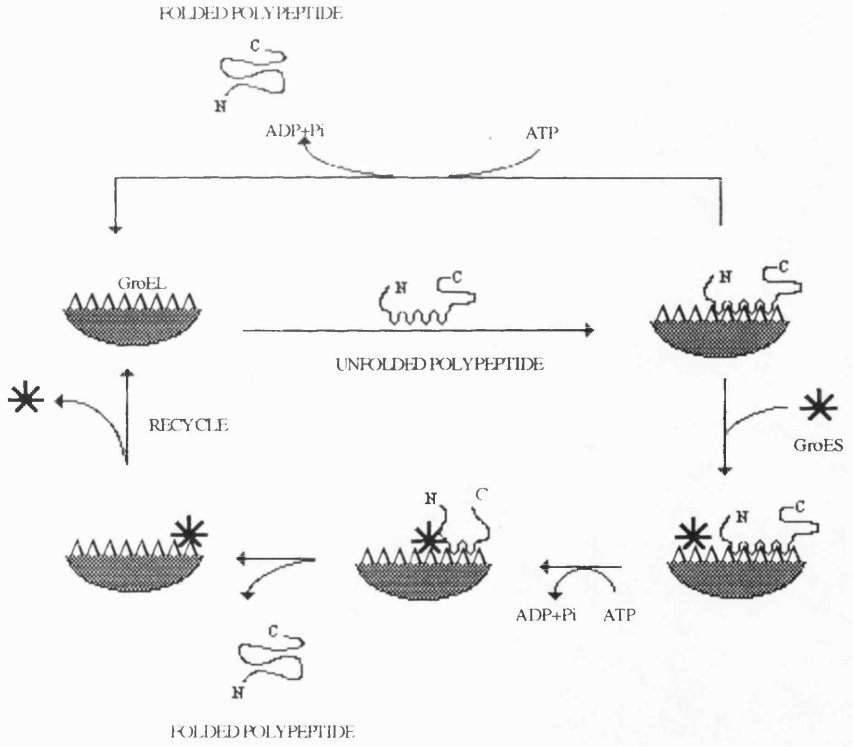


Figure from Ang *et al.*, 1991.

Like DnaK/hsp70, GroEL recognises and binds to unstructured peptide regions exposed in native or aberrant proteins and has a weak ATPase activity. The model takes into account that some proteins associate weakly with GroEL and will dissociate easily, whereas GroES is proposed to accelerate displacement of tightly bound proteins thereby ensuring efficient recycling of GroEL.

The GroEL protein is 54% identical at the amino acid level to yeast hsp60 coded by

the nucleus but found in the mitochondrial matrix (Reading *et al.*, 1989). It is also 48% identical to the Ribulose-1,5-bisphosphate (RuBisCo) binding protein also encoded by the nucleus but found in the chloroplast (Hemmingsen *et al.*, 1988). All of these proteins can assemble the RuBisCo enzyme (composed of 16 subunits) *in vitro*. In each case the presence of GroES and ATP was essential (Goloubinoff *et al.*, 1989). Not only did this suggest that the GroEL/GroES system was universal but it also predicted the existence, in mitochondria and chloroplasts, of a eukaryotic GroES. Such a protein has been recently discovered in mitochondria (Lubben *et al.*, 1990).

In yeast the hsp60 protein, the essential product of the *Mif4* gene, is required for the folding in the mitochondrial matrix of newly imported proteins (Cheng *et al.*, 1989). One of the hsp70 proteins (mt-hsp70) coded for by the *SSC1* gene, is also found exclusively in the mitochondria (see Table 1.2). It is thought that mt-hsp70 initiates the events leading to refolding of imported proteins. This is followed by completion of the import/assembly process either involving hsp60, as occurs with the β subunit F1-ATPase (Cheng *et al.*, 1989),or an alternative mechanism that can operate in the absence of hsp60, as for the ADP/ATP carrier (Ostermann *et al.*, 1990).

It is interesting that the genes for both mt-hsp70 and hsp60 are not only essential for growth at normal temperatures but are also heat inducible. It is known that during heat shock, mitochondria appear swollen with the intracristal spaces enlarged (Welch and Suhan, 1985). This is accompanied by a rapid reduction of ATP levels in mammalian cells (Findly *et al.*, 1983). The damage to integrity and function of the mitochondria may be partly due to denaturation of proteins. Increased levels of mt-hsp70 and hsp60 may act as damage limitation agents provided they are successfully imported into the mitochondria during stress; it should be noted that there is no report in the literature which analyses protein composition of mitochondria from unstressed and heat shocked cells (this is one question that is dealt with in chapter 4 of this thesis). Furthermore, these proteins could conceivably give mitochondria a "head start" during recovery by rapidly importing/refolding the influx of mitochondrial precursors that will replace irreversibly damaged proteins.

1.2.3 hsp90/HtpG.

In prokaryotes this family is represented by a single gene which is 40% identical to its eukaryotic counterparts. The only detectable phenotype of an *E. coli* strain in which the gene has been deleted is slow growth at high temperatures (Bardwell and Craig, 1988). In both eukaryotes and prokaryotes hsp90 is an abundant protein in exponentially growing cells, constituting 1-2% of cytosolic protein (Iwasaki *et al.*, 1989).

Yeast has two genes coding for hsp90. One is highly expressed at 25°C and induced by 1.5 - 2 fold at 39°C, the other being expressed at a much lower level at 25°C but exhibiting a 20 fold increase in expression at 39°C. With heat shock, hsp90

accumulates to nearly the same levels as the constitutively expressed hsc90 (Borkovich *et al.*, 1989). A similar pattern of expression is also exhibited by most higher eukaryotes. The genes of humans and mice have been designated *HSP90* α and β (Simon *et al.*, 1987; Minami *et al.*, 1991). Both are expressed at a basal level under unstressful conditions, transcription of *HSP90*α being enhanced by heat shock. Non-denaturing PAGE reveals that the prokaryotic and eukaryotic members of this family are dimeric (Welch *et al.*, 1990). Interestingly, studies on murine hsp90 reveal that this native complex is a homodimer (i.e. α/α or β/β) (Minami *et al.*, 1991). Among the reported biochemical properties of hsp90 are autophosphorylation and an ATP binding activity (Csermely and Kahn, 1991), phosphorylation by casein kinase II (Lees-Miller and Anderson, 1989[a]) and phosphorylation by a dsDNA-dependent kinase (Lees-Miller and Anderson , 1989[b]). The significance of these activities is unknown. Also it may be that they do not occur *in vivo* since concentrations of hsp90 used in some of these experiments are rather high and incubation times with [γ^{32}P]-ATP were long (ca. 45 min). None of the kinase catalysed phosphorylation activities have been reported in the yeast homologs. This is not surprising, since even though the proteins from yeast and mammals are highly homologous, the motifs involved are found in humans, mice, rabbits and chickens but not in *S. cerevisiae*, *Trypanosoma cruzii*, *E. coli* and three *Drosophila* species (Lees Miller and Anderson, 1989 [b]). This implies that properties of the protein have been modified during evolution.

Even though the two proteins have different patterns of expression in yeast they have equivalent and essential functions, since deletion of either of the hsp90 genes can be tolerated but deleting both is lethal (Borkovich *et al.*, 1989). Although deleting either gene impaired growth at higher temperatures (> ca. 36^{0}C) there was no effect on thermotolerance, whether or not cells were given an adaptive heat shock before the upshift to a lethal temperature (Borkovich *et al.*, 1989). Interestingly, a mouse cell line containing dramatically reduced levels of hsp90 (due to expression of anti sense RNA) showed reduced levels of growth at mildly elevated temperatures (in agreement with similar work on *E. coli* and yeast) but also exhibited reduced survival at highly elevated temperatures (Bansal *et al.*, 1991). The fact that reducing hsp90 levels in yeast did not give a similar result may be explained by comparing the extent to which hsp90 levels were reduced. Probing western blots with hsp90 antisera showed that levels of this protein (though dramatically reduced) were still easily detectable in strains of yeast where one of the hsp90 genes had been deleted (Cheng *et al.*, 1992), whereas the mouse cell line expressing the antisense RNA reduced hsp90 to barely detectable levels. More evidence for the role hsp90 might play in thermotolerance is provided by the finding that hsp90 is the only protein that is expressed at a significantly higher level in a thermotolerant variant of a Chinese hamster cell line (Yahara *et al.*, 1986).

Though little is known about the biochemical associations of hsp90 in yeast, the proteins of avian and mammalian cells are known to associate with a number of cellular

components, as evidenced by the extremely heterogeneous elution profile of hsp90 during gel filtration (Welch *et al.*, 1990). The most thoroughly studied interaction is that with glucocorticoid receptors. Hsp90 binds to steroid hormone receptors but only in the so—called non–transformed (i.e non DNA–binding) state (Baulieu and Catelli, 1989). Transformation of the receptor to the DNA binding state by addition of hormone co-incides with dissociation of hsp90 (Sanchez *et al.*, 1987). This led to the idea that hsp90 negatively regulated the receptor by capping its DNA binding site (Baulieu and Catelli, 1989). A number of recent publications indicate a more subtle interaction. hsp90 has now been found to bind within a sub region of the hormone binding domain (Howard *et al.*, 1990). Also, very little active receptor is made in the presence of very low levels of hsp90 (Picard *et al.*, 1990) while removal (*in vitro*) of hsp90 results in a high proportion of misfolded and therefore inactive receptors (Hutchison *et al.*, 1992 [a]). All of this suggests that hsp90 is necessary for the initial assembly and correct folding of the receptor, a role strongly reminiscent of that of hsp70.

Together with another protein of 50kDa (pp50), hsp90 has been shown to associate with several transforming kinases in virus infected cells. These kinases have little activity while in this complex. When the complex reaches the plasma membrane hsp90 and pp50 dissociate, the kinase becomes phosphorylated on tyrosines, and acquires full activity (Courtneidge and Bishop, 1982). Also, hsp90 co-purifies with and activates (Rose *et al.*, 1989 [b]) the heme-regulated kinase that phosphorylates the α sub unit of eukaryotic initiation factor 2 (eif-2α). Increased phosphorylation of eif-2α results in reduction of ribosomal initiation complexes (Duncan and Hershey, 1984), and may have something to do with the translational consequences of heat shock (see section 6.1).

It should be noted that the levels of hsp90 are significantly higher than would be needed to interact with steroid receptors and kinases only, implying that most of the hsp90 interactions remain unreported. The role that hsp90 plays during growth at higher temperatures, though important, is as yet unclear. High temperatures may disrupt the various protein interactions that hsp90 is involved in. Increased hsp90 levels with heat shock may serve to restore the equilibria of these associations.

Chapter 6 of this thesis provides preliminary data on hsp synthesis dramatically affecting certain autophosphorylation/kinase activities. In addition, it has since been shown that hsp90 influences this (see section 6.4). Reports of hsps influencing kinase activity are rare. Analysis of such phenomena may yield information regarding stress inducible responses (see section 6.1).

1.2.4 Functional co-operation/overlap between hsp60/70/90.

The three hsp classes discussed so far seem to have a collective purpose of protecting, preserving and recovering the function of various proteins. There is the

additional possibility that hsp90 and hsp70 may act co-operatively. Evidence for this includes: i) the $pp60^{src}$ multiprotein complex containing hsp90 and hsp70, as well as another 50kDa protein (Hutchison *et al.*, 1992 [b]); ii) steroid receptors which bind hsp90 also bind hsp70 (Smith *et al.*, 1990); iii) hsp90 exists in the cytosol in heteromeric complexes containing hsp70 and three other proteins one of which is also associated with steroid hormone receptors (Perdew and Whitelaw, 1991). This implies the existence of a complex containing both hsp70 and hsp90, as well as other proteins, which provides the unfoldase/assembly activities essential for producing active multimeric protein complexes.

1.2.5 hsps of M_r 100-110 kDa.

Most organisms, including yeast, humans and plants synthesise hsps in this size range, *Drosophila* being one of the few organisms that does not (Lindquist, 1986). Until recently, of all the major hsps, there were very few reports in the literature regarding the properties of this group of proteins. In *S. cerevisiae* the largest major hsp (hsp104) is not detectable at normal growth temperatures, but is induced during heat shock, transition to stationary phase and early in sporulation (Sanchez and Lindquist, 1990 and references threrein). In mammalian cells antibodies raised against this protein revealed localisation in or around the nucleoli; this staining could be eliminated altogether by ribonuclease (Subjeck *et al.*, 1983).

Given that this protein is one of the most abundant hsps, especially in yeast (see Fig. 1.2), it was surprising to find that the corresponding gene for this protein had not been cloned from any organism. Chapter 3 of this thesis presents a limited sequence (18 amino acids long) from the hsp104 protein of *S. cerevisiae*, generated by sequencing a peptide from a limited proteolytic digest. Entering this sequence into a protein sequence database revealed that this fragment was homologous to a region within the ClpB protein of *E. coli* (Gottesman *et al.*, 1990). This protein is also heat inducible (Squires *et al.*, 1991) and it follows that ClpB is the prokaryotic counterpart of hsp104.

A degenerate oligonucleotide based on this amino acid sequence was to be used to clone the corresponding gene. However, soon after this peptide sequence was obtained Lindquist's group published two articles describing gene cloning, gene deletion (Sanchez and Lindquist, 1990) and *in vitro* mutagenesis studies (Parsell *et al.*, 1991) on the gene encoding hsp104 from *S. cerevisiae*. From the gene sequence it was apparent that hsp104 and ClpB proteins are colinear and homologous throughout (Parsell *et al.*, 1991). Not surprisingly this gene hybridised to heat inducible mRNAs from *S.pombe* and human cells, of a size that would encode proteins of 100-110kDa (Parsell *et al.*, 1991).

Mutants lacking the gene for hsp104 grow at the same rate as wild type cells at 25°C and 37.5°C and reach similar stationary phase densities. Furthermore both mutant and wild type die at the same rate when exposed directly to lethal temperatures; however,

when given a mild pre-treatment (i.e a sub-lethal heat shock) the mutant cells acquired less thermotolerance on upshift to a lethal temperature (Sanchez and Lindquist, 1990). Hsp104 contains two putative nucleotide-binding domains; *in vitro* mutagenesis designed to inactivate these sites without disrupting overall structural stability of the protein showed that both are essential for function in thermotolerance (Parsell *et al.*, 1991).

The prokaryotic homolog of hsp104, ClpB, is part of the ClpA/ClpB family. The only member of the group which has been functionally characterised is the ClpA protein, which encodes the regulatory sub unit of the ATP-dependent Clp (Ti) protease (Gottesman *et al.*, 1990). It has been suggested that hsp104 may protect cells from heat shock by degrading proteins which have been irreversibly denatured, though a proteolytic defect in the strain lacking hsp104 has not been detected yet (Parsell *et al.*, 1991).

It should be noted that since deletion mutants retain a capacity to tolerate heat, other factors such as other hsps and metabolic/physiological changes must also play a role in induced thermotolerance. In addition, it has become apparent that there are two different ways by which the thermotolerance defect in cells lacking hsp104 can be largely suppressed (see sections 1.2.6 and 1.7.1). Thermotolerance is briefly reviewed in section 1.7.1. For a more thorough account, which includes a determinant of stress tolerance revealed by work presented in this thesis, see section 7.2.

1.2.6 The small hsps.

All organisms produce one or more small hsps. They are a heterogeneous group with respect to size (Sinibaldi and Storti, 1982). They are most abundant in stressed plants, a good illustration of this being the soybean which synthesises twenty different small hsps ranging from 14 to 27kDa (Schoffl and Key, 1982). *Drosophila melanogaster* synthesises four of these proteins, hsps28, 26, 23 and 22 (Lindquist, 1986), and *S. cerevisiae* produces a 26kDa protein (Bossier *et al.*, 1989) and a recently discovered protein of 12kDa (Praekelt and Meacock, 1990). Both of the corresponding genes in yeast are not transcribed at normal temperatures but are induced on heat shock and on entry into stationary phase. Where purified in its native form the protein is multimeric. In human cells, for instance, it exhibits a high native molecular-weight ranging from 200 to 2000 kDa (Welch *et al.*, 1989). All of these proteins have similar hydropathy profiles and most have a short region of homology with a sequence in mammalian α crystallin, a major component of the vertebrate eye lens (Craig, 1985).

Deletion of the gene coding for hsp26 in yeast has no effect on growth at any temperature in either rich or poor media, or in respiratory or fermentative metabolism. There is also no effect on acquired thermotolerance in mid-log or stationary phase cells and, even though the protein accumulates to high levels in sporulating cells there is no effect on sporulation or spore germination at any temperature (Petko and Lindquist, 1986). When

these experiments were carried out, hsp12 (a related protein) had not yet been discovered so strains lacking hsp26 may have exhibited no phenotype because hsp12 could compensate functionally. The reverse may also have been possible since strains lacking the gene for hsp12 also had no effect on growth or on the ability to acquire thermotolerance (Praekelt and Meacock, 1990). However preliminary experiments on an hsp12/hsp26 double deletion mutant indicated that its ability to survive at high temperatures is not compromised (cited in Praekelt and Meacock, 1990). These results are puzzling since it seems unlikely that a protein which has not only been conserved for millions of years, but is also made in such high amounts (see Fig. 1.2), has no function.

Research into small hsps has recently been given new impetus. Induction of murine hsp27, using an inducible expression vector driven by the metallothionein promoter, confers protection against the thermal killing of mouse cells; also, αB-crystallin is now known to be a heat shock protein in mammalian cells and its overexpression leads to acquisition of thermotolerance (Lavoie and Chepelinsky, respectively, cited in Hightower, 1991). In addition to this, phosphorylation of pre-existing murine hsp27 is an early event in the mammalian stress response and has been tentatively implicated in thermotolerance (Landry; cited in Hightower, 1991).

Incubations at high yet non-lethal temperatures, prior to still further upshift to lethal temperatures, will induce all hsps. Deleting the hsp26 gene might have a negligible effect on the acquisition of thermotolerance if there is functional overlap between different hsps. It follows that any role played by hsp26 of yeast in adaptive thermotolerance may only be detectable under conditions where factors that promote acquired thermotolerance (such as hsp104) are absent. A precedent for functional overlap of different hsps exists. The defect in acquired thermotolerance displayed by cells lacking hsp104 can be suppressed by over-expressing hsp70 (Parsell *et al.*, 1991).

1.2.7 Heat inducibility of components of proteolytic pathways.

Both prokaryotic and eukaryotic cells have at least one hsp that is involved in proteolysis (the Lon and ubiquitin proteins, respectively). This suggests that one role of the heat shock response is the degradation of proteins which have been irreversibly damaged during stress. Other hsps, notably DnaK/hsp70 and GroEL/hsp60, function to reverse heat damage. It is unclear what features of aberrant proteins determine whether they are degraded or salvaged.

The ATP-dependent Lon protease is a heat inducible protein of 94kDa. Mutations in the corresponding gene cause a 2-4 fold reduction in the rate of degradation of proteins containing amino acid analogs (Chung and Goldberg, 1981), though there is no eukaryotic homolog. However, various components of the degradative system based on the eukaryotic protein, ubiquitin, are heat inducible.

As its name implies ubiquitin, a 76 residue protein, is found in all eukaryotic cells. It is found either free or covalently linked to a variety of cellular proteins. The ATP-dependent ubiquitin conjugation-process is an essential precondition for selective degradation of intracellular proteins. Ubiquitin-protein conjugates are degraded by a specific multiprotein complex called the proteosome (for a review see Finley and Chau, 1991).

Ubiquitin genes have been isolated from several organisms. By far the most well characterised of these are the four genes in yeast . *UBI1*, *UBI2* and *UBI3* code for hybrid proteins in which ubiquitin is fused to unrelated amino acid sequences. The fourth gene, *UBI4* contains five consecutive ubiquitin-coding repeats; it is expressed at very low levels in growing cells but is induced by heat shock, nutrient deprivation (Finley *et al.*, 1987), and sporulation (Treger *et al.*, 1988). Genes for two ubiquitin conjugating enzymes *UBC4* and *UBC5* are also heat shock inducible (Seufert and Jentsch, 1990).

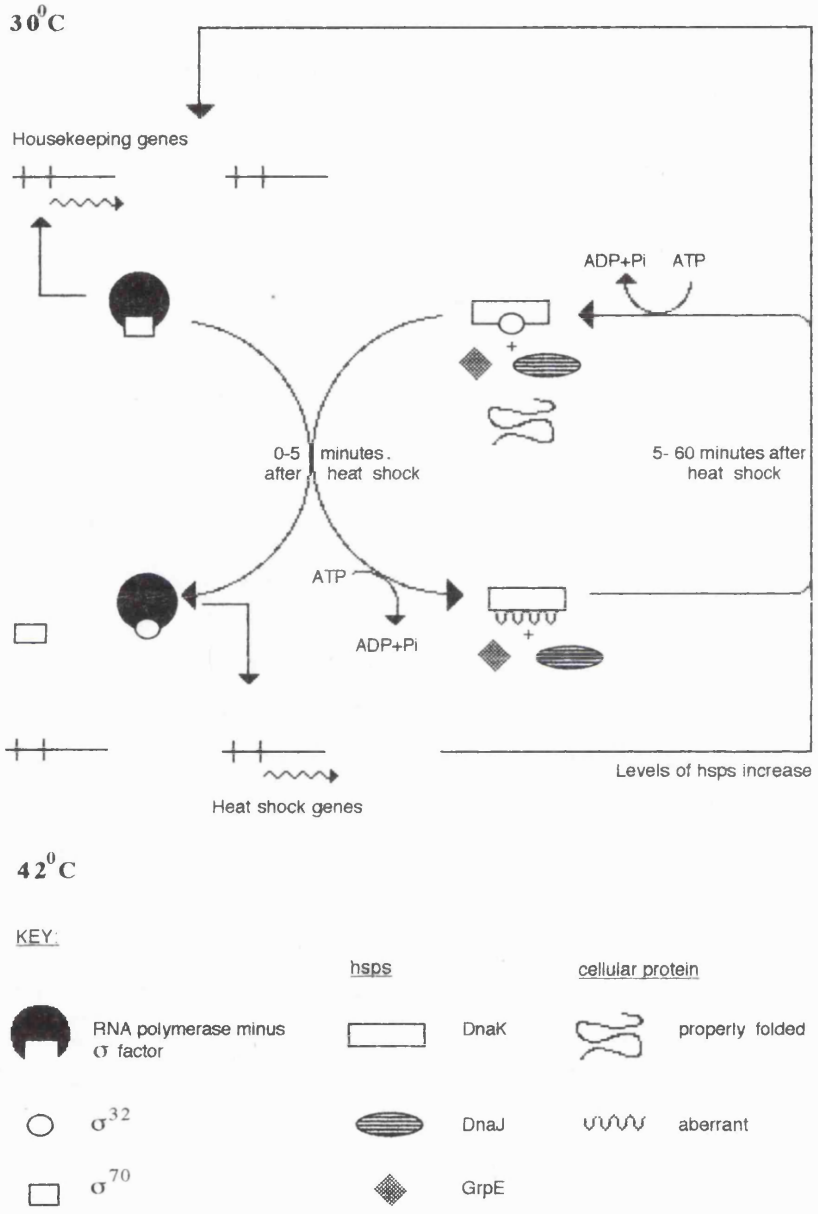
The importance of ubiquitination in the cellular response to stress is indicated not only by the stress inducibility of *UBI4*, *UBC4* and *UBC5*, but also by the stress sensitive phenotypes of mutations in these genes. Strains carrying *UBI4*, or *UBC4* plus *UBC5* deletions are far more sensitive than the wild type both to amino acid analogs and prolonged incubation at elevated temperatures (Finley and Chau, 1991). Though the *ubi4* deletion mutant grows at rates comparable to wild type strains between 23^{0}C and 36^{0}C, only 1-5% of mutant cells form colonies after 16 hours at 38.5^{0}C (a marginal growth temperature for yeast), whereas 60% of wild type cells survive (Finley *et al.*, 1987). In addition this deletion mutant is also more sensitive than the wild type to starvation for carbon and nitrogen (Finley *et al.*, 1987).

Ubiquitin has also been implicated in the regulation of the heat shock response. This arises from various observations, such as expression of certain heat shock genes at a non-permissive temperature in a mammalian cell line that has a temperature-sensitive ubiquitin-activating enzyme (Finley *et al.*, 1984). This and other possible mechanisms for hsp gene activation are discussed in section 1.3.2 .

1.3 Regulation of the response.

The study of how the heat shock response is regulated has provided a great deal of information about the molecular mechanisms involved in mounting a transient global response to an environmental stimulus. It is apparent that the response is orchestrated in such a way as to suit the specific requirements of different organisms. In *Drosophila*, regulation is exerted on both transcription and translation (Lindquist, 1981). This is not surprising given that mRNAs in *Drosophila* have half-lives of between 6-9 hours. To effect rapid changes in protein synthesis the cells must therefore block translation of pre-existing mRNAs. *E. coli* does not have this problem, consequently the response is controlled

Figure 1.4 Model for autoregulation of the heat shock response in *E.coli*.



Free DnaK binds σ^{32} sequestering it from RNA polymerase and/or presenting it to proteases. The $t_{1/2}$ of σ^{32} increases on heat shock due to a combination of factors, including the association of DnaK with aberrant protein. This allows the transcription factor to bind to RNA polymerase which leads to transcription from heat shock promoters. The ensuing increase of free DnaK levels lead to the recapture of σ^{32}, thereby re-establishing low levels of it, thereby dampening the heat shock response. This figure is compiled from data found in the references cited in section 1.3.1. Recent experiments have shown that purified DnaK can bind to the purified σ factor (Liberek *et al.*, cited in Hightower, 1991) hence the direct interaction between the two proteins shown above.

almost entirely at the level of gene transcription (Yamamori and Yura, 1980). In addition, there are examples of regulation arranged in such a way as to satisfy requirements of different cell types within the same organism. Regulation is primarily transcriptional in the somatic cells of *Xenopus* but translational in oocytes (Bienz and Gurdon, 1982). Due to their enormous size oocytes would require 10-100 days to synthesize an effective amount of heat shock mRNA. The hsp70 gene is therefore constitutively transcribed but translationally repressed. On heat shock the tranalational repression is lifted.

1.3.1 Regulation of transcription in prokaryotes.

In *E. coli* the heat shock response is transcriptionally regulated by the cellular concentration of σ^{32}, a sigma factor that binds to core RNA polymerase and redirects it to heat shock promoters (Grossman *et al.*, 1984). The fact that the promoters for heat shock genes are recognised by this complex, rather than by the predominant polymerase (containing σ^{70}), allows their regulation to be distinct from the vast majority of *E. coli* genes. Ordinarily, "housekeeping" genes are transcribed only because levels of σ^{32} (encoded by *rpoH*) are negatively regulated. Even though *rpoH* mRNA is relatively abundant, σ^{32} is synthesised at a very low rate (Gross et al, 1990). Also, this protein has a very short half life ($t_{1/2}$), ca. 45 seconds (Straus et al, 1987).

The intracellular concentration of σ^{32} increases 15 to 20 fold five minutes after a 30^{0}C to 42^{0}C upshift; levels declining thereafter. This is achieved by increased transcription of *rpoH*, and transient stabilisation of both *rpoH* mRNA and the *rpoH* product σ^{32} (Gross et al, 1990). This results in preferential transcription of hsp genes. It was anticipated that the transient nature of the response could be coupled to the function of hsps in proteolysis or the control of protein structure. Not surprisingly mutants in *dnaK*, *dnaJ* and *grpE* fail to switch off the heat shock response (Tilly et al, 1983; Gross et al, 1990). This was due to a defect in degradation of σ^{32}. In agreement with this, overproduction of DnaK leads to a dampening of the heat shock response (Tilly et al, 1983). These hsps therefore regulate their own synthesis, and the synthesis of other hsps, by increasing σ^{32} degradation thereby re-establishing the low levels of σ^{32} in the cell (see Figure 1.4).

Though some of the players in this negative regulatory loop are known, the proteins that degrade σ^{32}, the mechanism by which $t_{1/2}$ of σ^{32} is increased and the putative translational repressor of *rpoH* mRNA, remain unidentified. In addition to this, the discovery of another sigma factor (σ^{24} or σ^{E}) means that the regulation of the prokaryotic heat shock response may be more complex than previously thought. The heat shock gene *htrA* whose product is essential for *E. coli* viability at high temperatures (Strauch *et al.*, 1989) is under the exclusive control of σ^{24} (Erickson and Gross, 1989). Also, one of the four promoters of *rpoH* itself is recognised by σ^{24}, the induction it elicits being particularly

strong when cells are shifted to 50°C (Erickson and Gross, 1989).

1.3.2 Regulation of transcription in eukaryotes.

The induction of eukaryotic heat-shock genes in response to temperature upshift is mediated by the binding of a transcriptional activator, heat shock factor (HSF), to a short highly-conserved DNA sequence known as the heat shock element (HSE). HSEs are defined as an array of a variable number of the five bp sequence nGAAn arranged in alternating orientations upstream of the TATA box (for review see Sorger, 1991) . At least two nGAAn units are needed for high affinity binding of HSF; these may be arranged head to head (nGAAnnTTCn) or tail to tail (nTTCnnGAAn) (Perisic *et al.*, 1989).

Upon heat shock a pre-existing pool of inactivated HSF is converted to a form capable of stimulating transcription. In *S. cerevisiae* and *K. lactis* (budding yeasts) HSF is bound to DNA both before and during heat shock (Jacobsen and Pelham, 1991). HSF becomes highly phosphorylated on heat shock, the transcriptional activity of HSF closely following the extent of its phosphorylation over a range of temperatures (Sorger and Pelham, 1988). In contrast, the HSF of *S. pombe* (fission yeast), *Drosophila*, and humans binds to DNA only after heat shock. As in *S. cerevisiae*, this is accompanied by phosphorylation of the factor (Gallo *et al.*, 1991). In higher eukaryotes binding of HSF to the HSE *per se* appears to be insufficient for transcriptional activation. In murine erythroid-leukemia cells heat shock does not induce hsp70 transcription. Though HSF binds to DNA after the temperature upshift, it does not become phosphorylated in these cells (Hensold *et al.*, 1990).

The kinase responsible for HSF phosphorylation remains unidentified. Alternatively, elevated temperatures may stimulate HSF autophosphorylation. Even though the factor has been purified to homogeneity from several organisms, there have been no reports in the literature ascribing such an activity to it.

In *S. cerevisiae* the HSF gene is essential for viability (Sorger and Pelham, 1988). This is thought to be due to the requirement of basal levels of hsps during normal temperatures. There are several lines of evidence for this HSF binding sites in the promoter of one of hsp70 genes mediates 80% of non heat shock (basal) activity (Park and Craig, 1989). Also, overexpressing HSF in the absence of heat shock results in a four fold increase of the level of a major species of hsp70 (Sorger and Pelham, 1988). The sustained basal and transient heat-shock inducible-activities of HSF appear to be mediated by physically separatable regions of the polypeptide (Sorger, 1990).

1.4 The molecular nature of the cellular thermometer.

Though the transcriptional activators of heat shock genes in both prokaryotes and

eukaryotes have been identified, the precise molecular mechanism which senses changes in temperature is presently unknown. Recent work suggests that a homeostatic mechanism involving the level of free hsps in the cell provides a thermometer for detecting and reacting to temperature changes. Some hsps are known to interact with aberrant proteins, and in certain cases aid renaturation (Pelham, 1986). More specifically, they could bind to HSF and repress it at normal temperatures. Heat-shock -produced aberrant protein would compete for hsps, thereby freeing HSF and allowing it to i) be phosphorylated and (in organisms other than budding yeast) ii) bind to the heat shock element in hsp gene promoters. Ensuing overproduction of hsps will restore the free pool of these hsps to appropriate levels and HSF will again be inhibited. This is reminiscent of the way DnaK (the prokaryotic homolog of hsp70) autoregulates the heat shock response in *E. coli* (see Figure 1.4).

If this were true one would expect to see the following:

I. Strains which synthesise low levels of hsps would prematurely activate the heat shock response. In yeast, strains carrying deletions of two of the constitutively-expressed hsp70 genes (*SSA1* and *SSA2*) express hsps at high levels, even at 23 ^{0}C (Craig and Jacobsen, 1984). However, it should be noted that hsp70 is essential for cell viability,so reducing the levels of this protein may itself be stressful, causing activation of the heat shock response by a mechanism other than that described above.

2. One of the early events of heat shock must be a dramatic drop in the pool of free hsps. This is a feasible proposition given the increase in aberrant protein that will ensue, but it has only been reported for levels of free ubiquitin (which decrease by 75 %; Rose and Warms, 1987)

iii) HSF/hsp complexes must exist in unstressed cells. No such report exists in the literature. However, recombinant *Drosophila* HSF produced in *E. coli* will bind to HSEs with high affinity in the absence of heat (Clos *et al.*, 1990). In contrast to this, the same HSF will not bind to HSEs if produced in *Xenopus* oocytes. This suggests that HSF may interact with one or more negative regulators found in eukaryotic cells, possibly eukaryotic hsps.

Aberrant protein seems to be a recurring theme in studies designed to investigate the heat-shock response trigger. Firstly, many of the conditions known to induce hsp genes are thought to cause denaturation of intracellular proteins (see Table 1.1). Denatured λ repressor can induce the response in *E. coli* (Parsell and Sauer, 1989); injecting denatured protein (but not native protein) into *Xenopus* oocytes has the same effect (Ananthan *et al.*, 1986). Also, a mutant mouse cell line which cannot ubiquitinate proteins above a certain temperature exhibits abnormally high synthesis of hsps at such temperatures (Finley *et al.*, 1984). Finally, there is *in vivo* evidence of cellular protein denaturation within the

temperature range of hsp induction in both bacteria and mammalian cells (Lepock *et al.*, 1988, 1990).

Wu has suggested that HSF itself is a cellular thermometer (in Hightower, 1991). HSF from unshocked HeLa cells can be induced to bind HSEs *in vitro* by exposing nuclear extracts to elevated temperatures (Larson *et al.*, 1988) and other conditions that promote protein unfolding, such as nonionic detergents and increasing concentrations of urea (Mosser *et al.*, 1990). Such results, however, do not distinguish between models where HSF *per se* responds to environment by direct conformational change, from models where it is the interaction of HSF with other proteins that is affected.

One must also bear in mind that heat shock has many effects on the cell, as well as the build up of aberrant protein (see sections 1.8-1.8.5). For example, intracellular pH falls and levels of calcium rise (see section 1.8.3). These may also play a role in triggering the heat shock response. Evidence for this is the activation of HSF *in vitro* by decreasing the pH of buffers, and by increasing the levels of calcium (Mosser *et al.*, 1990). There may even be different induction pathways triggered by different cellular events. Yuzawa has isolated two groups of *E. coli* mutants, one defective in responding to unfolded proteins as inducers but still heat inducible the other unresponsive to both, suggesting the existence of at least two distinct induction-pathways (cited in Hightower, 1991).

Finally, the regulation of the heat shock response in eukaryotes has taken a new twist. Morimoto has described cloning and characterisation of two genes encoding murine heat-shock transcription factors (*HSF1* and *HSF2*) which respectively display heat inducible and constitutive HSE binding to DNA (cited in Hightower, 1991). This system may be widespread among higher eukaryotes. There are also two human HSFs, HSF1 exhibiting heat inducible binding (Rabindran *et al.*, 1991); the binding characteristics of HSF2 are unknown (Schuetz *et al.*, 1991). However it is well established that HeLa cells have two HSE binding activities one found in unshocked extracts, the other in stressed cells (Kingston *et al.*, 1987). The HSE protein complex in unstressed cells may correspond to DNA bound to HSF2. Different HSFs may have evolved to respond to different temperature thresholds or to other (chemical) stress signals. This, in addition to the emerging data suggesting the existence of separatable induction pathways, makes regulation of the response to stress far more complicated than previously thought.

1.5 Hsp induction caused by development.

As well as being constitutively expressed and heat inducible, heat shock genes are also switched on during the course of development. The best example of this is found in *Drosophila.* For example, messages for hsps26, 28 and 83 are induced at high levels in ovarian nurse cells and passed into developing oocytes; hsp70, 68, 22, and 23 mRNA is not produced and cannot be induced at this time (Zimmerman *et al.*, 1983). Similar events

also occur during the development of other eukaryotes (Lindquist, 1986) the reasons remaining unclear.

1.6 Hsp induction caused by progression through the cell cycle.

The lack of essential nutrients in the growth medium causes yeast cells to arrest in stationary phase. The cells enter a quiescent state often referred to as G_0, where they remain viable and metabolically active. In this state cells are primarily unbudded (Johnston *et al.*, 1977), accumulate storage carbohydrates (Lillie and Pringle, 1980), and exhibit resistance to heat shock (Schenberg-Frascino and Moustacchi, 1972) and cell wall degradative enzymes (Deutch and Perry, 1974). Also, 95% of proteins detected in log phase are repressed in their synthesis (Boucherie, 1985), and genes which were repressed in the presence of glucose become derepressed.

Another consequence of entry into stationary phase is an increase in the mRNAs of certain heat shock genes including mRNAs for hsp26 (Petko and Lindquist, 1986), hsp12 (Praekelt and Meacock, 1990), hsp104 (Sanchez and Lindquist, 1990), polyubiquitin (Finley *et al.*, 1987) and some of the members of the hsp70 family, (*SSA1/2* and *SSA3*; Werner-Washburne *et al.*, 1989).

These drastic physiological changes occurring on cell cycle arrest are thought to be controlled primarily by the decrease in levels of cAMP, which acts through the concomitant decrease in the activity of the cAMP-dependent protein kinases. The role of cAMP in yeast has been largely determined by analysing mutants in the biosynthetic pathway of cAMP or the cAMP-dependent protein-phosphorylation mechanism (Matsumoto *et al.*, 1982 and 1985).

That heat-shock gene induction coincides with the decrease in cAMP levels that occurs during the transition to slower growth is not proof positive of the involvement of cAMP. A more convincing demonstration of the involvement of this metabolite has come from the use of strains which are unable to elevate cAMP in response to high glucose levels. These strains grow more slowly than the wild type and synthesise many heat shock proteins constitutively. Among these strains are those that maintain low levels of adenylate cyclase by, for instance, a Ty transposable element insertion within the promoter of the gene coding for adenylate cyclase (Iida, 1988).

Even though many heat shock genes are induced by heat shock and in response to a decline in levels of cAMP, the mechanisms of their induction are distinct. Heat-shock gene activation cannot respond to a decline in cAMP since intracellular levels of cAMP increase two-fold with 24-36^{0}C (Boutelet *et al.*, 1985; Camonis *et al.*, 1986) or 25-40^{0}C (Hirst, 1990) heat shocks. Moreover, several heat shock genes are still capable of further activation by heat shock in cells with constitutively low levels of cAMP (Tanaka *et al.*, 1988). In addition heat-shock element (HSE) - lacZ reporter gene constructs give no cAMP

or growth-phase dependent changes in β-galactosidase synthesis in the absence of heat shock (Kirk and Piper, 1991). Analyses of the promoters of heat shock genes have located regions responsible for growth-phase dependent expression. In the *SSA3* promoter, for example, a 35 bp region upstream of the heat shock element (HSE) contains sequences capable of activating transcription after the diauxic shift. Although the HSE *per se* was incapable of driving transcription in response to decline of cAMP levels, removing it reduced expression from the *SSA3* promoter by 71% after the diauxic shift, implying a strong positive interaction between the HSE and the 35 bp sequence upstream of it (Boorstein and Craig, 1990).

1.7 Thermotolerance.

When cells of *S. cerevisiae* are shifted from a low growth temperature (20°C) to a lethal temperature (48°C) the cells are rapidly killed. However, if cells are first pre-adapted by incubation at a higher non-lethal temperature (ca. 37-40°C for 30 minutes) the rate of killing upon upshift to a lethal temperature is dramatically decreased (McAlister and Finkelstein, 1980). This phenomenon is exhibited by a variety of systems including bacteria, *Drosophila*, plant and mammalian cells (Hall, 1983 and references therein), and is referred to as **acquired thermotolerance**. Acquired thermotolerance has been extensively studied, especially in mammalian cells. This is largely because of the upsurge in interest in the use of heat as an adjunctive technique for the treatment of certain cancers in humans.

1.7.1 The role of hsps and other factors in acquired thermotolerance.

Many studies demonstrate that preferential synthesis of hsps occurs during the period when development of thermotolerance is at its maximal rate. The argument that hsps are involved is strengthened by the fact that other inducers of thermotolerance (apart from heat) also induce hsps. In addition, individual hsps have been implicated in acquired thermotolerance, including hsp70 (Angelidis *et al.*, 1991) and hsp104 (Sanchez and Lindquist, 1990). However the importance of hsps in acquired thermotolerance may be overestimated. For instance, an *S. cerevisiae* strain carrying a deletion of the gene coding for hsp104 may show defects in acquired thermotolerance, but they only manifest themselves after five minutes at the lethal temperature (De Virgilio *et al.*, 1991; Sanchez and Lindquist, 1990 and Parsell *et al.*, 1991). Furthermore, the reduction in acquired thermotolerance exhibited by this mutant almost disappears when the time of pre-adaptation at the higher non-lethal temperature is increased from 30 to 60 minutes (De Virgilio *et al.*, 1991). This may suggest a minor role for individual hsps in acquired thermotolerance. A major role for a combination of hsps may be inferred from this, but

studies with inhibitors of protein synthesis make this doubtful.

Many groups have investigated the development of thermotolerance by using inhibitors of protein synthesis to eliminate the participation of hsps. This has yielded mixed results. Some groups maintain that hsps do play a role (McAlister and Finkelstein, 1980; Li and Werb, 1982); others report that blocking hsp synthesis has no effect (Hall, 1983; Watson *et al.*, 1984; Widelitz *et al.*, 1986). Some of the latter reports may be dismissed because the effectiveness of the inhibitors had not been tested. However this does not apply in every case. Two of the more obvious explanations for such anomalies are; i) incorrect interpretation of thermotolerance data and ii) the selection of single time points at which to measure thermotolerance resulting in the participation of hsps being missed. For example, choosing early points (< 5 minutes) in the killing curves of *S. cerevisiae* will not reveal the role of hsp104 in acquired thermotolerance.

All available data lead to the conclusion that, though hsps may play a minor role in thermotolerance, the mechanisms that make the greatest contribution are poorly-understood hsp-independent events associated with heat shock. It is obvious that experiments designed to investigate such mechanisms (involving inhibitors of protein synthesis or transcription) have their drawbacks. For instance, the widely employed transcriptional inhibitor 1,10-phenanthroline enhances the abundance of certain heat-shock genes (Adams and Gross, 1991). These problems can be overcome by using the recently-discovered hsf1-m3 mutation which prevents activation of heat-shock transcription factor; and, even though it causes a general block in heat inducible transcription, has hardly any effect on inducible (acquired) thermotolerance (Smith and Yaffe, 1991).

The question arises as to the nature of these hsp independent mechanisms that confer thermotolerance to the cell. A useful perspective from which to study them is to determine the cellular changes that occur on heat shock (these are discussed fully in sections 1.8-1.8.5). One of the most dramatic consequences of heat shock is the increase in levels of the non-reducing disaccharide trehalose. This has been observed in *S. cerevisiae*, *S. pombe* and *Neurosporra crassa* (Hottiger *et al.*, 1987; De Virgilio *et al.*, 1990 and Neves *et al.*, 1991, respectively). Moreover, thermotolerance increases in parallel to trehalose accumulation and decreases in parallel to the trehalose levels when cells are shifted back to normal temperatures, irrespective of the presence or absence of cycloheximide (De Virgilio *et al.*, 1990). Accumulation of trehalose also occurs on exposure to other inducers of thermotolerance, such as ethanol and hydrogen peroxide (Attfield, 1987).

It is well established that physiological state has a dramatic effect on both basal and acquired thermotolerance. The level of cAMP-dependent protein kinase (protein kinase A) plays a major role here. Yeast in rapid fermentative growth has high levels of cAMP and low basal thermotolerance. In contrast, quiescent cells have low levels of cAMP and high levels of basal thermotolerance. Moreover, studies employing mutants of the yeast cAMP

system show that low protein kinase A activity correlates with high thermotolerance (Iida, 1988), whereas high protein kinase A activity results in low thermotolerance (Tanaka *et al.*, 1989).

Acquired thermotolerance in rapidly growing cells is also dependent on the activity of this kinase. Compared to its isogenic wild type, a mild heat shock will not induce acquired thermotolerance in a strain with constitutively high levels of protein kinase A activity (Shin *et al.*, 1987). Conversely, cells with constitutively low levels of cAMP are constitutively thermotolerant i.e cells in exponential phase of growth behave as if they have already experienced a sub-lethal, pre-adaptive heat shock (Shin *et al.*, 1987).

It has been found that, like stationary phase cells, wild type *S. cerevisiae* is transiently arrested at the pre-replicative phase of the cell cycle by a sub-lethal heat shock (23^0-36^0C) (Johnson and Singer, 1980). Paradoxically, the implied depression in cAMP level due to heat shock does not occur; levels of this metabolite rise instead (see section 1.6).

In addition to heat itself many agents can induce thermotolerance. In *S. cerevisiae*, for example, these agents include ethanol (Plesset *et al.*, 1982), osmotic stress (Trollmo *et al.*, 1988) and hydrogen peroxide (Collinson and Dawes, 1992). However the physiological overlap between some of these agents and heat shock is unidirectional i.e. heat conditioning does not confer tolerance to osmotic shock (Trollmo *et al.*, 1988) or oxidative stress (Collinson and Dawes, 1992). These phenomena are poorly understood, and understanding stress tolerance remains a future research goal.

1.8 Physiological and biochemical changes that occur in cells experiencing stress.

Many reviews on the heat shock response only discuss the changes in gene expression and fail to mention the biochemical and physiological changes elicited by stress. This is ironic given that hsps play only a minor role in the acquisition of thermotolerance (see section 1.7.1). Studying hsps is more likely to yield information about: i) the recovery of normal cell processes on temperature downshift and/or ii) mechanisms that allow growth at higher non-lethal temperatures; at least one hsp is required for growth at high temperatures (see section 1.2.3). Investigating the protein-synthesis-independent biochemical and physiological changes caused by stress may yield important information about the mechanisms that confer stress tolerance to cells. In addition, the mechanism by which the cell recognises stress may be revealed.

A consequence of heat shock is the abrupt arrest of cell growth and proliferation. However, after a period of time both bacteria and yeast apparently adapt to their new temperature, normal gene expression is re-established, and cells re-acquire their normal pro liferative activities (Lindquist, 1986). In contrast, in higher organisms where body temperature is tightly regulated, acclimation is not typically observed and cells continue to

synthesise hsps until cell death.

1.8.1 Changes in cell morphology.

Electron microscopy has revealed dramatic morphological changes caused by stress. One obvious effect is the accumulation of perichromatin granules throughout the nucleus. Since splicing of precursor mRNAs is compromised during heat shock it is likely that these granules represent aggregates of unprocessed mRNA (Yost and Lindquist, 1986). Also, heat shock elicits a loss of the granular components of the nucleolus (representing pre-ribosomes) (Welch and Suhan, 1985). Consistent with this are biochemical studies demonstrating an inhibition of proper pre-rRNA processing and a cessation of ribosome biogenesis (Bouche *et al.*, 1979). These changes in the morphology of the nucleolus, and of the nucleus as a whole, appear to be far less dramatic in cells first made thermotolerant by an incubation at a higher non-lethal temperature (Pelham, 1984; Welch and Mizzen, 1988). Hsp70 has been implicated in limitation of the damage to nucleoli as well as enhancing recovery to normal morphology on temperature downshift (Pelham *et al.*, 1985).

On heat shock, mitochondria appear swollen with very prominent cristae and enlarged intracristal spaces (Welch and Suhan, 1985). Not surprisingly this is accompanied by a reduction of cellular ATP levels in *Drosophila* and *Tetrahymena* (Leenders *et al.*, 1974 and Findly et al, 1983, respectively). However ATP levels in yeast do not change on heat shock (J. Dickinson, personal communication). This is expected given that little of the ATP produced by yeast during fermentative growth comes from oxidative phosphorylation.

1.8.2 Changes in the levels of trehalose.

Heat shock results in a rapid accumulation of the non-reducing disaccharide, trehalose. In *S. cerevisiae* growing exponentially on glucose, the trehalose content increases one hundred fold within one hour after a 27-40°C upshift (Hottiger *et al.*, 1987). In parallel, activity of trehalose synthase increases. Surprisingly, activity of trehalase also increases, implying a rapid turnover of the accumulating trehalose during heat shock (Hottiger *et al.*, 1987). Also, thermotolerance closely follows levels of this disaccharide (see section 1.7.1) (De Virgilio *et al.*, 1990). All of these changes occur in the presence of cycloheximide, indicating that they are independent of protein synthesis.

The close correlation between trehalose levels and resistance to heat, to freeze drying (Gadd *et al.*, 1987) and to osmotic stress (Mackenzie *et al.*, 1988) suggest that this disaccharide is primarily a stress protectant rather than a reserve carbohydrate as previously thought.

1.8.3 Changes in intracellular pH (pH_i) and levels of calcium.

One early consequence of heat shock is a rapid drop in pH_i (Weitzel *et al.*, 1987; Findly, 1983 and Drummond *et al.*, 1986). This is thought to be due to stress induced permeabilisation of the plasma membrane, resulting in the dissipation of ionic gradients existing across it. Heat shock also causes an increase in intracellular calcium (Ca^{2+}_i) levels (Calderwood *et al.*, 1988 and Drummond *et al.*, 1986). In HeLa cells the increases in Cai^{2+}_i occur in two "waves". The first is a ca. 3-5 fold increase occurring within minutes of heat shock and is due to a release of Ca^{2+} from an internal store probably the mitochondria and/or the endoplasmic reticulum. The second 'wave' is a further 3-5 fold increase *beginning* thirty minutes after heat shock and is due to an influx of Ca^{2+} from the extracellular medium (Calderwood *et al.*, 1988). This phenomenon may be widespread as it also occurs in *Drosophila* (Drummond *et al.*, 1986). In contrast, the intracellular free-ion concentrations of Na^+, K^+, Cl^- and Mg^{2+} do not change on heat shock (Drummond *et al.*, 1986).

Changes in pH_i and Ca^{2+}_i may mediate the activation of certain second messengers in the cell (e.g. those leading to the activation of gene transcription events). It is possible that they activate heat-shock gene transcription. However, manipulating cells in order to lower pH_i and increase intracellular levels of Ca^{2+} does not support this idea (Drummond *et al.*, 1986). In contrast to this, manipulating the pH and Ca^{2+} levels of nuclear extracts from HeLa cells can induce the binding of heat shock factor to the heat shock element (see section 1.4). Finally, to confuse the issue, there is a report describing stimulation of heat-shock protein synthesis by artificially *increasing* pH_i (Whelan and Hightower, 1985). One should note that the techniques used to measure pH_i can influence the outcome of such experiments. For instance, the ^{31}P NMR method of following pH_i must be carried out at very high cell densities. In yeast the method used most frequently to measure pH_i involves determining the distribution of radiolabelled weak acids across the cell membrane (e.g. Pena *et al.*, 1982; Cole and Keenan, 1987; Ramos *et al.*, 1989). The technique cannot be used to follow the decline in pH_i that occurs during heat shock. The use of weak acids needs several minutes for attainment of an equilibrium state and is therefore unsuitable for cells in metabolic change.

1.8.4 Protein phosphorylation and stress.

Even though considerable effort has been applied towards understanding cellular and biochemical responses to heat, the underlying processes occurring at the molecular level are still poorly understood. It is well established that protein phosphorylation of

proteins and the coupled signal transduction are major processes in the regulation of cellular functions. Changes in the phosphorylation state of ribosomal, nuclear and nucleolar proteins caused by stress have been reported and are reviewed in section 6.1. Results of preliminary studies designed to detect similar phenomena associated with proteins of i) the plasma membrane and ii) the cytosolic fraction, are presented in chapters 4 and 6 respectively.

1.8.5 The effect of stress on the plasma membrane.

All organisms can adapt to changes in their environmental temperature by altering the lipid composition of their membranes. In general, the higher the growth temperature the more saturated the membrane fatty-acid composition (Houslay and Stanley, 1982; for reviews on the effects of temperature on membrane lipids of *S. cerevisiae* see Watson, 1984 and 1987). This acts to offset the increase in membrane fluidity that occurs on temperature upshift. It is generally accepted that an increase in membrane fluidity has two effects. Firstly, there will be a partial dissipation of ionic gradients that exist across membranes leading, for example, to decreases in pH_i. Secondly, the activities of membrane bound enzymes will be affected.

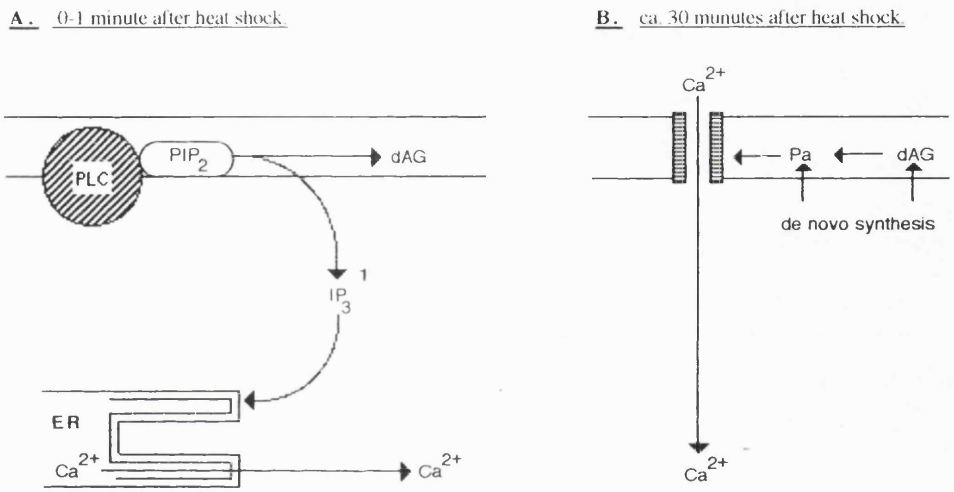
Though membrane lipid composition is slowly altered as a consequence of shifts in temperature, its purpose is to adapt the cell for long term survival. For instance, chilling *T. pyriformis* from 28 to 10°C (below the lipid phase separation temperature) results in the clustering of membrane proteins due to their exclusion from solid phase lipid. Within eight hours at 10°C the proteins are once again distributed evenly throughout the membrane due to desaturation of 50% of phospholipid acyl chains (Houslay and Stanley, 1982). This long term response is in sharp contrast to rapid events caused by shifts in temperature, such as hsp induction (1-5 minutes after heat shock), drop of pH_i, or the activation of second messenger systems (30s-1minute after heat shock, see below and Fig. 1.5).

Even though heat shock or other forms of stress must have marked effects on the activity or regulation of a number of key plasma membrane proteins, there are only a few examples in the literature of such events. Among them is the four fold decrease in activity of Na^+/K^+ ATPase when HeLa cells are shifted from 37°C to 45°C (Burdon and Cutmore, 1982). In addition, there is an overall decrease in the ability of ligands to bind to their receptors as in the case of epidermal growth factor (Welch, 1990). There are also examples of a fall in the number of receptors e.g. the insulin receptor (Calderwood and Hahn, 1983).

Calderwood *et al.*, (1987 & 1988) have demonstrated that heat can induce second messenger cascades. These, of course, have their initiating signals in the plasma membrane. Figure 1.5 presents a summary of these events. Rapid turnover of phosphatidyl inositol-4,5-bisphosphate due to heat (Fig. 1.5 A) is a widely occurring phenomenon,

observed in human, rat, murine and hamster cell lines. Additionally, ethanol and arsenite have a similar effect in hamster cells (Calderwood *et al.*, 1988 and references therein). An interesting aspect of this phenomenon is the large increase in levels of diacylglycerol that occurs within seconds of heat shock. This should stimulate protein kinase C (Nishizuka, 1988).

Figure 1.5 Second messenger cascades activated by heat shock.



A. Heat stimulates action of phospholipase C (PLC) which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (dAG). When IP_3 is produced as a consequence of PLC activation by various hormones and growth factors, it diffuses into the cytosol where it interacts with ER receptors, leading to liberation of stored Ca^{2+}. Heat shock is thought to have the same effect.

B. Phospahtidic acid (PA), another metabolite of the phosphoinositide pathway, accumulates later on during heat shock and shows an excellent correlation with the delayed heat-induced influx of Ca^{2+} from the extracellular environment.

1. Levels of IP_3 increase by 180% by 30 seconds after heat shock, remaining high for 3-5 minutes and then declining.

References: Calderwood *et al.*, 1987 and 1988.

1.8.6 Aims.

Compared to certain aspects of the response to stress, such as heat-shock protein induction, there are few reports in the literature describing events that occur at the plasma membrane. This is surprising given that the plasma membrane is bound to be the first region of the cell to be affected by environmental stress.

Two second-messenger cascades have been shown to be activated by early heat shock. A response to stress, the purpose of which is to protect the cell, should operate as quickly as possible. It seems logical that the plasma membrane should play a part in detecting stress as well as in the induction of protective mechanisms. Moreover, since the

plasma membrane and the components associated with it are damaged on heat shock, some of these protective mechanisms should be targeted to this region of the cell.

1.9.1 The putative role of plasma membrane ATPase during heat shock.

One of the consequences of heat shock in *S. cerevisiae* is the transcriptional induction of *PMA1* (Curran *et al.*, 1988) which codes for the plasma-membrane-associated H^+ translocating ATPase (H^+-ATPase). This enzyme is crucial for all fungal and plant cells (for a review see Serrano, 1988). The proton gradient it generates is responsible for uptake of nutrients, maintenance of intracellular K^+ levels and the regulation of pH_i. H^+-ATPase is under very tight control, as would be expected for an enzyme thought to hydrolyse upto 40% of all the ATP generated by the cell. *Levels* of the enzyme itself are subject to very tight regulation. Attempts to overexpress the *PMA1* gene (as with *GAL1* promoter-*PMA1* constructs) or maintain *PMA1* at multiple copies per cell are generally unsuccessful (Eraso *et al.*, 1987). The slight increases in overexpression of the gene that can be achieved invariably lead to slower growth. These restrictions to ATPase levels may be due to limitations over the amount of this enzyme that the plasma membrane can accommodate; this protein being generally the most abundant protein in the plasma membrane. Alternatively, higher levels of this protein may compromise cellular ATP levels. *Activity* of this enzyme is also under tight control;it is activated by glucose and by acidification of the medium (Serrano,1983; Eraso and Gancedo, 1987. However, the mechanisms by which this is controlled remain unknown.

One of the effects of heat shock is dissipation of the H^+ gradient that is maintained across the plasma membrane by H^+-ATPase. The observed induction of the gene coding for H^+-ATPase seems to be a logical consequence of heat shock since cytoplasmic acidification will be counteracted by action of this enzyme. Activity of H^+-ATPase has not previously been considered a possible influence on the tolerances or responses of fungi and plants to heat shock. Chapter 3 describes experiments where a series of strains, isogenic but for mutations at the *PMA1* locus, were used to obtain evidence as to whether H^+-ATPase action is indeed one of the determinants of stress tolerance.

1.9.2 Changes occurring in protein composition of the plasma membrane during stress.

It is well established that stress proteins are directed to the endoplasmic reticulum, golgi body, mitochondria, nucleus and nucleolus. Surprisingly, bearing in mind that the plasma membrane is damaged during stress, there have been no reports to date of eukaryotic stress proteins associating with this membrane. It is nevertheless conceivable

that cells target proteins with a damage limitation function to the plasma membrane as part of their inducible protective responses. Furthermore, though *PMA1* is transcribed during heat shock it is not known whether the corresponding protein is inserted into the plasma membrane during stress (or even if the *PMA1* message is translated under heat shock conditions). Changes in the level of H^+-ATPase in the plasma membrane during heat shock must surely affect the extent to which yeast cells counteract the intracellular acidification that occurs at elevated temperatures. This is an important aspect of cell physiology, given that yeast use a considerable proportion of cellular ATP in maintaining pH_i (see section 3.1).

This study investigated the protein composition of plasma membranes of stressed and unstressed yeast as an initial step towards establishing to what extent the plasma membrane or the enzymes and carrier systems associated with it are altered in response to heat shock and other types of stress (Chapter 4).

Chapter 2 Materials and methods.

2.1 Materials.

Standard reagents:	AR grade supplied by Sigma and BDH.
Microbiological media:	Supplied by Difco. Auxotrophic requirements supplied by Sigma.
Electrophoresis reagents:	Acrylamide and bis-acrylamide (Electran grade) and ammonium persulphate supplied by BDH. TEMED supplied by Sigma. Molecular weight markers : i) 29-205 kDa ii) 14.2-66 kDa iii) 14.3-66 kDa (prestained) all supplied by Sigma.
Enzymes:	Protease V8 from *Staphylococcus aureus* supplied by ICN immunobiologicals. α-Chymotrypsin type VII and RNAase A typeIIIA supplied by Sigma.
Protease inhibitors:	PMSF, TLCK, TPCK and Pepstatin A all supplied by Sigma.
Transfer membranes for protein (Western) blotting:	Genescreen (0.2μ pore size) supplied by Dupont (NEN research products). PVDF supplied by Millipore (0.4 μ pore size) and Bio-Rad (0.2μ pore size).
Immunological reagents:	Biotinylated goat anti-rabbit IgG, streptavidin-horse-radish peroxidase conjugate and 4CN all from Bethesda Research Laboratories Life Technologies, Inc (BRL).
Reagents for detecting glycosylated proteins:	Concanavalin A typeV from *Canavalia ensiformis* and horseradish peroxidase type VI-A supplied by Sigma.
Reagents for protein assay:	Dye reagent concentrate and BSA standard supplied by Bio-Rad.

Radiolabelled amino acids and nucleotides:	L-[^{35}S]-Methionine (1,000Ci/mmol; 10μCi/μl), and L-[4,5-^{3}H(N)]-Leucine (60Ci/mmol; 1μCi/μl) supplied by Dupont (NEN research products). [γ-^{32}P]-5'-Adenosine triphosphate (3,000Ci/mmol; 10μCi/μl) supplied by Amersham.
Other reagents:	Cycloheximide and Coomassie blue R-250 were supplied by Sigma. SDS (biochemical grade) was supplied by BDH. Triton-X114 was supplied by Boehringer Mannheim. Ecoscint A was supplied by National Diagnostics (Manville, New Jersey, USA). All buffer salts (Tris, MES and CAPS) supplied by Sigma.

Sources of any other materials are stated where appropriate in the text.

2.2 Yeast strains.

The strains of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* used in this study are given in Tables 2.1.A and 2.1.B respectively.

Table 2.1.A *S. cerevisiae* strains.

Name	Genotype	Source
Σ1278b	MATα *PMA1*$^{+}$ (prototrophic parent)	Ulaszewski *et al.*, 1983 (gift of Prof. A. Goffeau).
MG2129	" *pma1.1*	
MG2130	" *pma1.2*	
MG2131	" *pma1.3*	
MG2132	" *pma1.4*	
BJ2168	MATa *leu2, trp1, ura3-52, prb1-1122, pep4-3, prc1-407, gal2.*	Sorger and Pelham, 1987 (gift of Dr. H.R.B Pelham).
MW109	MATa *leu2, Δtrp1, ura3, his3, lys2.*	Werner-Washburne *et al.*, 1987 (gift of Dr. E.A. Craig).
MW123	MATα *leu2, Δtrp1, ura3, his3, lys2, (ssa1* :: HIS3) (*ssa2* :: LEU2)	

Table 2.1.B *S. pombe* strains.

Name	Genotype	Source
972	h^{-} *PMA1*$^{+}$ *ade7-413* (parent)	Ulaszewski *et al.*, 1986 (gift of Prof. A. Goffeau).
JV66	h^{-} *pma1.1 ade7-413*	

2.3 Growth media and culture conditions.

Yeast cultures were grown in rich media (YEPD) or minimal media plus glucose (MMG). The recipes for these are given below (all % values are w/v):

YEPD: 1% yeast extract,
2% bactopeptone,
2% glucose.

MMG: 0.67% yeast nitrogen base (without amino acids),
2% glucose,
plus one or more of the following auxotrophic requirements where appropriate; L-leucine (30mg/L), L-lysine (30mg/L), L-tryptophan (20mg/L), L-histidine (20mg/L) and uracil (20mg/L).

For solid media 2% bactoagar was added. Plates were left on the bench 2-3 days after pouring to dry.

Liquid media cultures were grown at 20°C with rapid agitation in conical flasks, media volume being 1/4 of the flask volume. Stocks of yeast strains were maintained at -70°C in 2xYEPD plus 15% glycerol.

2.4 Monitoring cell growth.

Yeast cell growth in liquid media was monitored by taking OD600nm readings at appropriate intervals and, where necessary, counting cells by using an improved Neubauer haemocytometer (Hawksley).

2.5 Assaying for stress tolerance.

All stress tolerance experiments were done on cells that had been in exponential growth (0.5-1x10^7 cells/ml) at 25°C in YEPD medium. Before each experiment *S. cerevisae* cultures were briefly sonicated for five seconds at 5μ, followed by five seconds rest (x5), a treatment which was just sufficient for no cell aggregates to be seen by light microscopy (a microprobe [tuned to 23KHZ] of an MSE Soniprep Ultrasonic Disintegrator was used). This step was not necessary with *Sch. pombe* cultures. The stress was then applied (2.5.1, 2.5.2 or 2.5.3) and cells were then spread on YEPD plates using dilutions designed to give 300 cells per plate. In all of the stress tolerance experiments killing was

measured from the colonies (including petites) on YEPD plates maintained 2-3 days at 28°C.

2.5.1 Assaying for thermotolerance.

Both basal thermotolerance (survival after a single step upshift to a lethal temperature) and acquired thermotolerance (survival at lethal temperatures after pre-adapting cells by incubating at a higher non-lethal temperature), were assayed. The non acute treatment used for induction of acquired thermotolerance was a rapid shift of part of each culture from 25°C to 38°C; cells being maintained at 38°C for 40 minutes prior to an upshift to 52°C for a variable period. An identical portion of each culture (the cells uninduced for thermotolerance) was immediately transferred from 25°C to 48°C for variable times. Killing of these "uninduced" cells at temperatures higher than 48°C was too rapid for accurate measurement. At intervals from 0 to 15 minutes, after the shift to 48°C or 52°C, aliquots were rapidly diluted into 5ml of YEPD (a 10x fold dilution) at room temperature (21-23°C) and the cells seeded onto YEPD plates within 20 minutes of the exposure to the high temperatures.

2.5.2 Assaying for tolerance to ultraviolet irradiation.

Cells were diluted appropriately in YEPD and 0.15ml aliquots were spread on YEPD plates. Immediately after plating, lids were removed from the petri dishes and the exposed surfaces were irradiated by an ultraviolet light source (2.6×10^5 ergs/sec/cm^2) for various times.

2.5.3. Assaying for tolerance to high ethanol and NaCl concentrations.

To cells appropriately diluted in YEPD were added absolute ethanol or 4M NaCl to give final concentrations of 12.5% (v/v) and 2.5M respectively. Aliquots were removed immediately (for zero time point) and then at subsequent intervals thereafter; these being diluted 100-1,000 fold prior to plating on YEPD plates.

2.6 Small scale pulse-labelling and protein extraction.

2.6.1 Pulse labelling.

Between 1×10^8 to 2×10^8 cells from exponential YEPD cultures maintained at 25°C were collected by centrifugation (3,000 xg, 5 minutes), resuspended in 10ml MMG

medium, recentrifuged as before, resuspended again in 10ml MMG medium and incubated for 20 minutes at 25^{0}C prior to heat shock. During this 20 minute incubation the cultures were subdivided into 1ml aliquots in glass tubes. To heat shock the cells, aliquots were shifted to the appropriate temperatures and, at the stated variable times after this upshift, were labelled by addition of either 1,000Ci/mmol L-[^{35}S]-Methionine (to 10μCi/ml) or 60Ci/mmol [^{3}H]-Leucine (to 30μCi/ml). A control aliquot was pulse labelled for 15 minutes at 25^{0}C without any heat shock. Incorporation of radiolabelled amino acid was stopped after the appropriate time by rapidly chilling the aliquots on ice, transferring them to 1.5ml eppendorfs and pelleting the cells by a 30 second spin at high speed in a microcentrifuge (ca.5,500 xg). The supernatant was discarded and the pellets rapidly frozen in dry ice and stored at -70^{0}C.

2.6.2 Extraction of total cell protein.

The labelled cell pellets were thawed on ice. Two pellet volumes of acid-washed glass beads (BDH, 40 mesh) plus sufficient protein-extraction buffer to just cover the pellet and glass beads was then added (ca. 150μl). Protein extraction buffer was: 500mM Tris-HCl pH 8.0, 2% SDS, 2mM EDTA, with the following additions made just before use: 2.5% β-mercaptoethanol, 0.5mg/ml RNAase, 1mM PMSF, ImM TPCK, 0.5mM TLCK and 2μg/ml pepstatin A (the last 4 constituents were from stocks described in section 2.9.1). After vortexing for 30 seconds followed by chilling on ice for 30 seconds (x8), glass beads and unbroken cells were pelleted by a 10 second spin in a microcentrifuge (5,500 xg). The supernatants were transferred to eppendorf tubes precooled on ice, and stored at -70^{0}C. If the samples were to be repeatedly freeze/thawed the addition of 100μl of 50% glycerol was found to limit fragmentation of proteins during the freeze/thaw process.

2.7 Measuring radioactivity incorporated into cellular protein.

Protein from a 20μl aliquot of each sample of total cell protein was precipitated by addition to 5ml 5% (w/v) TCA, 0.1% (w/v) bactopeptone. After 15 minutes on ice, precipitated protein was filtered by suction onto glass fibre filters (Whatman GF/C, 2.5cm diameter). The filters were washed once with 5ml of cold 5% TCA, 0.1% bactopeptone and twice with cold absolute ethanol, dried (120^{0}C, 15 minutes) and placed into 5ml scintillation vials containing 4ml of Ecoscint A scintillation fluid. Radioactivity precipitated onto the filters was counted by a Packard Tri-carb Liquid Scintillation Analyzer using the appropriate standards, channel, and gating settings for the appropriate radioisotope.

2.8 Protein assays.

Protein concentrations were determined by the dye-binding assay of Bradford (1976), using the Bio-Rad Protein-Assay Kit and BSA as standard. Both the standard and microassay procedures were as described in the manufacturer's instructions.

2.9 Sub-cellular fractionation.

There are several procedures by which one can isolate plasma membrane, mitochondrial and cytosolic fractions from yeast. A number of them were unsuitable for studies concerning the effects of stress on the protein composition of these fractions and for reasons detailed in section 4.2, a modified version of the procedure described by Serrano (1978) was used. All steps, including centrifugation runs, were carried out at 4°C.

2.9.1 Cell disruption.

A 1L culture was harvested by centrifugation (5 min, 5,000 xg, Sorvall GS3 rotor) and the resulting cell pellet resuspended in homogenisation buffer (HB). HB was 25mM Tris (adjusted to pH 7.0 with HCl), 6% sorbitol and 1mM EDTA, the following protease inhibitors being added just before use: 1mM PMSF and 1mM TPCK (both from 100mM stocks in ethanol), 0.5mM TLCK (added fresh) and 2μg/ml pepstatin A (from a 2.5mg/ml stock in methanol). The suspension was divided between two 50ml polycarbonate tubes and the cells were once again pelleted by centrifugation (10 min, 5,000 xg, Sorvall SS34 rotor).

To each pellet were added two volumes of acid-washed glass beads (BDH, 40 mesh), followed by a volume of HB that just covered the cells and glass beads. Cells were ruptured by vortexing on a whirlimixer for five minutes. This was just sufficient to disrupt 90% of the cells, as judged by light microscopy.

The homogenate was diluted 3x in HB and pH was adjusted from 5-6 to 7.0 with 1M NaOH. Glass beads, cell walls and non ruptured cells were removed by centrifugation (10 min, 530 xg, Sorvall SS34 rotor) and discarded. The supernatant was centrifuged for 20 min at 22,000 xg (SS34 rotor) to obtain a pellet (P1) composed of plasma membranes and mitochondria. The supernatant from this spin was then centrifuged for 1 hour at 100,000 xg (Beckmann 70 Ti rotor), to give a pellet (P2) and a final supernatant, the soluble or S100 fraction. This S100 fraction was stored at -20°C after the addition of glycerol (to 20% v/v).

2.9.2 Separation of plasma membranes and mitochondria.

The P1 pellet from 1L of yeast cells was resuspended in 2.0ml of HB by vortexing on a whirlimixer for 30 seconds. Resusupending the pellet in smaller volumes was avoided since although the plasma membranes and mitochondria formed bands during the subsequent density gradient centrifugation, they did so as solid discs which were impossible to remove from the centrifugation tubes.

Discontinuous sucrose gradients were prepared by overlaying three 4ml layers of 2.25M, 1.65M and 1.10M sucrose in 14 x 89mm Beckmann ultra-clear tubes. The sucrose solutions were prepared in HB buffer (without sorbitol). Resuspended membranes (1ml) were loaded onto each gradient, and the tubes centrifuged for 14 hours at 80,000 xg (Beckmann SW 41 Ti rotor). As discussed in section 4.3 plasma membranes and mitochondria band at the 2.25/1.65 and 1.65/1.10 interphases respectively. Both bands were collected with a pasteur pipette, diluted four times with HB, and pelleted by centrifugation for 40 minutes at 30,000xg (Beckmann 70 Ti rotor). Each pellet was resuspended by 1ml of HB and further purified by another density-gradient spin identical to the preceding one. Plasma membranes and mitochondria were collected and pelleted as before. Finally, the plasma membrane and mitochondrial pellets were resuspended in, 25mM Tris-HCl pH 7.0, 0.1mM EDTA, 50% glycerol, 1mM PMSF and 2μg/ml pepstatin A and were stored at -20°C.

2.9.3 *In vivo* protein pulse-labelling prior to sub-cellular fractionation.

To study plasma membrane and mitochondrial proteins labelled *in vivo* both before and during heat shock two 1L YEPD cultures were grown to mid-exponential phase (5 x 10^7 cells/ml) at 20°C. Cells (5x10^8,10mls) were collected from each culture by centrifugation (5 min, 3,000 xg, Sorvall SS34 rotor), resuspended in 50ml MMGa, recentrifuged and resuspended again in 100ml MMGa (MMGa; MMG plus appropriate auxotrophic requirements). Both MMGa cultures were incubated at 20°C for 20 minutes. This was followed by pulse labelling for 40 minutes at 20°C or at 10-50 minutes after a 20-40°C heat shock using either 1,000Ci/mmol L-[^{35}S]-Methionine (to 10μCi/ml) or 60Ci/mmol [^{3}H]-Leucine (to 30 μCi/ml). At the end of the 40 minute pulse-labelling period, cells maintained at 20°C were returned to the remaining 800ml of the original YEPD culture which had been maintained at the same temperature; the heat shocked cells were returned to the other YEPD culture which had been subjected to an identical heat shock treatment. This was immediately followed by sub-cellular fractionation as described in sections 2.9-2.9.2, the unlabelled cells providing extra 'carrier' membranes during the membrane purification steps.

2.9.4 Assaying the purity of plasma membrane and mitochondrial fractions.

The H^+-ATPase of the mitochondrial membrane is inhibited by sodium azide but not by orthovanadate. Conversely, the H^+-ATPase of the plasma membrane is inhibited by orthovanadate but not sodium azide (Anraku *et al.*, 1989). Yeast vacuoles also contain an ATPase the activity of which is not diminished by either inhibitor (Uchida *et al.*, 1985). For these reasons it is generally acknowledged that one of the best methods of assessing the purity of plasma membranes from yeast is to assay the proportion of their ATPase activity that is inhibited by orthovanadate (Rose and Veazey, 1988), contamination by mitochondria being determined from the proportion of the ATPase activity being inhibited by sodium azide. Other compounds which various workers use are N,N'-dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol (Des). Neither of these was included in the marker enzyme assays since DCCD inhibits all three enzymes (albeit to different extents) and diethylstilbestrol, though a potent inhibitor of plasma-membrane H^+-ATPase, also inhibits the vacuolar enzyme (Uchida *et al.*, 1985, also see Table 4.1). It should be noted that the H^+-ATPases referred to thus far (plasma membrane, mitochondrial, vacuolar) are each members of distinct classes of ion pumps, distinguishable at many levels e.g. structure, catalytic mechanism and H^+/ATP stoichiometry (see section 4.3 and Table 4.1).

ATPase activity was assayed by following the release of inorganic phosphate from ATP, as described by Serrano (1983), by a medium containing 50mM MES/Tris pH 5.7, 0.2 mM ammonium molybdate (to inhibit acid phosphatase), 10mM $MgSO_4$, 50mM KCl, 10μg of protein (from purified plasma membranes or mitochondria) and 6mM ATP. The reaction was started by addition of ATP and terminated after 10 minutes at 30°C (reaction rates were linear for at least 15 minutes). Initial assays used to determine the purity of the isolated plasma membrane and mitochondrial fractions also contained 0.1mM sodium orthovanadate or 5mM sodium azide. The following controls were run: i) without enzyme, which revealed a negligible rate of non-enzymatic ATP hydrolysis (identical to the rate seen when boiled plasma membranes or mitochondria were included); and ii) without ATP, revealing no increase in inorganic phosphate, as expected.

Results from the assays described above confirmed that the material collected from the 2.25/1.65M and 1.65/1.10M interphases on the sucrose gradients described in section 2.9.2, correspond to fractions containing plasma membranes and mitochondria, respectively (see section 4.3). Given that the sub-cellular fractionation described above (sections 2.9-2.92) yield plasma membranes of high purity, the activity of the associated H^+-ATPase was assayed by following the release of inorganic phosphate by the medium described above, in the presence of 5mM sodium azide to inhibit the low amounts of contaminating mitochondrial H^+-ATPase (Serrano, 1983).

2.10 Separation of proteins by gel electrophoresis.

2.10.1 SDS-PAGE.

Proteins were fractionated by size using the discontinuous electrophoresis system as described by Laemmli (1970). Gels were cast between two glass plates and run on a Studier-type slab-gel apparatus as described in Hames (1990). Briefly, the Laemmli system is composed of the following: (A) Stacking gel, 0.125M Tris-HCl (pH 6.8), 0.1% SDS, and 4% polyacrylamide. (B) Resolving gel, 0.375M Tris-HCl (pH 8.8), 0.1% SDS, and a polyacrylamide concentration that will give the best separation of the proteins in question. (C) Electrode buffer (pH 8.3) - 0.025M Tris, 0.192M glycine and 0.1% SDS. (D) Protein sample buffer, 0.125M Tris-HCl (pH 6.8), 10% glycerol (v/v), 5% β-mercaptoethanol (v/v), 2% SDS (w/v) and 0.013% bromophenol blue (w/v). The 30% acrylamide stock used to prepare solutions A and B was composed of 29.2% acrylamide and 0.8% bis-acrylamide. Gels were polymerised by the addition of 0.05% (v/v) of TEMED and 0.7% (w/v) of ammonium persulphate from a 10% (w/v) solution. Samples were prepared for electrophoresis by addition of an equal volume of solution D, incubation for 15 minutes at 40^{0}C, then loading into adjacent gel wells alongside protein marker samples of the appropriate size range (see section 2.1).

2.10.2 High-resolution two-dimensional electrophoresis.

This involved: i) separation of proteins according to their iso-electric points by non-equilibrium pH gradient-electrophoresis (NEPHGE) followed by ii) separation according to size using conventional SDS-PAGE (section 2.10.1), as described in O' Farrell *et al.*, (1977).

2.11 Analysis of gels following electrophoresis.

Upon completion of electrophoresis gel plates were separated and the stacking gels discarded. The resolving gels were analysed by one of the following ways:

2.11.1 Direct staining.

Abundant proteins (1μg or more) were detected by staining with Coomassie Blue R-250. Gels were incubated for 4 hours at room temperature in 0.05% (w/v) Coomassie blue, 50% (v/v) methanol, 10% (v/v) acetic acid. Destaining was by incubation at room temperature with gentle agitation in 5% (v/v) methanol and 7.5% (v/v) acetic acid, this solution being changed periodically. To visualise proteins that were present in low amounts

(10ng or more) a neutral silver-staining procedure was used (Harlow and Lane, 1988 [a]).

2.11.2 Gel autoradiography and fluorography.

Gels were dried onto Whatman 3MM paper under vacuum using a Bio-Rad Laboratories gel dryer, as described by Harlow and Lane (1988a). Dried gels were placed in direct contact with Fuji RX film in light proof cassettes for the appropriate length of time at -70^{0}C. For [^{32}P]-labelled proteins sensitivity was increased where necessary by placing a calcium tungstate intensifying screen against the side of the X-ray film not in contact with the dried gel.

Separated [^{3}H] or [^{35}S] labelled proteins were visualised by fluorography. Gels were soaked in 1M sodium salicylate (pH 5-7) for 20 minutes (Chamberlain, 1979), dried, then exposed to X-ray film as described above. Typically, 150,000 cpm of resolved total cellular-protein labelled with ^{35}S or ^{3}H could be visualised by an overnight or a 3 day exposure, respectively. X-ray film was developed according to manufacturers instructions.

2.11.3 Transfer of proteins from gels to membranes.

Separated proteins from a gel were blotted onto thin support matrices by electrophoretic transfer (Western blotting). A nylon blotting membrane was used (Genescreen) due to its superior protein-binding capacity and its physical strength. Also, nylon membranes do not change size during subsequent processing steps. The membrane was prepared for blotting according to manufacturer's instructions and electrophoretic elution was achieved by complete immersion of a gel-membrane sandwich in a buffer tank with steel-plate electrodes followed by electrophoresis at 50V, as described by Harlow and Lane (1988 [b]). After Western blotting, efficiency of transfer was determined by staining the gel with Coomassie blue. Also, prestained markers were run on gels to serve as internal markers for transfer and molecular weight.

The transfer buffer is typically of low ionic strength (25mM Tris, 150mM glycine [pH 8.3]) and contains 20% methanol to minimise swelling of the gel as blotting progresses. This buffer was found to be unsuitable for the transfer of plasma membrane proteins (see section 5.2.2). The best results were achieved by i) using a buffer with a high pH and a low concentration of methanol (35mM CAPS-NaOH pH 11, 5% methanol), and ii) blotting for 5 hours at 50 volts. However, substantial changes of blotting conditions still did not improve the very poor transfer of one particular plasma-membrane protein (see section 5.2.2).

2.11.4 Detection of specific proteins by immunostaining.

Specific proteins immobilised on nylon membranes were visualised by probing with the appropriate anti-sera followed by incubation with the various components of the BRL streptavidin marker system. The procedure described below is a combination of the methods reported by Sambrook *et al.*, (1989) and the BRL Products for Immunodetection Applications Guide. Solutions containing immunological regents or chromogenic substrate were freshly prepared. All incubations were at room temperature with gentle agitation on a platform shaker. Also, incubations of blots with primary antibody, goat anti-rabbit IgG and peroxidase were performed in heat-sealed plastic bags in a final volume of 0.1ml per cm^2 of nylon membrane. Preliminary experiments were conducted to determine the optimal amounts of primary antibody, while quantities of immunodetection reagents used were as recommended by individual product profiles.

After electrophoretic transfer (see section 2.11.3) remaining protein-binding sites on the blot were blocked by incubation for one hour in a blocking solution of 5% (w/v) non-fat dried milk in Tris buffered saline (TBS: 50mM Tris-HCl pH 7.5, 150mM NaCl), followed by incubation for 2 hours with blocking solution plus an appropriate amount of the primary antibody. The blot was then washed for 30 minutes in 3 changes of TBS. This was followed by a one hour incubation in blocking solution plus the appropriate quantity of biotinylated goat anti-rabbit IgG. Blots were then washed with TBS as before, followed by a one hour incubation with TBS plus the appropriate amount of streptavidin-horseradish peroxidase conjugate. The blots were washed with TBS as before and bound peroxidase was detected by incubation with TBS containing 0.01% H_2O_2, and 0.06% 4CN. Within 5-10 minutes the polypeptides to which the primary antibody had bound appeared blue against the white background of the blot. The stained blots were washed for 10 minutes in 3 changes of ddH_2O and stored in the dark.

2.11.4.1 Origin of antisera used in the work described in this thesis.

Polyclonal antisera were raised in rabbits as described by Harlow and Lane, (1988 [c]). Essentially, this involved subcutaneous injection of polyacrylamide gel fragments from multiple one-dimensional SDS-gel fractionations of protein from heat shocked yeast. Antisera crossreacting with both isoforms of hsp90, and, antisera crossreacting with hsp26, were prepared by L. Cheng and J.E. Coleman, respectively. The same method (carried out in the same laboratory) was used in an effort to raise antisera against hsp30 (see Chapter 5). A polyclonal antiserum raised against whole plasma membrane H+-ATPase was the gift of R. Serrano (Serrano *et al.*, 1986).

2.11.4.2 Quantifying signals on immunodecorated blots.

Densitometric traces of immunodecorated western blots were obtained by reflectance densitometry as described by Gifford *et al.* (1971) using a Joyce-Loebl Chromoscan 3 densitometer.

2.11.5 Detection of glycoconjugates.

Glycoproteins immobilised on nylon membranes were detected by their binding to the plant lectin concanavalin A. The procedure used was a combination of methods devised by Hawkes (1982) and Vai *et al.*, (1986) and is described below. All incubations were carried out at room temperature with gentle agitation on a platform shaker. Solutions containing concanavalin A, peroxidase and chromogenic substrate were all freshly prepared.

After electrophoretic transfer (see section 2.11.3) remaining protein-binding sites on the blot were blocked by incubation for two hours in solution A (composed of 2.5% (w/v) BSA in TBS). The blot was then placed in a heat-sealable plastic bag into which was introduced concanavalin A (50μg/ml) in solution A (0.1ml/cm^2 of blot). The bag was sealed, leaving as few air bubbles as possible. After a 30 minute incubation the blot was washed for 15 minutes in 3 changes of TBS followed by a 30 minute incubation with horseradish peroxidase (50μg/ml) in TBS, again in a heat-sealable plastic bag. The blot was then washed as before. Bound peroxidase was detected by the addition of 0.01% H_2O_2 and 0.06% 4CN in TBS. Within 1-5 minutes the concanavalin A binding polypeptides appeared blue against the white background of the blot.The stained blots were washed in ddH_2O and stored in the dark.

2.12 Peptide mapping by limited proteolysis.

This technique, which is especially suitable for analysis of a protein band in a gel slice, involved partial enzymatic proteolysis in the presence of SDS followed by analysis of the cleavage products by SDS-PAGE. The pattern of peptide fragments produced is characteristic of the protein substrate and the proteolytic enzyme used, and is highly reproducible. The procedure described below is modified from Cleveland *et al.* (1977).

After SDS-PAGE (section 2.10.1) using 1mm thick gels, proteins were visualised by staining with Coomassie Blue R-250. However to avoid possible acid hydrolysis, staining/destaining conditions were not the same as those described in section 2.11.1. Instead, proteins were visualised as quickly as possible by staining for 1 hour in 0.1% (w/v) Coomassie Blue R-250 in methanol : acetic acid : ddH_2O (5:1:4 v/v/v) followed by

rapid destaining (45minutes) in methanol : acetic acid : ddH_2O (5:1:4 v/v/v). Even under these mild staining conditions there was significant protein hydrolysis at room temperature (typically, the 110kDa plasma-membrane H^+-ATPase was cleaved into 3 fragments), this probably being due to the peptide bond between aspartate and proline being particularly susceptible to acid hydrolysis (Matsudaira, 1990). This problem was eliminated by carrying out these staining and destaining steps at 4°C. Bands from SDS-PAGE gels stained with Coomassie Blue were cut out with a scalpel blade and then soaked for 30 minutes with occasional swirling in 10ml of solution A: 0.125M Tris-HCl (pH 6.8), 0.1% SDS and 1mM EDTA. At this point gel slices could be stored at -20°C.

A second 1mm thick SDS gel was prepared as described in section 2.10.1 but with a stacking gel which: i) contained 1mM EDTA; and ii) that was twice as long as usual. Also, the final concentration of polyacrylamide in the resolving gel was 15%. The sample wells of this gel were filled with solution A. The bands from the first SDS gel were conveniently digested, without prior elution, by placing them in the sample wells of this second SDS gel followed by overlaying each slice with 20µl of solution A containing 10% glycerol, 0.01% bromophenol blue and an appropriate concentration of protease. Electrophoresis was performed in the normal manner with the exception that the current was turned off for 30 minutes when the bromophenol blue dye neared the bottom of the stacking gel. Bearing in mind that each gel slice contained only 1-2µg of protein, the pattern of peptide fragments generated on the second gel was visualised by silver staining. Alternatively, if the gel slices contained pulse-labelled protein the peptide fragments were visualised by autoradiography as described in section 2.11.2.

Of the 10 or so proteases that are commonly used for peptide mapping (such as trypsin and chymotrypsin), endoproteinase from *Staphylococcus aureus* V8 (protease V8) is probably one of the most ideal and was used for the experiments in Chapter 3. It has the additional advantage that the extent of proteolysis can be controlled by manipulation of the digestion buffer as well as by concentration of protease. In phosphate buffer protease V8 cleaves peptide bonds on the carboxy-terminus of either aspartate or glutamate, whereas in phosphate free buffer the enzyme acts specifically at glutamoyl bonds (Bond, 1989). Phosphate free conditions were used here to promote limited proteolysis. Protease V8 from the manufacturers was resuspended in 0.125M Tris-HCl pH 6.8 to give a final concentration of 5mg/ml (2.6 units/µl) and stored at -20°C. According to the product profile supplied by the manufacturers, 1 unit is that amount of enzyme required to cause a change in extinction coefficient at 280nm of 0.001 absorbance units/minute under conditions of the assay.

2.13 Limited N-terminal sequence analysis.

Polypeptides which were to be sequenced were prepared by blotting them onto PVDF membrane. However, several modifications to the usual SDS-PAGE and Western blotting protocols were found to be required to minimise N-terminal blocking. These were collated from a variety of published procedures (Matsudaira, 1987; Flannery *et al.*, 1989 and manufacturers instructions for use of PVDF) and are described below.

SDS-PAGE gels were cast as described in section 2.10.1 except that resolving gels were allowed to polymerise completely by letting the gel stand for 4 days at room temperature. The stacking gel was then cast and allowed to stand for 1 day (also at room temperature), prior to use. Gels were pre-electrophoresed for 2 hours at 8mA using the standard electrode buffer at the anode and standard buffer plus 50μM reduced glutathione at the cathode. The cathodic buffer was then discarded and replaced with standard buffer plus 100μM thioglycolic acid. Samples were loaded and electrophoresis was carried out as usual.

Electrotransfer to PVDF was performed using the standard Western-blotting protocol described in section 2.11.3 with the following modifications: i) PVDF was prepared for blotting by immersion in 100% methanol until the membrane became translucent and then equilibrated in transfer buffer (see section 2.11.3) for 5 minutes; and ii) after transfer, the blot was rinsed with ddH_2O and stained for 5 minutes in 0.05% (w/v) Coomassie Blue dissolved in 50% (v/v) methanol, followed by destaining for 10-15 minutes in 50% methanol.

The stained blot was rinsed in ddH_2O, air dried and stored at -20^{0}C. The appropriate band was cut from the membrane with a clean scalpel blade and trimmed to give a 2 x 4 mm segment. A limited N-terminal sequence was determined using repeated automated cycles of the Edman degradation using an Applied-Biosystems 470A Gas-Phase Protein-Sequencer equipped with an on-line phenylthiohydantoin amino acid analyser (this was carried out by Dr. Brian Coles, CRC molecular toxicology unit).

It was important to determine whether proteins that could not be sequenced were naturally N-terminally blocked or blocked due to electrophoresis or Western blotting procedures. For this reason 10μg of rat glutathione transferase 3-3 (donated by B. Coles) was treated as described above. This protein is not blocked *in vivo* and gave a high yield (>90%) of the expected N-terminal sequence.

In order to obtain limited amino-acid sequence-data from blocked proteins, peptide mapping as described in section 2.12 was scaled up. The second gel, bearing the peptide fragments, was run and blotted as described above. Peptides of low molecular weight, <20kDa, are easily washed from PVDF during sequencing reactions, so they were covalently attached to the membrane using entrapment chemistry as described by Coull *et al.*, (1991). The entrapment reactions and subsequent N-terminal sequencing were carried

out by D. Raman, Protein sequencing laboratory, ICRF.

Around 10-100pm of material is required for sequencing (corresponding to 0.5-5μg of a 50kDa protein). Hence amounts of the appropriate protein used were around 5 times this, in an attempt to overcome sample loss and partial N-terminal blocking during the procedures described above.

2.14 Phase partitioning of membrane proteins.

Integral and peripheral membrane proteins were separated by temperature-induced phase separation in Triton-X114 as described by Bordier (1981).

2.15 A general assay for plasma-membrane-associated kinase activities.

Plasma membranes were purified from yeast subjected to various conditions (see figure legends). Kinase activity associated with these membrane preparations was investigated using a modification of the procedure described by Kolarov *et al.*, (1988). Purified plasma membranes (50μg of protein) were incubated for 10 minutes at 30°C in 50mM MES-KOH (pH 6.0), 6mM $MgCl_2$ and 15μM ATP (composed of 15μM Na_2ATP mixed with 1μCi of 3,000 Ci/mmol [γ-^{32}P] ATP). The final reaction volume was 30μl. Reactions were started by the addition of ATP and stopped by adding an equal volume of Laemmli-gel protein sample-buffer. Samples were submitted to SDS-PAGE as described in section 2.10.1 and phosphorylated proteins were visualised by autoradiography as described in section 2.11.2.

2.16 A general assay for kinase activities associated with the S100 fraction.

The soluble (S100) fractions from yeast cultures subjected to various stresses (see figure legends), were isolated as described in sections 2.9-2.9.1. Endogenous ATP was removed in either of two ways:

(A) Dialysis. Visking dialysis tubing (Scientific Industries International Inc, U.K) was pre-treated as described by Findlay (1990). Aliquots of the S100 fractions were dialysed at 8°C, for the length of time indicated in figure legends, against 5 changes of a buffer composed of 10mM MES-Tris (pH 6.0), 50mM KCl, 1mM $MgCl_2$, 1mM DTT and 10% glycerol (referred to as dialysis buffer from now on). Prior to storage at -20°C, PMSF and pepstatin A were added to the dialysed samples, to final concentrations of 1mM and 2μg/ml respectively

(B) Centrifugal concentration. 2ml aliquots of the S100 fractions were concentrated to 50μl by using Centricon-3 centrifugal microconcentrators (Amicon) as described by

manufacturers instructions. To the 50μl retentate was added 900μl of dialysis buffer. This was followed by concentration and addition of dialysis buffer as described before. Addition of protease inhibitors and storage were as for the dialysed samples.

In vitro phosphorylation assays were performed by incubating 10μg (typically 5μl of S100 fraction) for various times at 30°C with 50mM MES-Tris (pH 6.0), 6mM $MgCl_2$ and 20μM ATP (composed of 20μM Na_2ATP mixed with 1 μCi of 3,000Ci/mmol [γ-^{32}P] ATP). Reactions were started and stopped as described in section 2.15 and phosphorylated proteins visualised by autoradiography as described in section 2.11.2.

Similar experiments, but with ultra-low concentrations of ATP were also carried out; this being achieved by using high specific-activity radioactive-ATP (see above) only, giving a final concentration of 10nM.

CHAPTER 3 Activity of the plasma-membrane-associated H+-ATPase is one of the determinants of stress tolerance.

3.1 Introduction

If an organism is to be moved from a cold to a warm environment the probability of its survival is vastly enhanced if the move is not made in one step but gradually. In other words providing the organism with the opportunity to adapt to a small change in temperature leads to the acquisition of additional thermal resistance. This phenomenon, referred to as acquired thermotolerance, increases the temperature range for survival. Acquired thermotolerance has been demonstrated in bacteria, yeast and mammalian cells in culture, and also in complex organisms. For instance, humans survive fevers that reach temperatures that will kill unadapted cells *in vitro*. Clearly, it is of considerable importance to understand how cells tolerate stress. Determining which of the stress inducible responses contribute towards thermotolerance along with how these phenomena are regulated may eventually enable us to control the survival of organisms exposed to stressful conditions. For example, thermotolerance has been studied extensively in mammalian cells largely because of the upsurge of interest in the use of heat as an adjunctive technique for the treatment of certain cancers. Furthermore, cells which survive these heat treatments are more resistant to certain anti-cancer drugs; this must be a consequence of the various processes induced by the initial heat shock (Hahn and Li, 1990).

The ability of cells to withstand cytotoxic agents or stressful situations is intimately affected both by physiological state and by their capacity to induce various types of protective response. An example of the latter is growth phase. In *S. cerevisiae* thermotolerance is low in cells undergoing rapid exponential growth and high in stationary phase cells (see section 1.7.1). The stress inducible responses are reviewed in Chapter 1. The most extensively studied of these is the induction of hsps (sections 1.2-1.2.7). Though a great deal is known about these responses, exactly how they contribute to stress tolerance remains poorly understood. Also, stress must influence the rates of enzyme catalysed reactions which will influence the flux of metabolic pathways. This may hasten cell death or even contribute towards stress tolerance in some cases. Work presented in this chapter suggests that the activity of a plasma-membrane-associated ion pump is one of the determinants of tolerance to high temperatures as well as other types of stress.

The *PMA1* gene, coding for the plasma-membrane-associated H^+ translocating ATPase (H^+-ATPase), was recently identified as one of the few in *S. cerevisiae* to be still transcribed efficiently after heat shock to 42^{0}C (approx. 3^{0}C above the optimum growth temperature). Polyadenylated RNA pulse labelled *in vitro* at 42^{0}C hybridises to relatively few sequences, one of them being the *PMA1* transcribed region (Curran *et al.*, 1988).

H^+-ATPase is an electrogenic H^+ pump, crucial to all fungal and plant cells. The electrochemical gradient it generates is essential for such important cellular functions as the uptake of nutrients, maintenance of K^+ levels and the regulation of intracellular pH (pH_i) (Serrano, 1988). This enzyme has been extensively characterised and the corresponding gene cloned from the yeasts *S. cerevisiae* and *Sch. pombe*, as well as from the ascomycete fungus *Neurospora crassa* (Serrano, 1988). All show extensive sequence homology as well as similar hydrophobicity profiles. The enzyme belongs to a large class of cation pumps called the P-type ATPases. All members of this class are characterised by sensitivity of enzyme activity to vanadate, formation of an aspartyl phosphate intermediate during catalysis, an M_r of the single polypeptide chain of ca. 110kDa and a catalytic mechanism involving cycling between two distinct conformational states (Perlin *et al.*, 1989). Other members of this group (also showing significant homology to the yeast enzyme) include the H^+-ATPase in the plasma membranes of higher plants (Harper *et al.*, 1989; Pardo and Serrano, 1989; Boutry *et al.*, 1989), various cation pumps found in mammals, such as the Ca^{2+}, Na^+/K^+ and H^+/K^+ ATPases, and the K^+-ATPase found in the plasma membrane of *E. coli* (Serrano, 1989). The characteristics of these P-type pumps make them readily distinguishable from the other two types of cation pump found in biological membranes, namely the F-type pumps found in the plasma membranes of eubacteria, the inner membranes of mitochondria and chloroplasts and the V-type pumps, found in endomembranous systems of eukaryote cells including the vacuoles, lysozomes, the golgi and coated vesicles (Nelson and Taiz, 1989). Table 4.1 lists the distinguishing characteristics of these pumps and illustrates their different sensitivities to various inhibitors which allows assay of each pump *in vitro*.

In *S. cerevisiae* the H^+-ATPase is the most abundant plasma-membrane protein accounting for 15-20% of total plasma-membrane protein, and consumes 40-60% of the cellular ATP (Eraso *et al.*, 1987; Serrano, 1984). Activity of the H^+-ATPase does not only take part in maintaining survival of the cell. Action of the enzyme has also been implicated as one of the signals required to turn on cell growth. A common early response of yeast (Gillies *et al.*, 1981) and cells of higher eukaryotes (Nuccitelly and Heiple, 1982) to stimuli which activate their proliferation, is an increase in pH_i. This intracellular alkalinisation is caused by activation of the Na^+/K^+ antiporter in animal cells (Perona and Serrano, 1988) and by the H^+-ATPase in fungal and plant cells (Serrano, 1989). The obvious question is whether the rise in pH_i is a coincident with activation of growth or a bona fide regulator. Expression of the yeast H^+-ATPase in fibroblasts suggests the latter, since the transformed fibroblasts exhibit an elevated pH_i and acquire tumorigenic properties (Perona and Serrano, 1988). In addition, intracellular pH changes controlled by H^+-ATPase also seem to be involved in breaking of dormancy during germination of fungal spores and plant seeds

(Serrano, 1989). A final aspect is the suggested participation of the pump in development of polarity in growing cells and organs such as roots, root hairs, pollen tubes and fungal hyphae. In all these systems polar growth correlates with a spatial concentration of pumps in non-growing parts, and leaks (involving K^+ and Ca^{2+} channels, and H^+ symports) in growing tips. This results in transcellular or transorgan ion currents (Serrano, 1989).

The crucial physiological roles of this enzyme require strict regulation. Regulation always seems to occur at the level of specific activity which can vary by as much as 10 fold under different conditions. In plants the enzyme is regulated by almost all factors that modulate physiology such as hormones, phytotoxins and light (Roldan *et al.*, 1991). In *S. cerevisiae* glucose metabolism (Serrano, 1983) and medium acidification (Eraso and Gancedo, 1987) are the best characterised factors that increase activity of H^+-ATPase. Though the molecular basis of these phenomena remain unclear, it is well established that they are independent of protein synthesis and involve stable modifications that alter various kinetic parameters of the enzyme. Control of H^+-ATPase activity will be discussed more fully at the end of Chapter 4, which not only adds heat shock and salt stress to the list of factors that influence activity of this enzyme but also shows that levels of this protein are dramatically altered during heat shock. This is the first time that large scale changes in the levels of this enzyme have been shown.

One of the effects of heat shock is the dissipation of the H^+ gradient initially established by the H-ATPase; this leads to a decrease in pH_i. This has been demonstrated in *Drosophila* (Drummond *et al.*, 1986) and *Tetrahymena* (Findly *et al.*, 1983) as well as in *S. cerevisiae* (Weitzel *et al.*, 1987). It is well established that in media with a pH lower than that of the cytoplasm passive movement of H^+ across the plasma membrane would tend to acidify the cytoplasm; in glucose fermenting cells extracellular pH can reach values as low as 1.6, whereas pH_i is not less than 6.0 (Serrano, 1984). Remarkably, yeast cells are able to maintain their pH_i between 6.0 and 7.5 when cells are resuspended in media the pH of which varies from 3.5 to 9 (Eraso and Gancedo, 1987). This pH gradient across the cell membrane -which can be as large as 4 pH units- is created and maintained mainly by the action of the H^+-ATPase. Sustaining pH_i is therefore of obvious importance, and cells expend a large proportion of cellular energy to achieve this. Within 45 minutes of a sub-lethal heat shock (23-40°C), pH_i of *S. cerevisiae* drops by 0.6 of a pH unit. A more severe heat shock (23-50°C) leads to a decrease in pH_i of 1.7 (Weitzel *et al.*, 1987). In both cases the decline in pH_i can be detected within minutes. This cytoplasmic acidification should be counteracted by action of H^+-ATPase, so it is not surprising that *PMA1*, the corresponding gene, is one of the few still transcribed after heat shock. The action of this enzyme has not previously been considered as a possible influence on the tolerances or responses of fungi and plants to heat shock.

In plants there is a good correlation between activity of the H+-ATPase and resistance to salinity (see section 7.3). Ion fluxes across membranes under such circumstances are likely to be high. The electrochemical gradient generated by H+-ATPase is believed to serve as a driving force for the transmembrane flux of other ions by uniport, H+-symport and H+-antiport mechanisms. Consequently it is not surprising that H+-ATPase activity is thought to play a role in tolerance to high salt levels. The use of a series of yeast strains that are isogenic but for mutations at the *PMA1* locus (as described below) would conclusively show whether or not H+-ATPse activity influences resistance to salinity or thermotolerance.

S. cerevisiae pma1.1, *pma1.2*, *pma1.3* and *pma1.4* along with *Sch. pombe pma1.1* were isolated as spontaneous mutants showing resistance to Dio-9, a non-specific inhibitor of the H+-ATPase (Ulaszewski *et al.*, 1983, 1986). They exhibit several similar changes in the *in vitro* properties of the enzyme which is why they were investigated in parallel in this study. These changes include i) a lower specific activity; ii) a modified K_m for MgATP; iii) strong resistance to vanadate (Ulaszewski *et al.*, 1983 for *S. cerevisiae* and Ulaszewski *et al.*, 1986 and 1987[a] for *Sch. pombe*). In addition, the H+-ATPase from the *pma1.1* mutant of *S. cerevisiae* is more resistant to thermal inactivation *in vitro* (Ulaszewski *et al.*, 1983). At the time at which the experiments reported in this chapter were performed, it was known that the *Sch. pombe pma1.1* mutation corresponded to a single amino acid substitution in the H+-ATPase, namely Gly to Asp at residue 268, within a highly conserved region of the so called transduction or phosphatase domain (Ghislain *et al.*, 1987). Similar information for the four *S. cerevisiae* mutants was not available. This chapter describes experiments whereby these mutants, along with the corresponding isogenic wild types, were used to obtain evidence as to whether H+-ATPase action influences tolerance to elevated temperatures and other forms of stress.

The H+-ATPases of: *Neurospora crassa*, *S. cerevisiae*, and *Sch. pombe* all display extensive sequence homology as well as similar hydrophobicity profiles, thought to indicate 8-10 transmembrane domains (Aaronson *et al.*, 1988). H+-ATPases from flowering plants also have upto 36% sequence homology with the fungal enzymes (Harper *et al.*, 1989; Pardo and Serrano, 1989; Boutry *et al.*, 1989). Given that this enzyme is so conserved in structure and function, should it be shown to influence stress tolerances in one organism there would be grounds to suspect that its action might also determine stress tolerance levels in diverse fungi and plants.

Results of the following experiments, which reveal that the action of H+-ATPase is indeed one of the determinants of stress tolerance, have been published (Panaretou and Piper, 1990; a copy of this article has been provided, see Appendix I).

3.2 Results.

3.2.1 Influence of the *pma1.1* mutations of *S. cerevisiae* and *Sch. pombe* on thermotolerance.

When *S. cerevisiae* or *Sch. pombe* cells in exponential growth at 25°C are placed at 48°C they rapidly lose viability. Neither organism can synthesise hsps above 42-43°C (see discussion). In both species, *pma1.1* caused higher thermotolerance levels at 48°C, as manifested by the greater survival of the mutant as compared to wild-type cells in Figs. 3.1 (a) and 3.2 (a). Similar results were obtained with *S. cerevisiae pma1.2*, *pma1.3* and *pma1.4* (data not shown). If, prior to exposure to lethal temperatures, the *S. cerevisiae* or *Sch. pombe* cultures were given a non-acute heat shock (a 25-38°C temperature upshift then a 40 minute incubation at 38°C) this effect of *pma1.1* on thermotolerance was reversed; Figs. 3.1 (b) and 3.2 (b). This effect was also demonstrated with *S. cerevisiae pma1.2*, *pma1.3*, and *pma1.4* (not shown). The non-acute heat shock to 38°C enables the induction of stress inducible responses such as hsp synthesis, associated with a marked elevation of thermotolerance (see section 1.7). In both *S. cerevisiae* and *Sch. pombe* cells induced for this heat shock response, *pma1.1* reduced viability at high temperature. Comparison of Fig. 3.1(a) with Fig. 3.1(b), and of Fig. 3.2(a) with Fig. 3.2(b), reveals that *pma1.1* reduces the increase in thermotolerance that is usually elicited by a 25-38°C upshift.

Figure 3.1 The effect of *pma1.1* on thermotolerance of *S. cerevisiae*. High temperature tolerance of the *S. cerevisiae pma1.1* mutant strain, MG2129 (o) and its wild-type parent, Σ1278b (•). Cultures were either (*a*) uninduced for thermotolerance, being shifted to 48°C from 25°C; or (*b*) had their thermotolerance elevated by prior heat shock from 25°C to 38°C for 40 minutes before being shifted to 52°C.

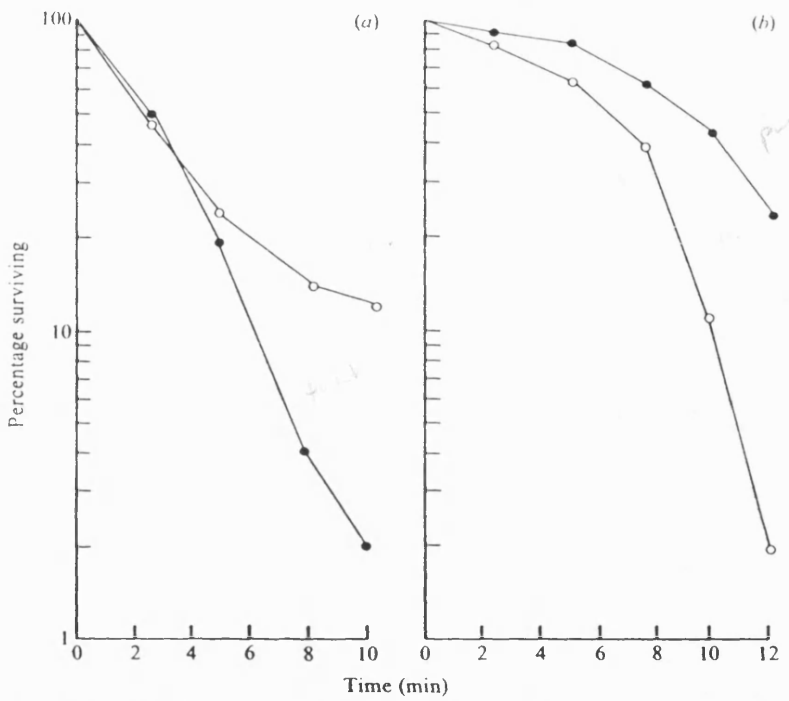
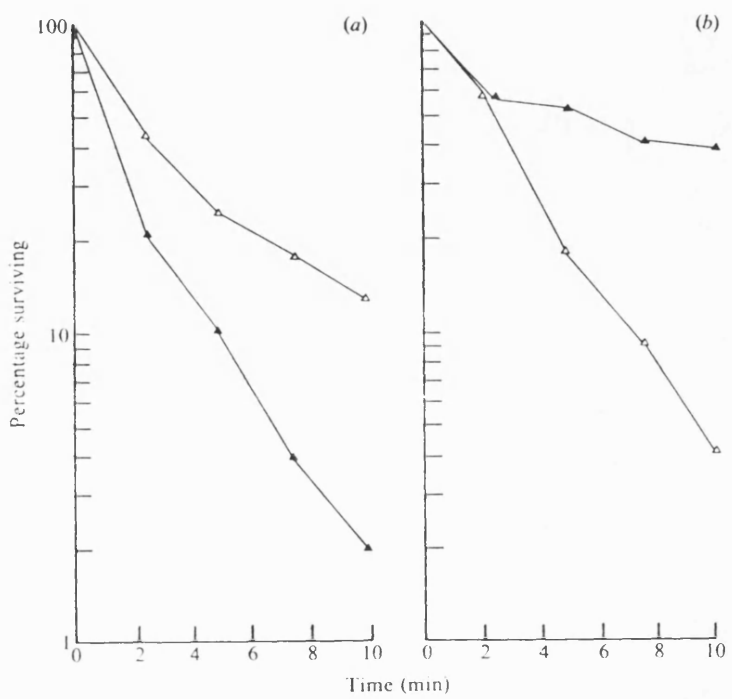


Figure 3.2 The effect of *pma1.1* on thermotolerance of *Sch. pombe*. High temperature tolerance of the *Sch. pombe pma1.1* mutant strain, JV66 (Δ) and its wild-type parent, 972 (▲). Cultures were either (*a*) uninduced for thermotolerance, being shifted to 48°C from 25°C; or (*b*) had their thermotolerance elevated by prior heat shock from 25°C to 38°C for 40 minutes before being shifted to 52°C.



3.2.2 Influence of *pma1.1* on hsp synthesis in *S. cerevisiae.*

S. cerevisiae strains MG2129 (carrying the *pma1.1* mutation) and Σ1278b (the isogenic wild type) were labelled with [^{35}S] methionine both before, and at intervals after, a 25-38^{0}C heat shock (as described in section 2.6.1). Their labelled proteins were then separated on a one-dimensional SDS gel (Fig. 3.3). After this relatively mild heat-shock, cells resumed an almost normal pattern of protein synthesis within about 1 hour (Fig. 3.3). However, synthesis of the major hsps was dramatically reduced in the *pma1.1* mutant strain. Therefore a partial suppression of the heat-shock protein induction in mutant, compared to wild type cells, is one of the probable reasons for the lowered induction of thermotolerance by 25-38^{0}C heat shock in *pma1.1 S. cerevisiae* (Fig. 3.1). Diminished induction of other poorly-understood heat-inducible responses (see sections 1.8-1.8.4) may also be involved. In other words, wild type cells were better equipped to survive the subsequent lethal heat shock.

When this labelling was repeated under conditions of considerably more severe (25-42^{0}C) heat-shock a rather different result was obtained (Fig. 3.4). The temperature is about 3^{0}C above the maximum for growth, and close to the maximum temperature at which *S. cerevisiae* will still display synthesis of hsps (Piper *et al.*, 1986, 1988). Normally, after cells are shifted to 42^{0}C, an initial burst of hsp synthesis is followed by a progressive cessation of all protein synthesis over about 1 hour. This is apparent from the labelling of the wild type Σ1278b strain in Fig. 3.3. Hsp synthesis in the *pma1.1* mutant at 42^{0}C, although initially similar to that of the wild type cells, was not subject to such rapid inhibition and was sustained for at least 70 minutes at 42^{0}C (Fig. 3.4).

These experiments indicate that, depending on the severity of the heat shock, *pma1.1* can affect either the extent or duration of heat shock protein synthesis in *S. cerevisiae.* They were not repeated on *Sch. pombe* strains 972 (the *pma1.1* mutant) and JV66 (the isogenic wild type) because growth in these strains in minimal medium is severely restricted by their adenine auxotrophy, even in the presence of exogenous adenine (Ulaszewski *et al.*, 1987 [a]).

Figure 3.3 The suppression of hsp synthesis at 38°C due to *pma1.1*. Proteins pulse-labelled in heat-shocked cells of *S. cerevisiae* Σ1278b and *pma1.1* mutant MG2129 before and after heat shock to 38°C. Cells were pulse labelled with [^{35}S] methionine 10-25 minutes (2), 25-40 minutes (3), 40-55 minutes (4), or 55-70 minutes (5) after temperature upshift. Proteins were separated on 12.5% gels and visualised by autoradiography. Track 1 shows proteins labelled in unstressed cells at 25°C, during a 15 minute incubation with [^{35}S] methionine. All gel samples contained protein from the same number of cells so the relative intensity of the bands on the autoradiographs indicates relative protein labelling under these conditions. Major hsps are indicated on the right.

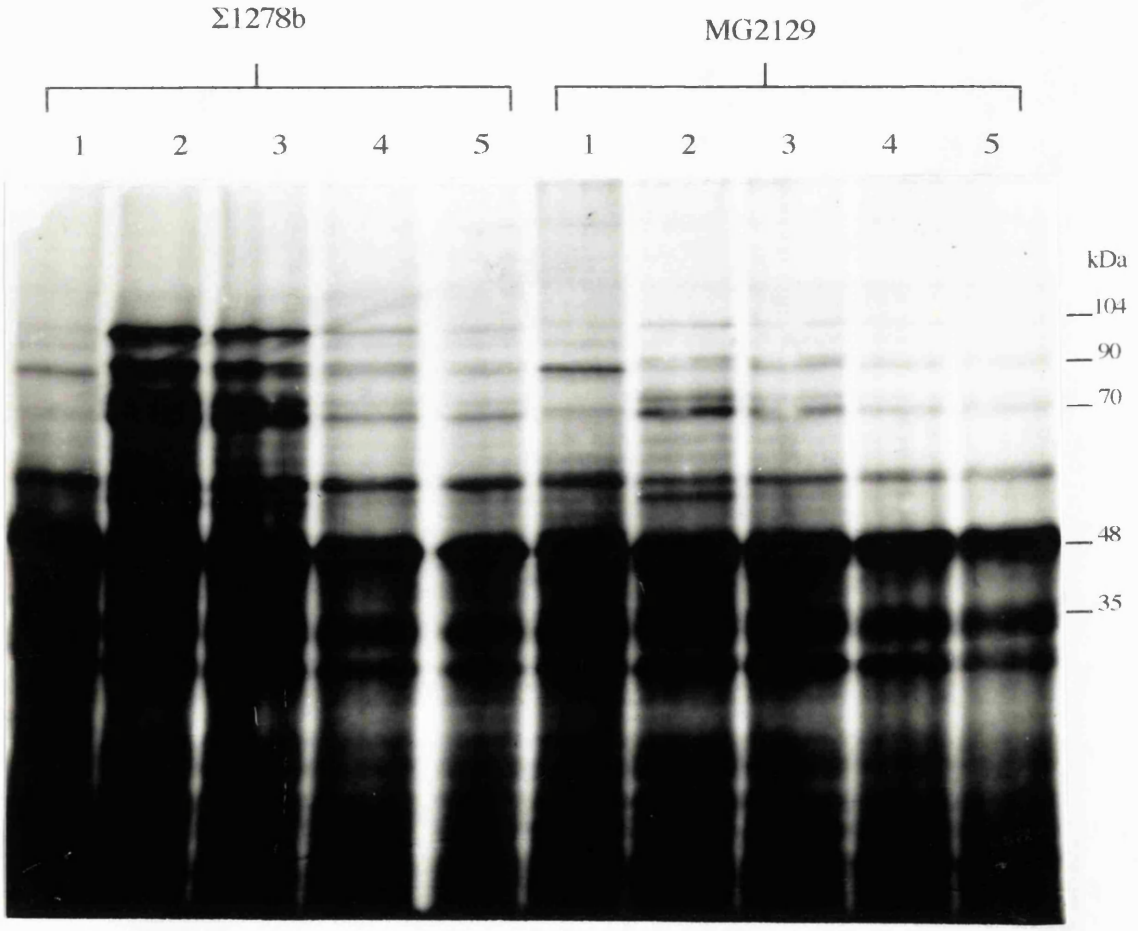
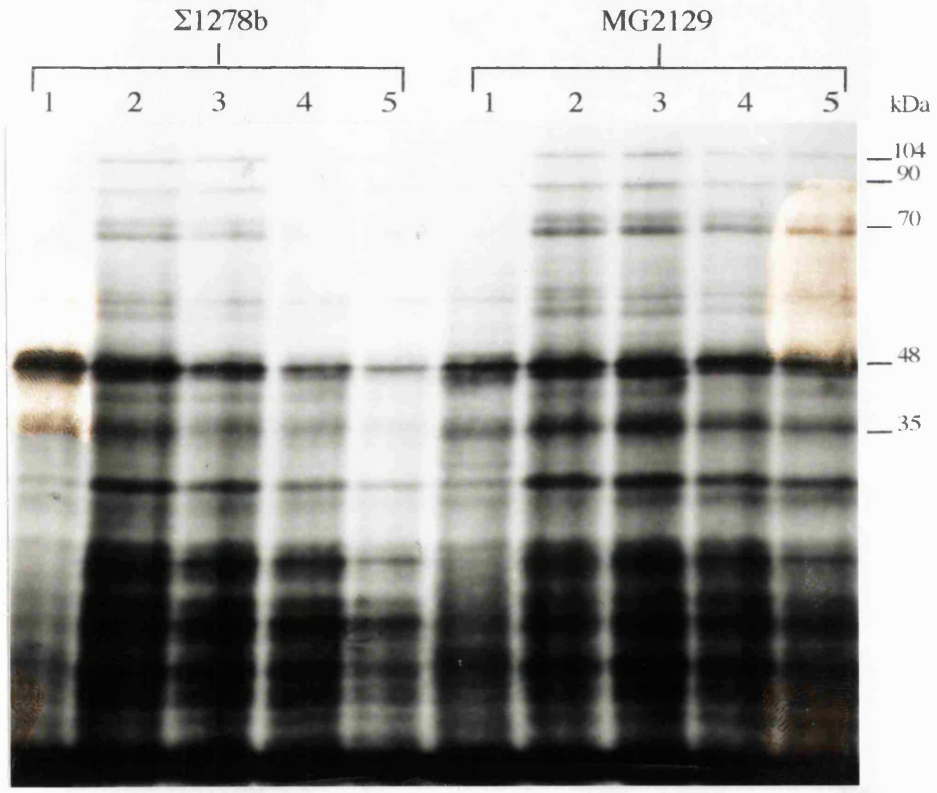


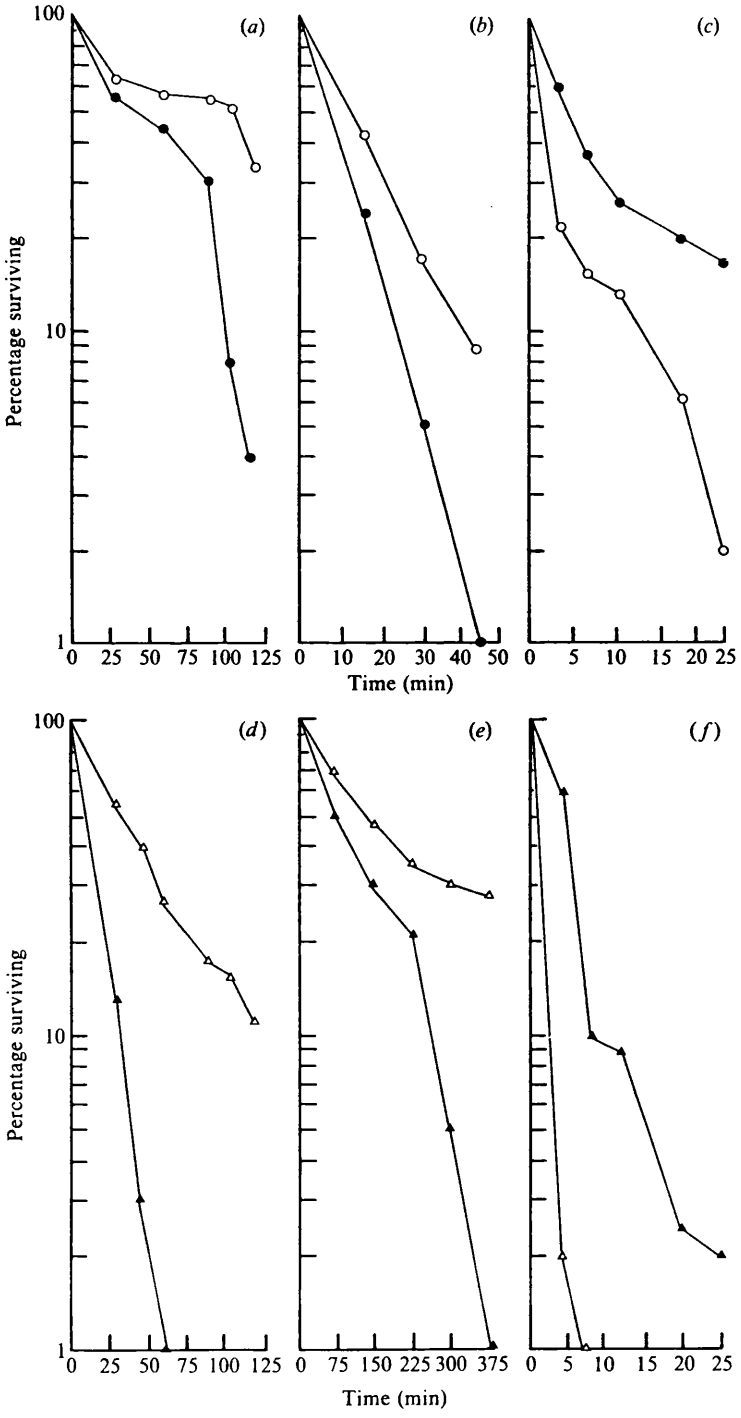
Figure 3.4 The effect of *pma1.1* on hsp synthesis at 42°C. Proteins pulse-labelled in heat-shocked cells of *S. cerevisiae* Σ1278b and *pma1.1* mutant MG2129 before and after heat shock to 42°C. Cells were pulse labelled with [^{35}S] methionine 10-25 minutes (2), 25-40 minutes (3), 40-55 minutes (4), or 55-70 minutes (5) after temperature upshift. Proteins were separated on 12.5% gels and visualised by autoradiography. Track 1 shows proteins labelled in unstressed cells at 25°C, during a 15 minute incubation with [^{35}S] methionine. All gel samples contained protein from the same number of cells so the relative intensity of the bands on the autoradiographs indicates relative protein labelling under these conditions. Major hsps are indicated on the right.



3.2.3 Influence of *pma1.1* on ethanol tolerance, osmotolerance and tolerance to ultraviolet irradiation.

The *pma1.1* mutations of *S. cerevisiae* and *Sch. pombe* increased tolerance of brief exposures to high ethanol and high salt concentrations, and decreased tolerance to ultraviolet irradiation (Fig. 3.5). Similar results were obtained with *S. cerevisiae pma1.2*, *pma1.3* and *pma1.4* (not shown). Unstressed cells initially in exponential growth at 25^{0}C growth on YEPD were used for these tolerance experiments (see section 2.5).

Figure 3.5 The effect of *pma1.1* on tolerance to lethal concentrations of ethanol, NaCl and UV irradiation. Survival of yeast exposed to (*a*,*d*) 12.5% (v/v) ethanol; (*b*,*e*) 2.5 M NaCl; and (*c*,*f*) ultraviolet irradiation (2.6 x 10^5 ergs/sec/cm^2). (*a-c*) Survival data for *S. cerevisiae* Σ1278b (●) and *pma1.1* mutant MG2129 (o); (*d-f*) data for *Sch. pombe* 972 (▲) and *pma1.1* mutant JV66 (Δ).



3.3 Discussion.

PMA1, the gene coding for H+-ATPase, is one of the few genes still transcribed efficiently after heat shock in *S. cerevisiae*. One of the consequences of heat shock, namely the drop in pH_i, might be counteracted by action of this enzyme. This prompted the investigation of the possibility that H+-ATPase action influences stress tolerance.

Inhibitors would have proved invaluable in such a study. However, in the past, the study of energetic and regulatory functions of the H+-ATPase has been complicated by the lack of inhibitors specific enough for *in vivo* experiments. The most effective inhibitors are vanadate, diethylstilbestrol and dicyclohexylcarbodiimide (Serrano, 1984). All of them have side effects on whole cells. Vanadate inhibits acid and alkaline phosphatases (Gallagher and Leonard, 1982 and Bowman and Bowman, 1982, respectively), diethylstilbestrol inhibits and uncouples respiration (Balke and Hodges, 1977) and also inhibits the vacuolar H+-ATPase (Uchida *et al.*, 1985), and dicyclohexylcarbodiimide acts on mitochondrial, vacuolar and plasma membrane H+-ATPases, albeit to different extents (Rose and Veazey, 1988). This has been overcome by molecular genetic techniques which enables manipulation of enzyme activity (per cell) by introduction of extra copies of the gene, or control of gene expression by antisense methodologies. In yeast, due to the high frequency of homologous recombination, there is the additional aspect of deleting the gene of interest or replacing it with an *in vitro* mutagenised copy. The latter has enabled dissection of functional and regulatory domains of H+-ATPase (Portillo and Serrano, 1988; Portillo and Serrano, 1989; Portillo *et al.*, 1991).

Deleting the *PMA1* gene is lethal (Serrano *et al.*, 1986), and overexpressing (Eraso *et al.*, 1987) or underexpressing the gene (Vallejo and Serrano, 1989) is toxic to the cell; which explains why, *in vivo*, H+-ATPase is solely regulated by mechanisms based on modulation of enzyme activity rather than alteration of gene expression. To determine whether action of the enzyme was a determinant of stress tolerance, mutations at the *PMA1* locus which altered activity (and not expression) of H+-ATPase were used (Ulaszewski *et al.*, 1983, 1986).

H+-ATPase mutants, isolated as spontaneous mutations showing resistance to the aminoglycoside antibiotic Dio-9, were used to obtain evidence as to whether action of this enzyme exerts a major influence on tolerances to elevated temperatures and other forms of stress. The fact that mutation to Dio-9 resistance causes identical changes to several tolerances in two distantly-related species (Figs 3.1, 3.2 and 3.5) shows that the profound metabolic changes that ensue from alteration to H+-ATPase activity are an important determinant of these tolerance levels. Also, protein pulse-labelling in *S. cerevisiae* indicated that *pma1.1* can affect the extent and duration of at least one heat inducible response, namely hsp synthesis (Figs. 3.3 and 3.4). Since the *pma1* mutants were selected

as spontaneous mutants and not using mutagenesis, it is most unlikely that mutations in genes other than *PMA1* caused the differences in i) catalytic properties of H+-ATPase (Ulaszewski *et al.*, 1983, 1986) or ii) stress tolerance (shown here). Also Ulaszewski *et al.*, (1987 [b]) have shown that a 5kb fragment carrying the wild type H+-ATPase structural-gene restores normal drug sensitivity of a *pma1* mutant after integrative transformation. Furthermore, Dio-9 resistance leads to similar changes to tolerance levels in both *S. cerevisiae* and *Sch. pombe*. This is compelling evidence that any difference shown by these mutants are due to mutations at the *PMA1* locus. Also, it should be noted that *PMA1* is not the only H+ transporter of the fungal cell membrane. Schlesser *et al.*, (1988) have cloned and sequenced a closely related gene (*PMA2*). However there is little doubt that *PMA1* encodes the primary H+-ATPase of *S. cerevisiae*, given the pronounced phenotypes of *pma1* mutants (Ulaszewski *et al.*, 1983, 1986) and the demonstration that disruption of *PMA1* is lethal (Serrano *et al.*, 1986).

When *S. cerevisiae* or *Sch. pombe* cells in active growth at 25°C are shifted to 48°C there is a rapid arrest of protein synthesis. No hsps are synthesised (pulse-labelling studies have shown that *S. cerevisiae* cannot synthesise hsps above 42-43°C [Piper *et al.*, 1986]). In addition it is most likely that other heat-inducible responses are only weakly induced, if at all. These cells, uninduced for thermotolerance, quickly die. At 48°C *pma1.1* conferred higher thermotolerance (Figs. 3.1 (a) and 3.2 (a)). This result was not entirely unexpected for *S. cerevisiae*, since *pma1.1* causes slightly slower growth of prototrophic strains on YEPD, and thermotolerance levels are generally thought to be inversely related to the growth rate of this organism (see section 1.7.1). Overall the importance of heat inducible responses must be limited in this case since the cells were not given the opportunity to adapt to higher temperatures. In these circumstances factors intrinsic to the cell will be of more importance. An obvious candidate for such a factor is the thermal stability of he H+-ATPase. *In vitro* the mutant enzyme is more resistant to heat inactivation; at 50°C the inactivation time of 50% of H+-ATPase activity is more than twice that for wild type enzyme (Ulaszewski *et al.*, 1983). If this is reflected at high temperatures *in vivo* then it seems likely that rates of H+ efflux in the mutant will be higher (the opposite of the situation at 25°C). Accordingly, mutant cells would be more efficient at limiting the drop in pH_i that occurs during heat shock. Given that a 23-50°C upshift causes a drop in pH_i of 1.7 (Weitzel *et al.*, 1987) and that sustaining pH_i is of obvious importance to the cell, the changes in pH_i caused by heat shock and the rate at which they are limited could be a factor that influences stress tolerance. It would be interesting to determine whether pH_i changes are indeed lower in the mutant cell after a 25-48°C upshift. The method of choice for determining pH_i involves studying the distribution of a non-metabolisable weak acid across the cell membrane (Pena *et al.*, 1982). However this is unsuitable in the case of heat shock

as weak acids need several minutes to attain a steady state distribution and are hence unsuitable for cells in metabolic change. Effects of heat shock on pH_i are invariably studied by [^{31}P] NMR, using the pH-dependent chemical shifts of the intracellular inorganic and sugar phosphates (Busa and Nuccitelli, 1984). but this method cannot determine pH values lower than pH 6.

Figs. 3.1 (b) and 3.2 (b) show survival of the same *S. cerevisiae* and *Sch. pombe* cultures after they had been shifted from 25°C to 38 °C for 40 minutes and then exposed to a considerably higher temperature (52°C). This non-acute 38°C heat shock induces the synthesis of hsps (among other responses), and causes a marked elevation of thermotolerance (for evidence of this in *S. cerevisiae* see Piper *et al.*, 1986, 1987, 1988). However the increase in this thermotolerance was less for *pma1.1* than for wild type cells given the same heat shock, in both *S. cerevisiae* and *Sch. pombe*. As a result, *pma1.1* cells were killed more rapidly in such cultures induced for thermotolerance, the converse of the situation found in the uninduced cultures. Both this observation and the reduction in hsp synthesis (Fig 3.3) are consistent with *pma1.1* causing a partial suppression of the heat shock response normally caused by a 25-38°C upshift. Other heat inducible responses may also have been partially suppressed. Consequently, wild type cells were better equipped to survive the subsequent upshift to a lethal temperature. This could be tested by repeating the experiment in the presence of cycloheximide which will eliminate the contribution of hsp synthesis. However, other heat inducible responses, such as the dramatic elevation of trehalose levels (section 1.8.2), would be unaffected since they are protein synthesis independent.

The *pma1.1* mutants of *S. cerevisiae* and *Sch. pombe* exhibit greater tolerances of ethanol and high salt than the wild type; the opposite being true in the case of tolerance to UV irradiation (Fig 3.5). Low cyclic AMP levels in *S. cerevisiae* also cause higher ethanol tolerance (Iida, 1988). Ethanol diffuses rapidly into *S. cerevisiae* cells (Guijarro and Lagunas, 1984) and effects membrane order, as shown by Walker-Caprioglio and Parks (1987), using the lipophilic probe 1,6-diphenyl-1,3,5 hexatriene. This change in membrane order causes enhanced H^+ influx (Leao and VanUden, 1985), and ethanol therefore has marked effects on the maintenance of pH_i and H^+-coupled transport of solutes across the plasma membrane. It has since been shown that ethanol, in concentrations that affect growth and fermentation rates (3 to 10% v/v), activate the H^+-ATPase *in vivo*; the maximal activation being 3x the basal activity (Rosa and Sa-Correia, 1991). Furthermore, a correlation has been established between H^+-ATPase activity in cells grown with ethanol and their ethanol tolerance (Rosa and Sa-Correia, 1992). Finally, ethanol also causes a slow induction of thermotolerance (Coote *et al.*, 1991).

In contrast to ethanol tolerance, osmotolerance may primarily reflect intracellular levels of osmoregulatory solutes. Fungi that grow in media of high osmotic strength

synthesise polyols (e.g glycerol, arabitol) at high levels; these increase osmotolerance by functioning as compatible solutes. Their levels change with growth state and are thought to be major determinants of osmoregulation. Glycerol, probably the most important osmotic effector in exponentially-growing salt-stressed yeast (Reed *et al.*, 1987), is made at higher levels in yeast exposed to hypertonic media (Maiorella *et al.*, 1984). Another school of thought maintains the most important component of halotolerance is the one dealing with the avoidance of the toxicity promoted by the Na^+ ion in the cytoplasm, rather than the osmotic problem (see section 4.5.3). Efflux of Na^+ could be ultimately controlled by the electrochemical H^+ gradient which is itself created and maintained by the H^+-ATPase.

In the case of *S. cerevisiae* four *pma1* mutants were used in the stress tolerance experiments described above. All four mutants responded identically, i.e. exhibiting the same differences in tolerance relative to the isogenic wild type, and no differences with respect to each other. For this reason only data pertaining to *pma1.1* were presented. Not surprisingly it has since been found that the four independently-isolated *pma1* genes contain the same base-pair modification that results in amino acid substitution of Ala608 by Thr in the nucleotide binding domain (Van Dyck *et al.*, 1990). The *Sch. pombe pma1.1* mutation corresponds to a single amino-acid substitution in the H^+-ATPase, namely Gly to Asp at residue 268, within a highly conserved region of the so called transduction or phosphatase domain (Ghislain *et al.*, 1987).

The importance of H^+-ATPase in heat shock has since been confirmed in an independent study by Coote *et al.*, (1991). Furthermore this group showed that factors which perturb pH_i such as sorbic acid and ethanol induce thermotolerance; this acquisition of thermotolerance being independent of hsp synthesis.

The rate of H^+ efflux from yeast cells increases by around 50% at sub-lethal temperatures that induce thermotolerance. This suggests activation of the enzyme per se or an increase in its levels (Coote *et al.*, 1991). The latter seems likely since the *PMA1* gene is one of the few still transcribed efficiently after heat shock. However, translation of the *PMA1* mRNA and insertion of the resulting polypeptide into the cell membrane during heat shock has not been demonstrated. This can be investigated by following the changes in the protein composition of the plasma membrane caused by stress. In addition changes in activity of this enzyme can be followed by *in vitro* assays. Both of these aspects are dealt with in Chapter 4.

The reduction in H^+-ATPase activity due to *pma1.1* causes a slight reduction in growth rate in rich medium and a considerable reduction in growth on minimal medium containing ammonia as nitrogen source. Amino acid and purine auxotrophies reduce the growth rate still further (Ulaszewski *et al.*, 1987 [a]). Physiological changes due to adaptation of *pma1.1* cells to slower growth presumably cause some of the tolerance effects observed, but may not be the only cause of the observed differences. Thus *pma1.1* might

even allow higher H^+-ATPase activity under high ethanol or dehydrating conditions, just as it renders this enzyme less susceptible to both vanadate inhibition and thermal inactivation (Ulaszewski *et al.*, 1983). The available evidence highlights the need to relate tolerance measurements to other physiological studies (e.g. those measuring pH_i shifts under stress conditions) and *in vitro* studies of H^+-ATPase action. However, the results with *pma1.1* mutant strains described here show that the H^+-ATPase action cannot be ignored as a factor affecting tolerances to a variety of potentially cytotoxic agents (Figs. 3.1, 3.2, and 3.5) and as a determinant of the extent and duration of at least one inducible stress response (Fig 3.3).

3. 4 Limited N-terminal sequence from a fragment of hsp104.

3.4.1 Generation of a peptide map of hsp104.

PMA1, encoding the plasma membrane associated H+-ATPase, is one of the few genes still transcribed after heat shock (Curran *et al.*, 1988). This had been demonstrated in earlier work by M.W Davies as follows:

1. A λ recombinant library of yeast genomic-DNA sequences was screened for those hybridising strongly to transcripts still made by yeast cells at 42°C.
2. Recombinants isolated were annealed to known heat-shock gene clones.
3. By elimination, one sequence was obtained that had not been previously identified as a heat shock sequence.
4. A small part of the transcibed region was sequenced and found to be identical to a region of the *PMA1* gene.

At the time at which this work was carried out all of the genes coding for the major hsps had been cloned apart from that coding for hsp104. The above screen yielded only one heat inducible sequence that had not been previously characterised, suggesting that the H+-ATPase and hsp104 were encoded by the same gene. Consistent with this, the apparent molecular masses of H+-ATPase and hsp104 are similar. Though H+-ATPase migrates as a slightly diffuse band of ca. 110-120 kDa relative to size markers on low percentage SDS gels (i.e ca. 6,000 kD higher than hsp104) the discrepancy may have been due to a post translational modification of H+-ATPase. It has since been shown that H+-ATPase is subject to limited glycosylation (see Fig. 5.4 and Serrano *et al.*, 1991).

On the other hand, it is well established that levels of H+-ATPase are very tightly controlled, attempts to overexpress or limit expression of the *PMA1* gene invariably having a detrimental effect on the cell (Eraso *et al.*, 1987; Vallejo and Serrano, 1989). During a 25°C-40°C heat shock hsp104 accumulates to high levels (see Fig. 3.6.A lane 3). Furthermore, newly synthesised H+-ATPase has catalytic activity prior to its arrival at the plasma membrane (Chang and Slayman, 1990). This enzyme consumes 40-60% of all ATP synthesised by the cell (Serrano, 1984). Consequently, dramatic overexpression of the corresponding gene would surely compromise the energy charge of the cell. This suggests that hsp104 and H+-ATPase are unrelated.

To determine whether there was any similarity between these two proteins, peptide maps were produced by limited proteolytic digests followed by comparison of the 'fingerprints' thus generated. Degree of similarity can be calculated using a formula described by Calvert and Gratzer (1978) which takes into account the total number of

peptides plus the number of peptides of equal molecular weight.

In addition to this, if the two proteins are unrelated then a partial N-terminal sequence from one of the peptides of an hsp104 digest would enable construction of a degenerate oligonucleotide, which could be used to clone the corresponding gene by genomic library screening.

Both H^+-ATPase and hsp104 were isolated from cells in exponential phase, growing at 20^{0}C. The H^+-ATPase was obtained from purified plasma membranes which were prepared by density gradient centrifugation as described in sections 2.9-2.9.2. Hsp104 was obtained from the cytosolic (S100) fractions, isolated as described in section 2.9.1, from cells subjected to a 40^{0}C heat shock for 40 minutes. Prior to digestion with protease V8 both H^+-ATPase and hsp104 were to be excised from SDS-PAGE gels. Low percentage (7%) gels were used to give the best possible resolution. A number of steps were taken to ensure that i) the correct proteins were excised and that ii) there were no proteins co-migrating with the proteins of interest (which would give mixed peptide maps). The latter being particularly important in the case of hsp104 since, unlike the H^+-ATPase, it was being isolated from a relatively crude fraction.

H^+-ATPase was easily located on gels since it is well established that it has an M_r of ca.110kDa and is the most abundant protein in the yeast plasma membrane (this is apparent in Fig. 3.6 A lane 1 and Fig. 4.2 lane A). In addition, this protein strongly cross-reacted with anti-H^+-ATPase anti-sera (4.2 lane A*).

The precautions listed below were taken to ensure that the bands excised from gels on which cytosolic fractions from heat shocked cells had been resolved (see Fig. 3.6.A lane 3) contained hsp104 only:

1. S100 extracts from unstressed and heat shocked yeast were run alongside each other on the preparative gels (Fig. 3.6.A). hsp104 was abundant in the heat shock fraction (as visualised by staining with Coomassie blue). There were no bands at the corresponding position in the unstressed fraction. Therefore, it was unlikely that the band corresponding to hsp104 in the heat shock fraction contained another protein which co-migrated with hsp104.

2. To confirm this one could take advantage of the fact that during heat shock only hsps incorporate radiolabelled amino acids. Proteins synthesised during non-stressful conditions only would be poorly labelled. Fig. 3.7.B shows a peptide map of hsp104 excised from a gel on which pulse labelled proteins from heat shocked cells had been resolved. The resulting peptide map (visualised by autoradiography) is identical to that in Fig. 3.6 B, generated by locating hsp104 using Coomassie blue and visualising the resultant peptide map by silver staining.

3. The above assumes that there is only one hsp with a molecular weight of

104 kDa. This was confirmed by two-dimensional electrophoresis (Figure 3.7.C) which displayed only one form of this protein (unlike hsp70 which was resolved into several isoforms).

Both H^+-ATPase and hsp104 were excised from a 7% gel like that shown in Fig. 3.6.A and were partially digested by protease V8 as described in section 2.12. The fragments were run on a 15% SDS-PAGE gel and visualised by silver staining. Figure 3.6 B shows that the polypeptides give completely different peptide profiles and are hence unrelated. Figure 3.7.B (hsc/hsp 90 tracks) is a positive control, showing the virtually identical 'fingerprints' that would be expected from digests of two proteins that are highly homologous at the amino acid level (97% in this case; Borkovich *et al.*, 1989).

Figure 3.6 Comparison of the H+-ATPase and hsp104 polypeptides of *S. cerevisiae* by peptide mapping.

A. Isolating H+-ATPase and hsp104. Sub-cellular fractionation was carried out on cultures of the auxotrophic strain Σ1278b which had been grown to exponential phase at 20°C (as described in sections 2.9-2.9.2). Proteins of the plasma membrane fraction (**1**) and the post-ribosomal supernatant fractions from unstressed cells (**2**) and from cells subjected to a 40 minute 20-40°C heat shock (**3**), were separated on a 7% gel and visualised by staining with Coomassie blue. (**1**) was loaded with 100μg of protein, (**2**) and (**3**) were loaded with 60μg of protein. (**M**) molecular mass markers, sizes of which are indicated on the left. Positions of H+-ATPase and hsp104 are indicated on the left and right, respectively.

B. Peptide maps of H+-ATPase and hsp104*. Both proteins were excised from the gel shown in **A**. The gel slices were applied to a second SDS gel (15% acrylamide) in the prescence of *Staphylococcus aureus* V8 protease, as described in section 2.12. Peptide 'fingerprints' generated were visualised by silver staining. **4-6**, peptide maps of H+-ATPase ; **7-9**, peptide maps of hsp104. **4&7**, **5&8**, **6&9** are peptide maps produced by digestion with 20ng, 50ng and 500ng of protease@ respectively. (**10**), 50ng of protease only♦.

C. Negative control. Gel slices not treated with protease were not included in peptide mapping gels since it was found that proteins within them were susceptible to digestion once they had entered the gels. For this reason H+-ATPase (**11**) and hsp104 (**12**) excised from a gel like that in **A** were electrophoresed into a separate 15% gel. Subsequent staining with Coomassie blue showed that the proteins are intact and therefore were not fragmented by the various steps involved in isolating them.

* Each lane in B represents a limited digest of approximately equal amounts of protein. Lanes 4, 5 and 6 were each loaded with 1 gel slice containing H+- ATPase whereas lanes 7, 8 and 9 were each loaded with 3 gel slices containing hsp104 (excised from gels identical to that shown in **A**).

@ 522 units/mg (manufacturers define one unit as the amount of enzyme required to cause a change in extinction at 280nm, of 0.001 absorbance units /minute at pH 7.8 if casein is the substrate).

♦The M_r of protease V8 is 11.4kDa (Drapeau *et al.*, 1972). The protein is not visualised on this gel since polypeptides with an M_r <14 kDa are not resolved by conventional SDS-PAGE (Hames, 1990).

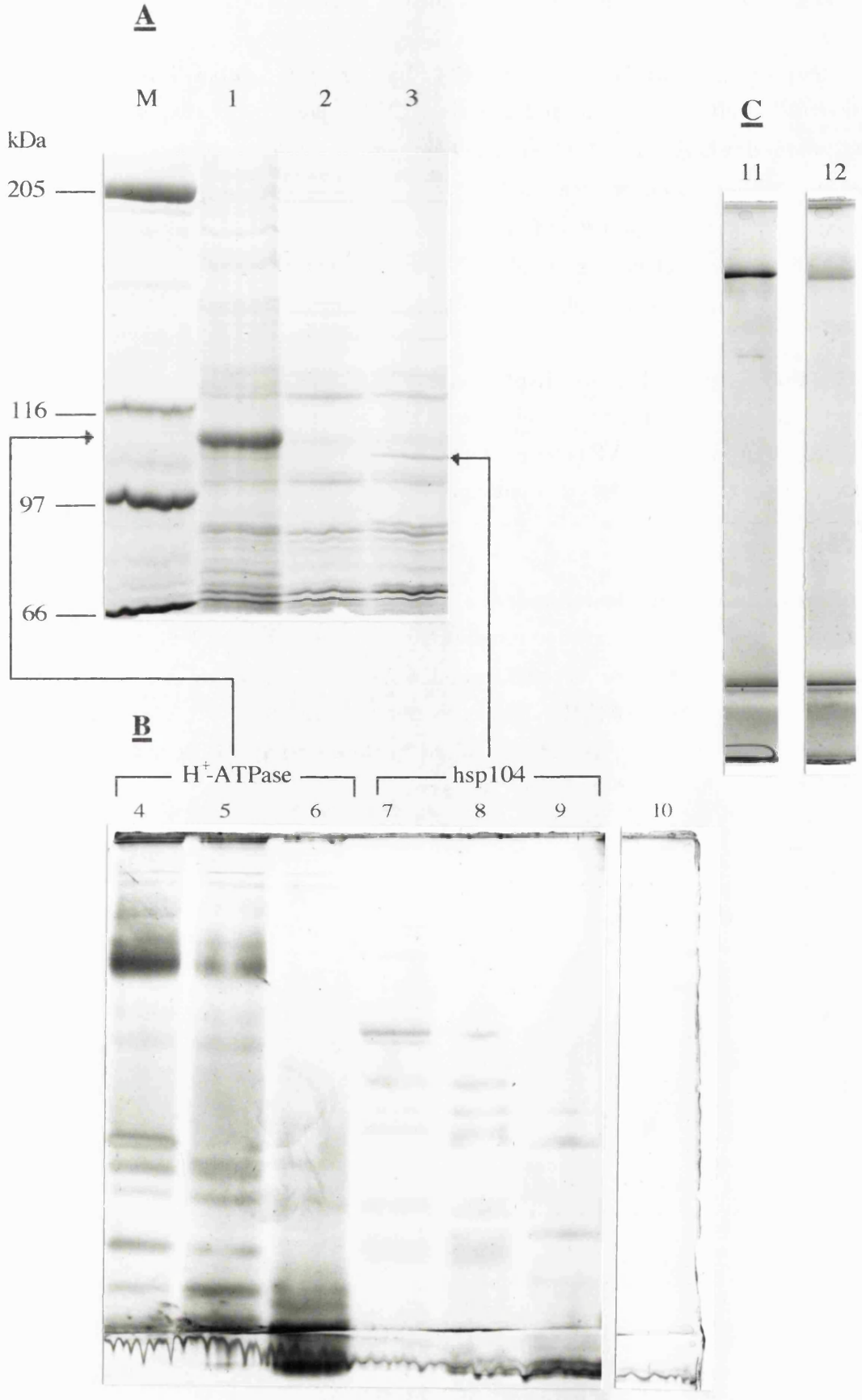
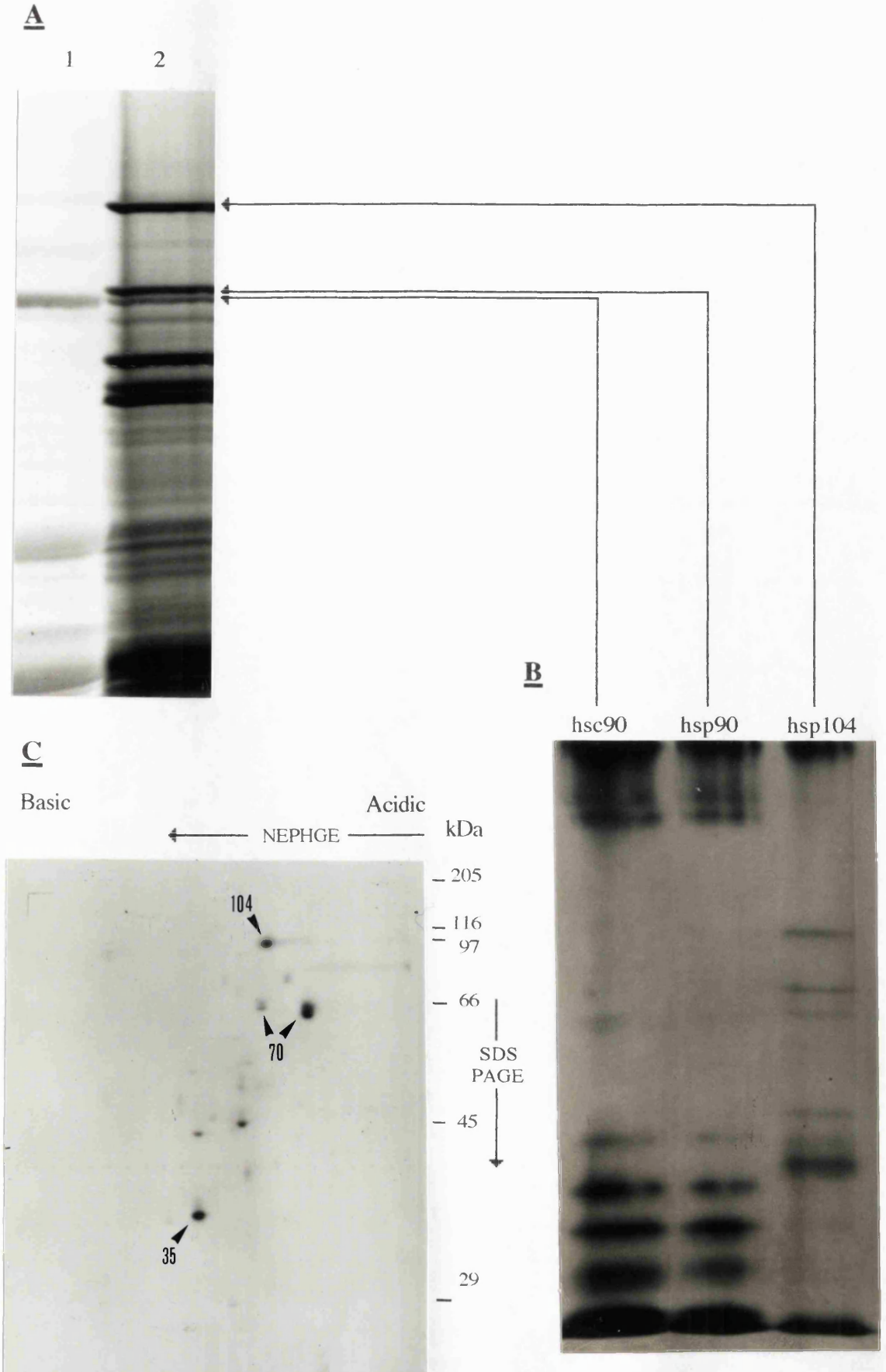

A
M
1
2
3
kDa
205
116
97
66
C
11
12
B
H+-ATPase
hsp104
4
5
6
7
8
9
10

Figure 3.7 Bands excised from gels on which soluble fractions from heat shocked cells have been separated contain hsp104 only.

A. Preparing radiolabelled hsp104 by *in vivo* pulse labelling of *S. cerevisiae*. Cells of the auxotrophic strain Σ1278b were grown to exponential phase at 20°C and pulse labelled with [^{35}S]-methionine either for 40 minutes at 20°C or 10-50 minutes after a temperature upshift to 40°C (as described in section 2.6). Labelled proteins of the post-ribosomal supernatant from the unstressed (**1**) and heat shocked (**2**) cells were separated on a 10% gel and visualised by fluorography. Both lanes were loaded with 5x10^5 cpm of acid precipitable material.

B. Peptide maps of hsp104, hsp90 and hsc90. All three proteins were excised from the gel shown in **A**. The gel slices were applied to a second gel (15%) in the presence of *Staphlococcus aureus* V8 protease as described in section 2.12. Peptide 'fingerprints' generated were visualised by fluorography. Each protein was digested with 50ng of protease.

C. Two-dimensional electrophoresis showing the existence of only one hsp with an M_r 104kDa. Labelled proteins from the post-ribosomal supernatant of heat shocked cells (from the same preparation used in **A**) were separated by non-equilibrium pH gradient electrophoresis (**NEPHGE**) as described in section 2.10.2, followed by SDS-PAGE on a 15% gel. Proteins were visualised by fluorography. Molecular mass markers are indicated on the right. Arrows indicate hsps.


A
1
2
B
hsc90
hsp90
hsp104
C
Basic
Acidic
NEPHGE
kDa
205
116
97
66
45
29
SDS
PAGE
104
70
35

3.4.2 hsp104 is N-terminally blocked.

Excellent resolution of hsp104 can be achieved simply by 7% SDS-PAGE (see Fig. 3.6.A lane 3). This can be exploited in an attempt to obtain a limited N-terminal sequence of the polypeptide. Polypeptides which are to be sequenced in a gas phase sequencer may be easily prepared by blotting the polypeptide band directly onto polyvinylidene difluoride (PVDF) membrane. The membrane is stained and the band simply cut out and sequenced directly. Several precautions to the usual SDS-PAGE and blotting methods are required to minimise N-terminal blocking during these procedures; they are described in section 2.13.

Proteins from S100 fractions from heat shocked and unstressed cells resolved by gel like that shown in Fig. 3.6.A, were blotted onto PVDF. The hsp104 band was easily visualised by staining with Coomassie blue. Accordingly, there was at least 1μg of the polypeptide present per band, constituting at least 10pm of hsp104. Up to 7 such bands can be loaded into a sequencing cartridge thereby providing more than enough material for sequencing (see section 2.13). However, no sequence was obtained (not shown). This was not due to the polypeptide being blocked during SDS-PAGE or western blotting. A sample of glutathione transferase 3-3 was subjected to the same procedure. This protein is not blocked *in vivo* (B. Coles personal communication) and gave a high yield (>90%) of the expected N-terminal sequence (not shown). This strongly suggests that hsp104 is naturally N-terminally blocked.

Since hsp104 was N-terminally blocked it was cleaved enzymatically to generate internal peptides with unblocked N-termini. The polypeptide was subjected to limited proteolysis by protease V8 as described in section 2.12. Because of the additional steps involved, more starting material was required. Accordingly, 5 times as much protein (300μg) was loaded onto 7% SDS gels 3 times as thick as usual (3mm). Proteins were resolved as described in section 2.10.1 and hsp104 was visualised and subjected to peptide mapping as described in section 2.12. Precautions designed to limit N-terminal blocking (section 2.13) were employed during the final steps only i.e resolution of peptide fragments on 15% gels and subsequent Western blotting.

The resulting peptide fragments resolved on a gel like that shown in Figure 3.8 were blotted onto PVDF. Three fragments of relatively high M_r (between 29 and 45kDa, see Fig. 3.8) were not amenable to sequencing. Consequently, these fragments probably possessed the natural (blocked) N-terminus. This was not surprising given that protease V8 cleaves glutamoyl peptide bonds at their C-terminal end. However, a smaller fragment (of M_r >29kDa, referred to as fragment D, see Fig. 3.8) was sequencable.

Since small peptides may elute from the membrane surface during sequencing reactions there are various protocols available by which they can be covalently attached to the membrane. Therefore, 4 fragments of type D were conventionally sequenced, (carried out by B. Coles, CRC molecular toxicology unit) and 4 were sequenced after they had been

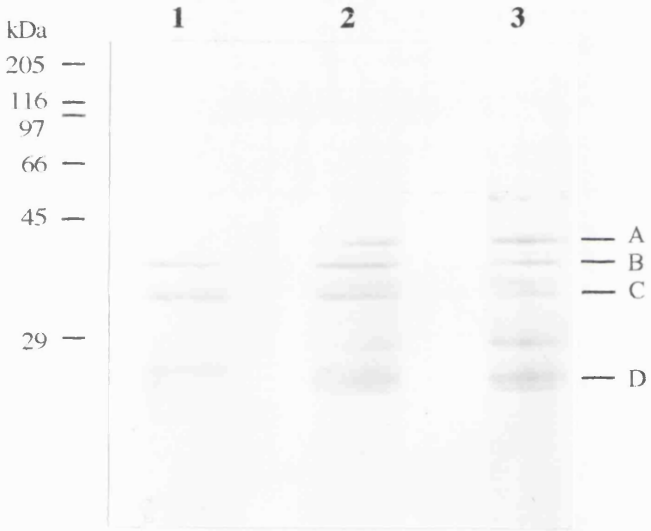
covalently attached to the PVDF membrane by the entrapment reactions described by Coull *et al.*, 1991 (carried out by D. Raman, ICRF, Protein sequencing laboratory). Non attached fragments yielded a sequence 16 amino acids long whereas attached fragments yielded a sequence only 2 residues longer. Only the phenylthiohydantoin amino acid analyser traces from the Edman degradation carried out on the non attached fragments are shown (Fig. 3.9). The sequence from the covalently attached fragments is presented in Fig. 3.10).

3.4.3 A limited N-terminal sequence from a fragment of hsp104.

The sequence from fragment D (see Fig. 3.8) is given in Fig. 3.10. As expected, no identical sequences were found in the ICRF OWL database. The existing sequence found to be the most similar corresponded to a region in the ClpB polypeptide of *E. coli* i.e 8 identical residues, 6 conserved residues, 4 mismatches (Gottesman *et al.*, 1991). Interestingly, this polypeptide (like hsp104) is heat inducible (Squires *et al.*, 1991).

A degenerate oligonucleotide, based on the limited N-terminal sequence presented in Fig. 3.10, was to be used to isolate the gene coding for hsp104. However, soon after the N-terminal sequence described here was obtained a report describing the cloning of this gene and the effects of gene disruption appeared in the literature (Sanchez and Lindquist, 1990). The gene sequence was not included and subsequently appeared in a second report describing the effects of *in vitro* mutagenesis of the coding region (Parsell *et al.*, 1991). Not surprisingly, a region of the amino acid sequence of hsp104 (Parsell *et al.*, 1991) is identical to the limited sequence obtained from fragment D (see Fig. 3.10). Furthermore, homology between hsp104 and ClpB is not limited to the short sequence obtained by work presented here. These polypeptides are co-linear and homologous throughout (Parsell *et al.*, 1991). The functions of hsp104 and ClpB, and the consequences of disrupting the gene encoding hsp104 are described in section 1.2.5.

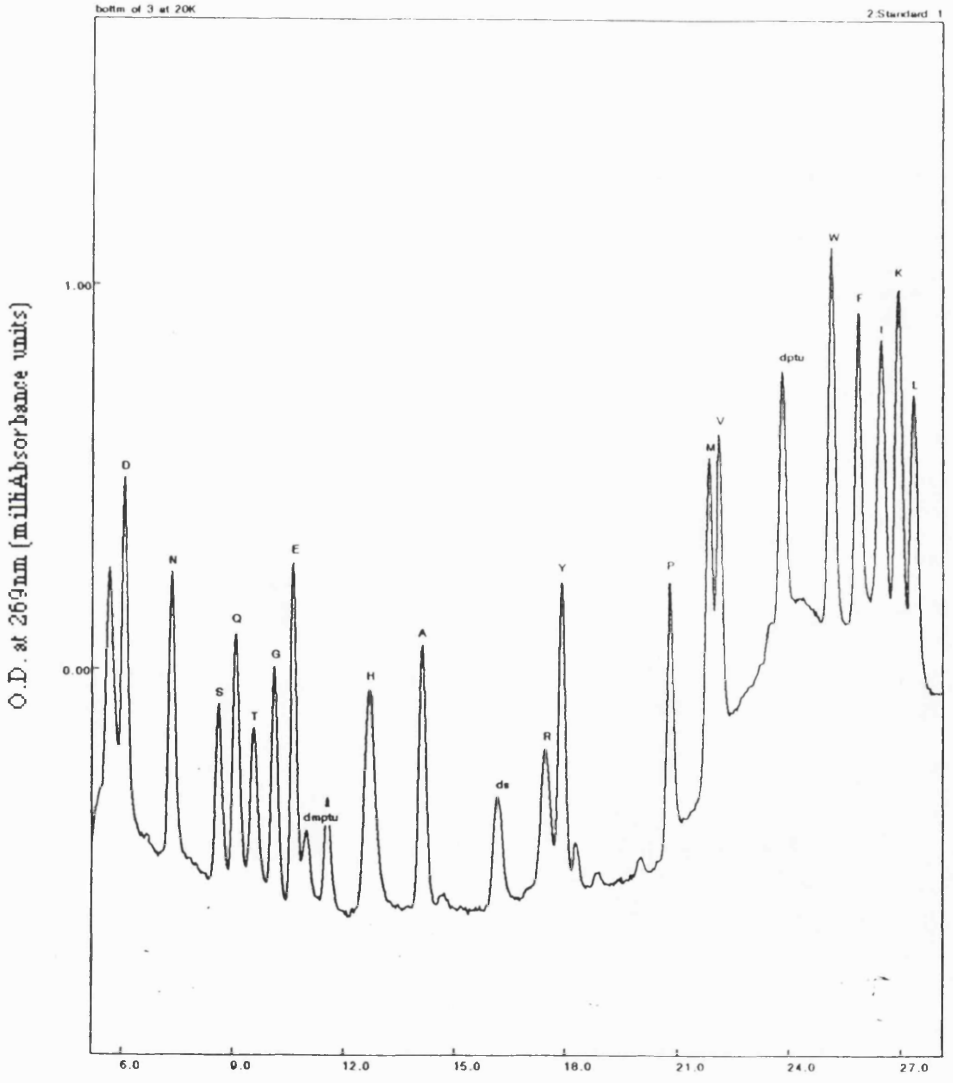
Figure 3.8 Large-scale peptide map of hsp104. A 7% SDS gel 3 times thicker than usual (3mm) was used to resolve hsp104 from heat shock S100 extracts. Each lane was loaded with 300μg of protein. Bands coresponding to hsp104 were excised and subjected to limited digests with *Staphylococcus aureus* V8 protease as described in section 2.12. The 15% gel used to resolve the peptide fragments thereby generated is shown below. Peptides were visualised by Coomassie blue. Each lane was loaded with three hsp104 bands prepared as described above. (**1**), (**2**), and (**3**) are peptide maps produced by 600, 225 and 75ng of protease V8, respectively. Fragments selected for N-terminal sequencing are indicated on the right*, molecular mass markers are indicated on the left.



* Gels similar to this one (but not stained with Coomassie blue) were blotted onto PVDF. Fragments of the M_r indicated above were selected for sequencing. For each fragment, four bands were loaded into a sequencing cartridge.

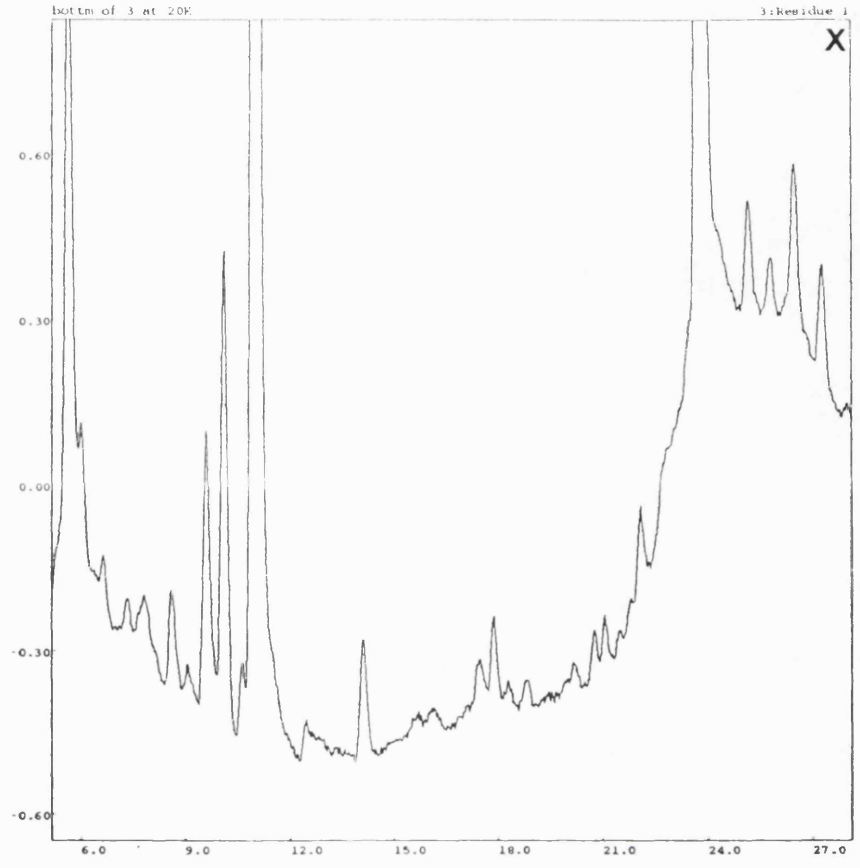
Figure 3.9 Sequence of one of the low molecular–mass fragments derived from proteolytic cleavage of hsp104. Four PVDF bands on which fragment D (M_r >29kDa, see Fig. 3.8) had been blotted, were loaded into an automated gas-phase protein–sequencer. Traces from the on line phenylthiohydantoin (PTH) amino acid analyser are provided. (**A**), calibration of the system by separation of PTH-amino acid standards: (**1-18**), consecutive cycles of the automated Edman degradation carried out on fragment D. Where possible, the released PTH-amino acid is indicated. On all traces amino acids are indicated by their single letter codes. Vertical and horizontal axes, and by products of the PTH derivitisation process are labelled on trace **A** only.

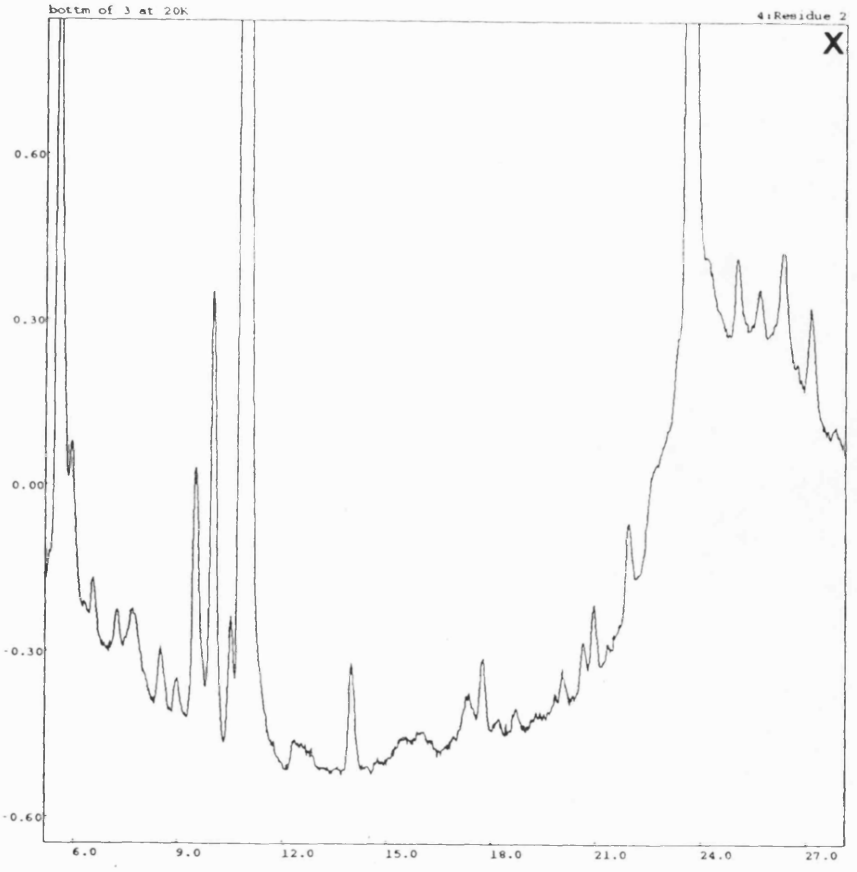
A

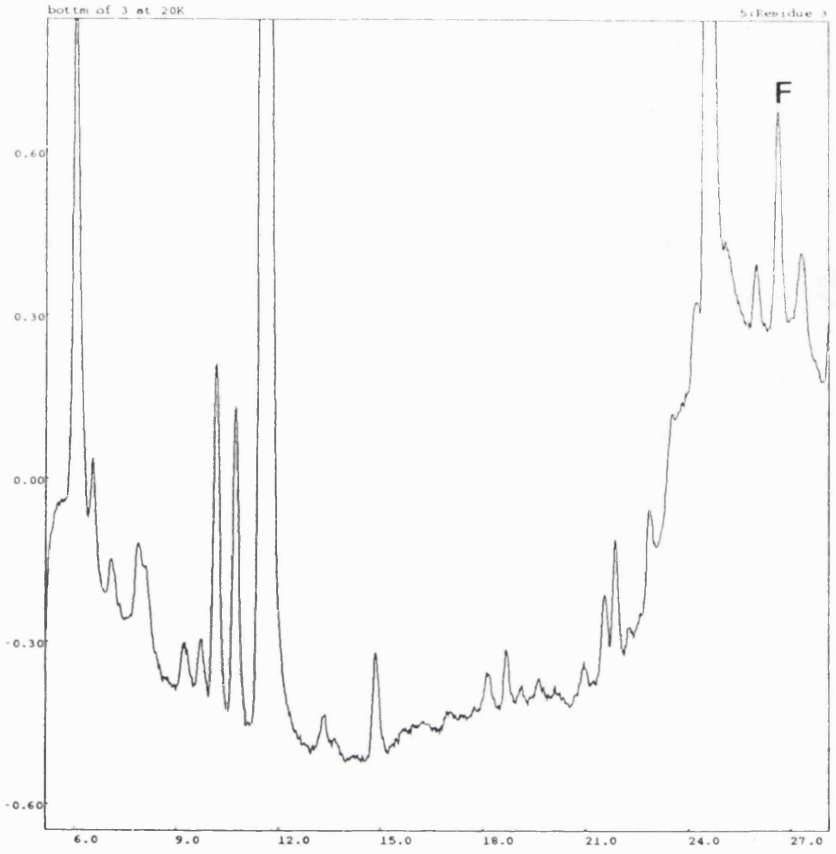


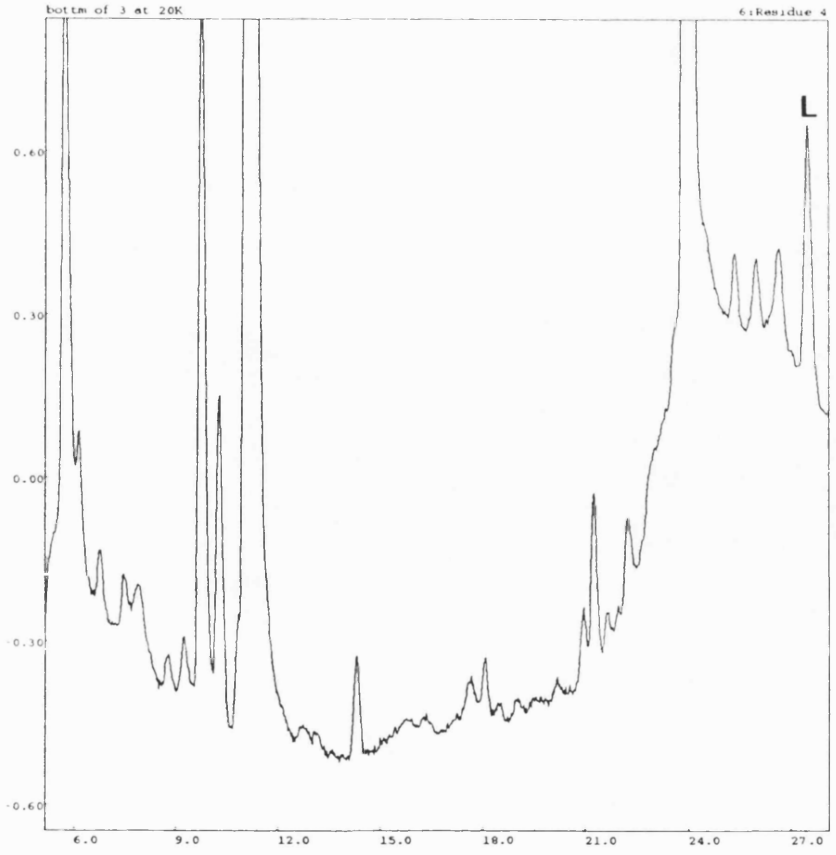
Retention time of PTH-amino acids on H.P.L.C. column (min)

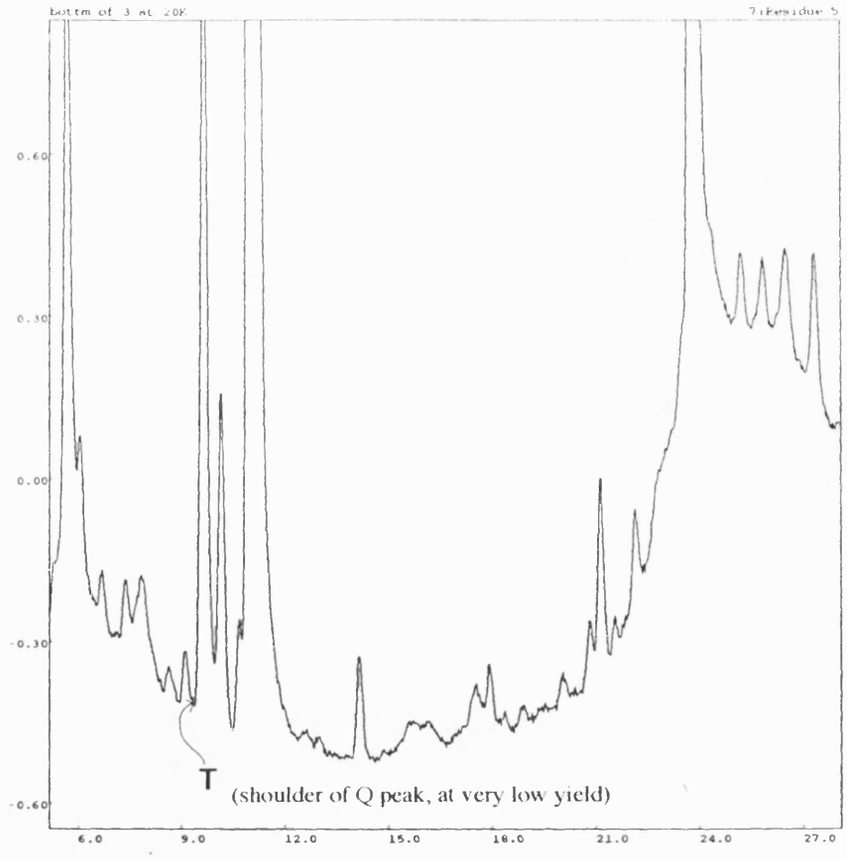
dmptu: dimethylphenylthiourea; ds: dithiothreitol-serine adduct; dptu: diphenylthiourea.
single letters (apart from X): amino acid codes (see page 17).
X: identity of the amino acid released in this cycle is unclear.

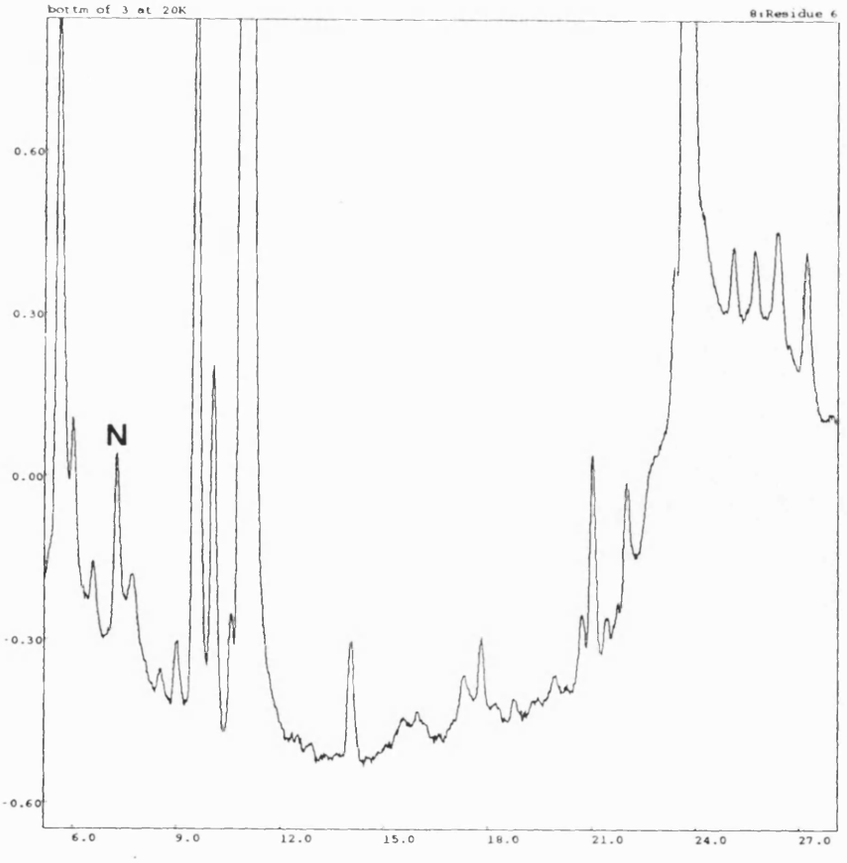

bottm of 3 at 20K
3:Residue 1
X
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

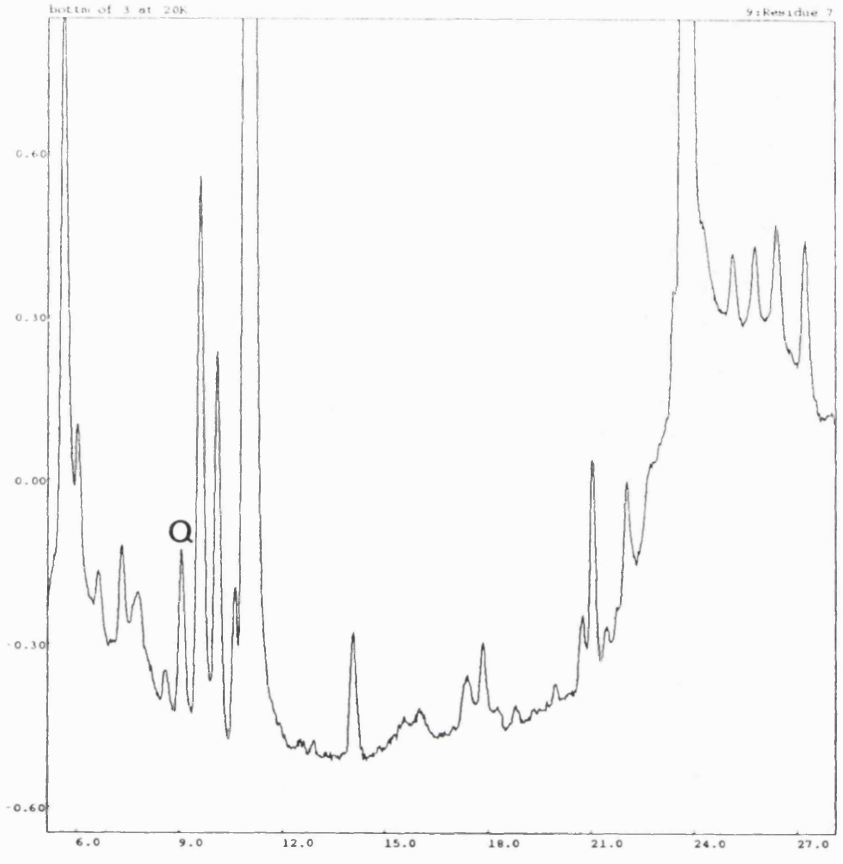

bottm of 3 at 20K
4:Residue 2
X
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

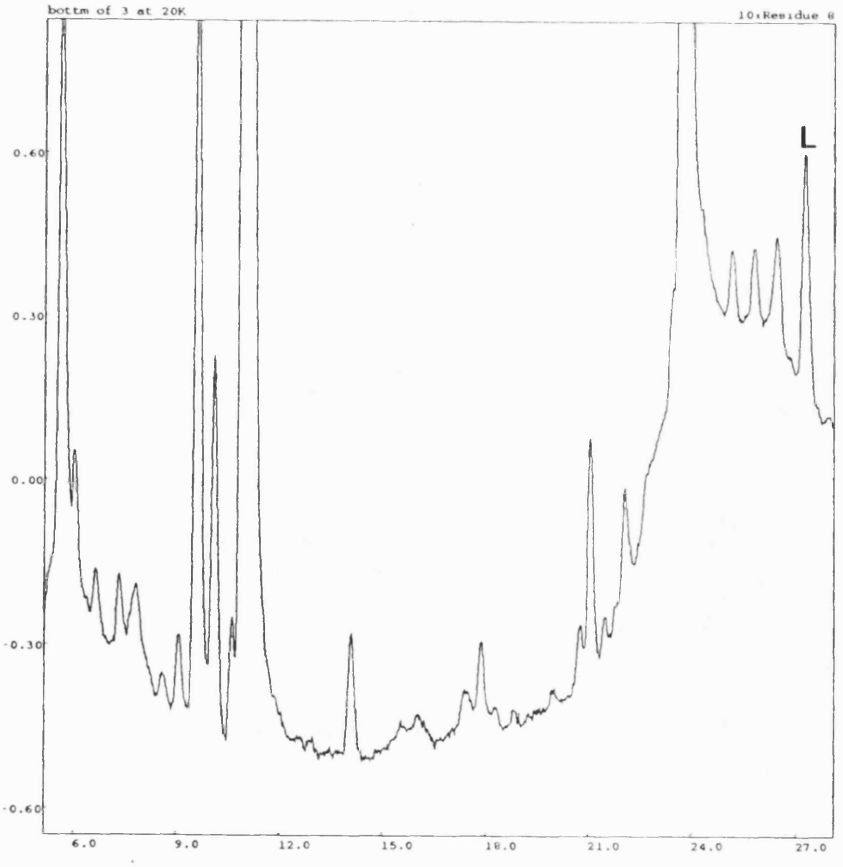

bottm of 3 at 20K
5:Residue 3
F
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

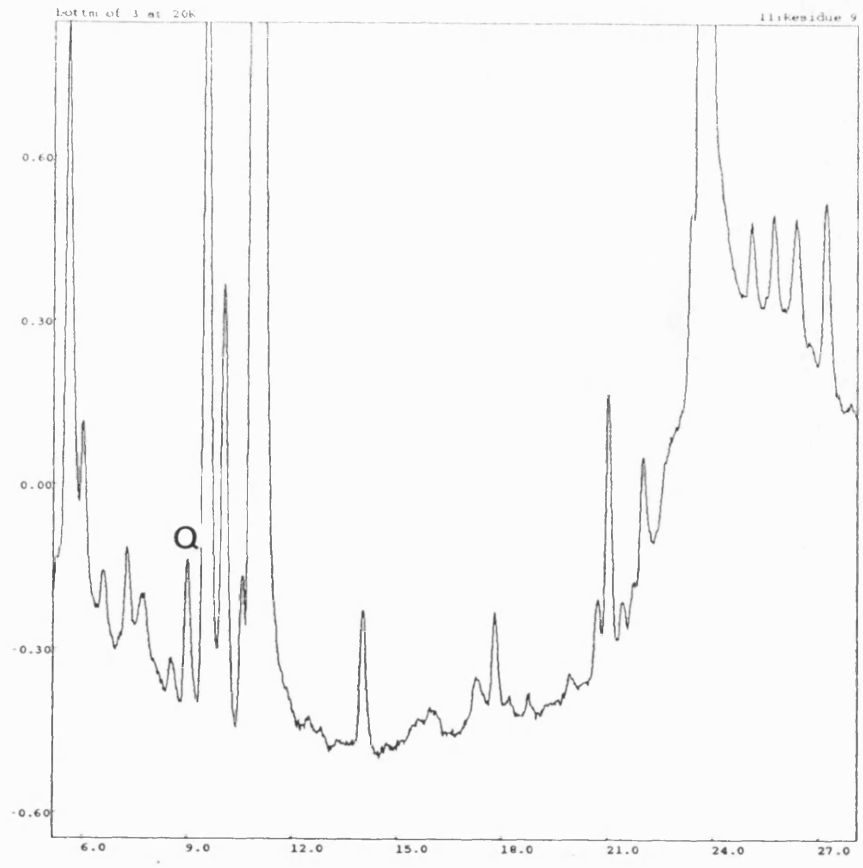

bottm of 3 at 20K
6:Residue 4
L
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

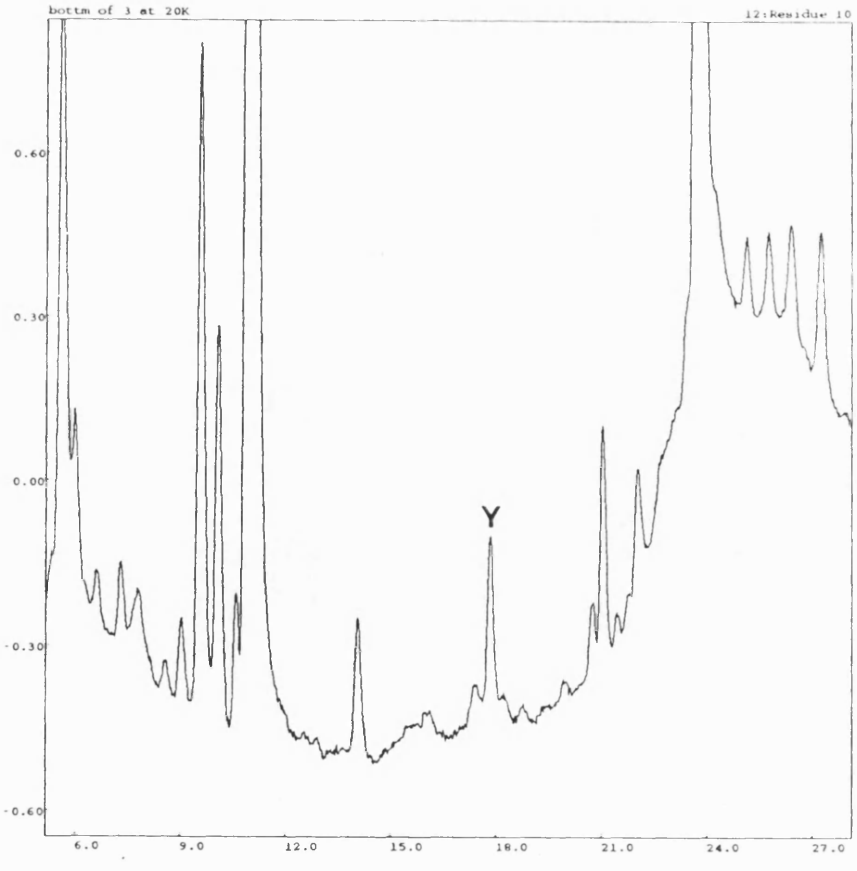

bottm of 3 at 20K
7:Residue 5
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
T
(shoulder of Q peak, at very low yield)

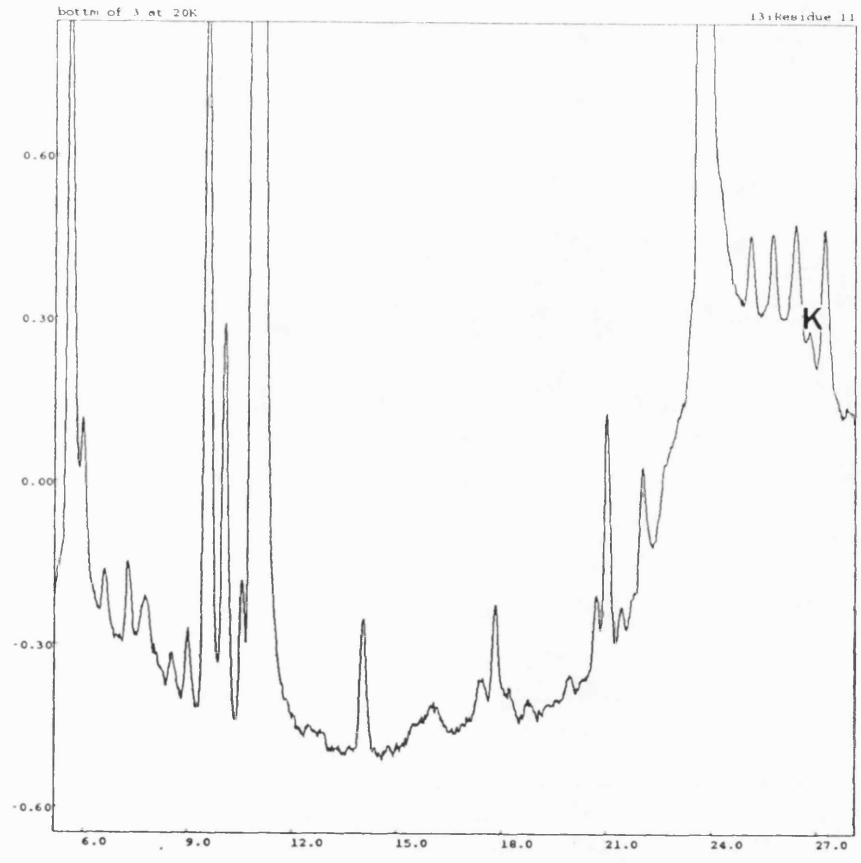

bottm of 3 at 20K
8:Residue 6
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0
N

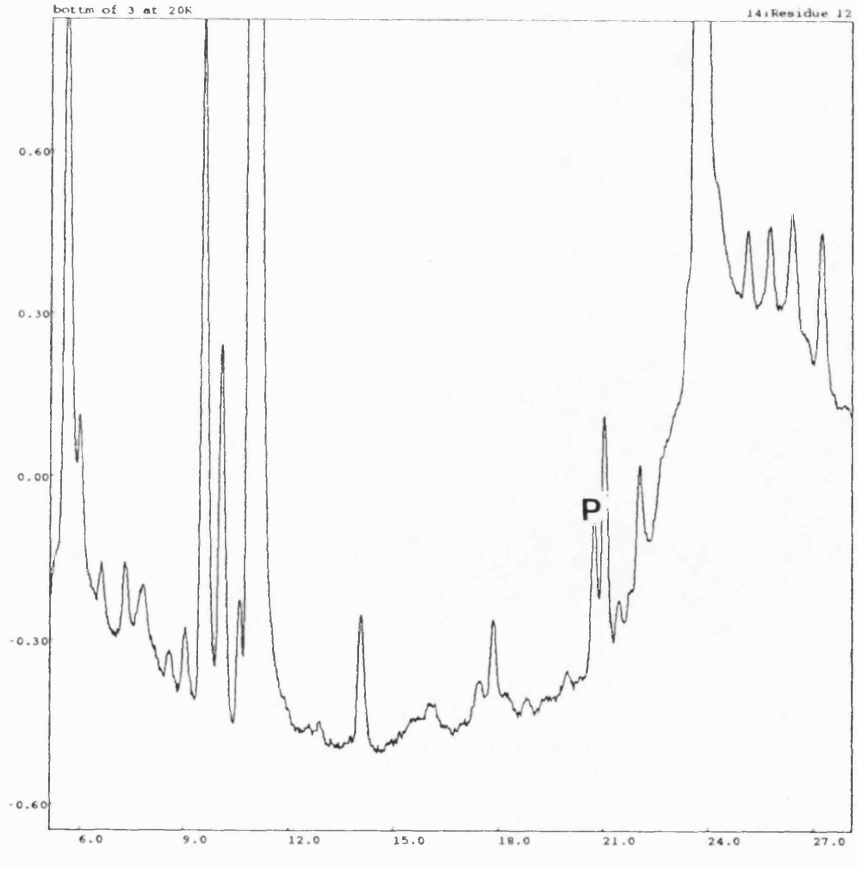

bottm of 3 at 20K
9:Residue 7
0.60
0.30
0.00
-0.30
-0.60
Q
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

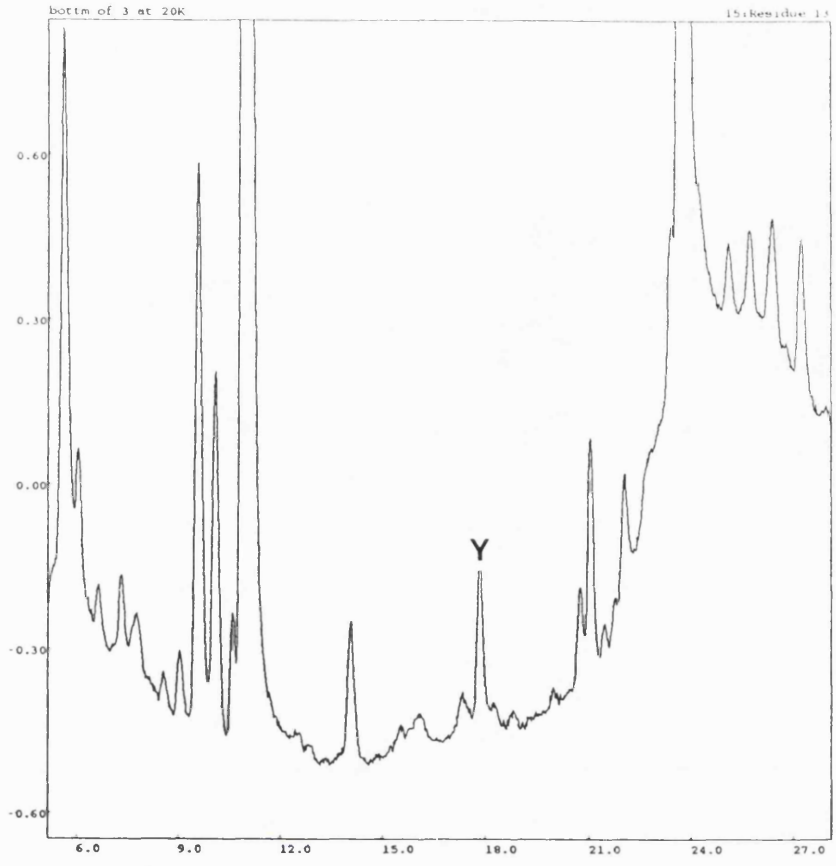

bottm of 3 at 20K
10:Residue 8
0.60
0.30
0.00
-0.30
-0.60
L
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

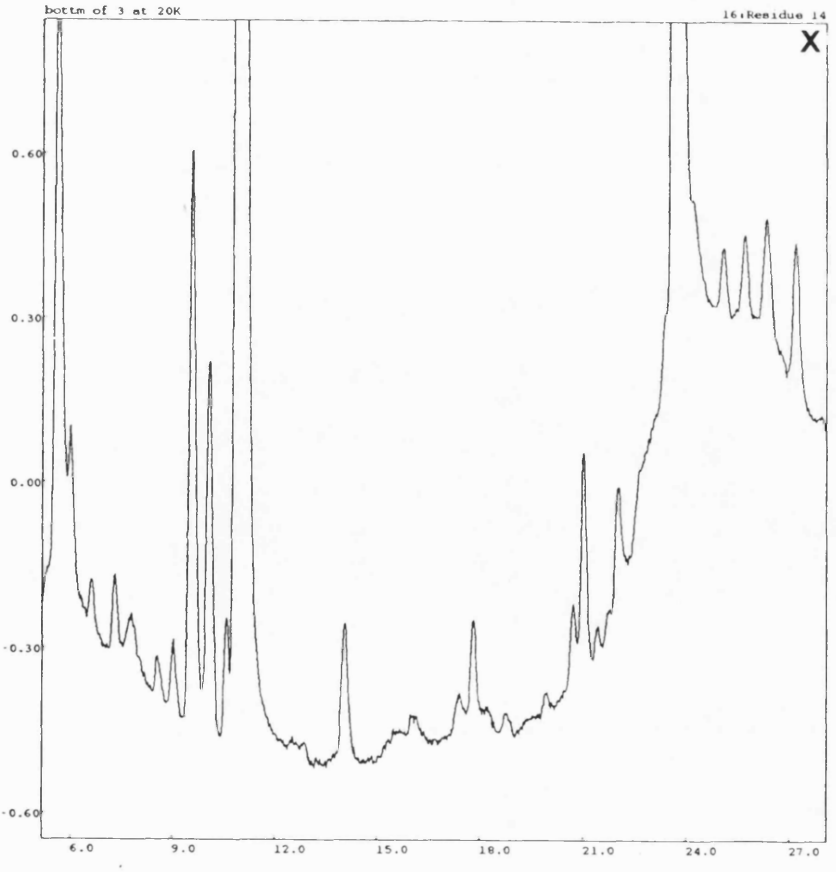

bottm of 3 at 20K
11:Residue 9
0.60
0.30
0.00
-0.30
-0.60
Q
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

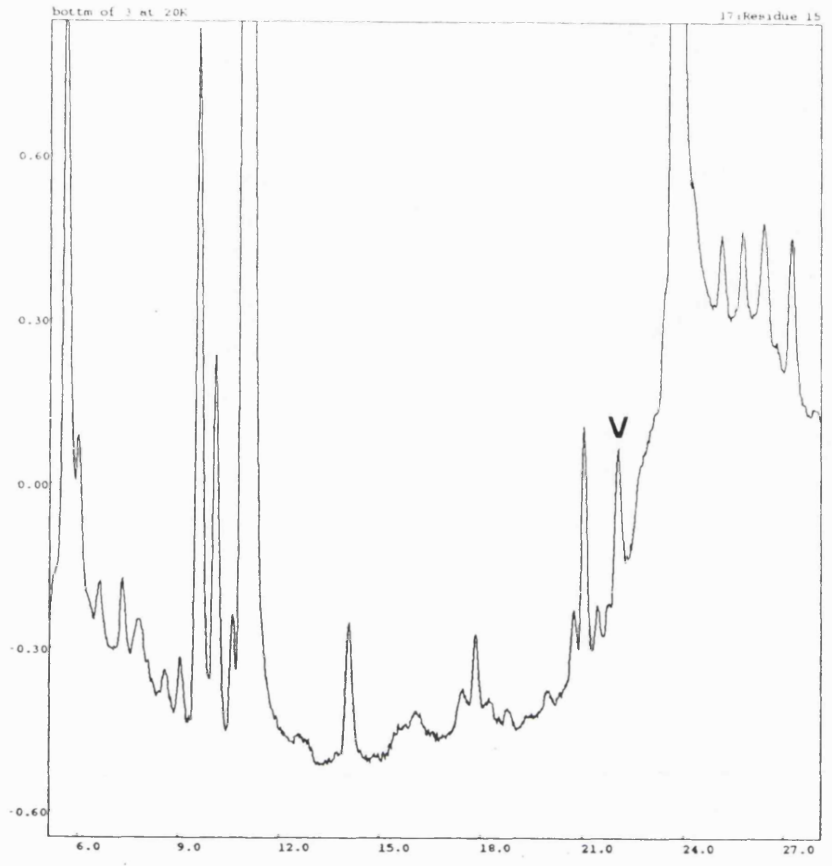

bottm of 3 at 20K
12:Residue 10
0.60
0.30
0.00
-0.30
-0.60
Y
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

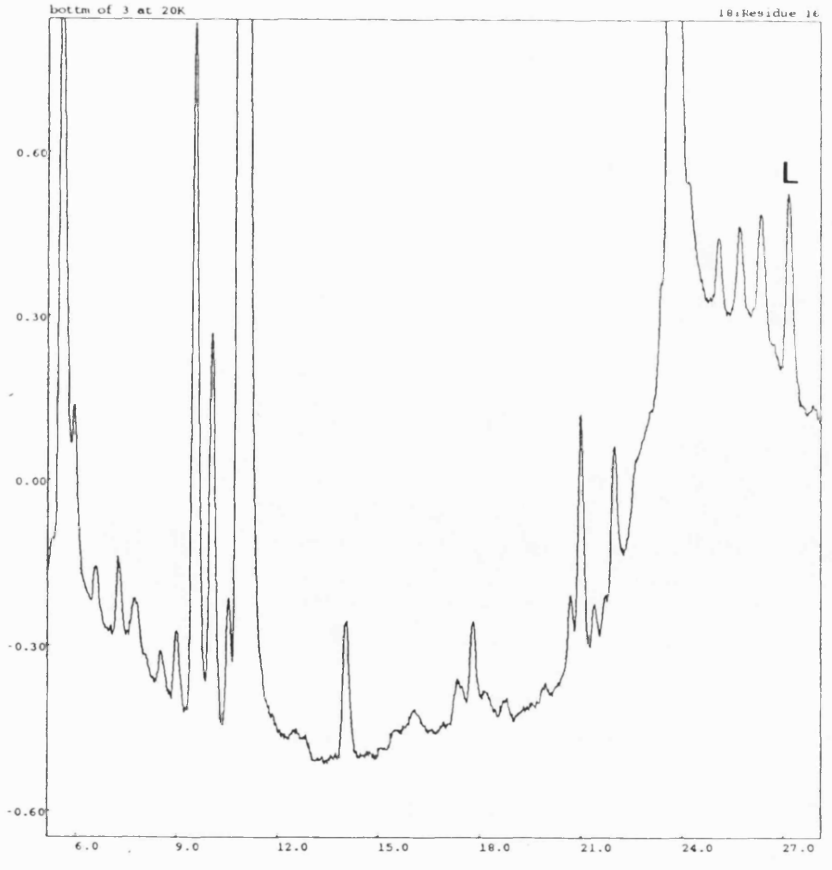

bottm of 3 at 20K
13:Residue 11
K
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

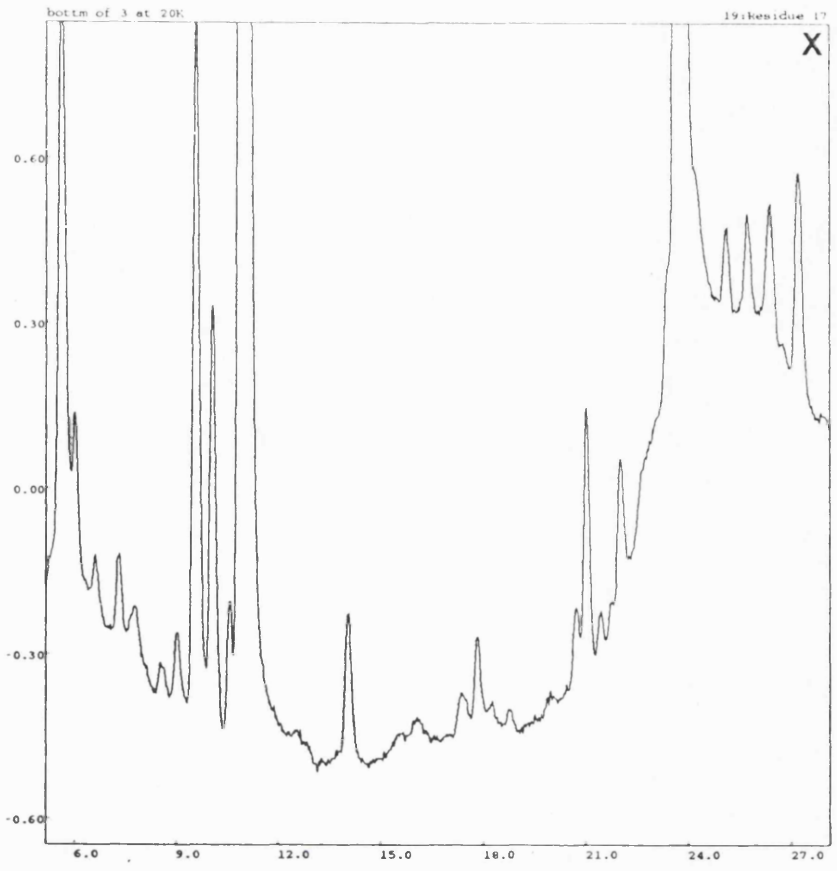

bottm of 3 at 20K
14:Residue 12
P
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0


bottm of 3 at 20K
15:Residue 13
0.60
0.30
0.00
-0.30
-0.60
Y
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0


bottm of 3 at 20K
16:Residue 14
X
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0


bottm of 3 at 20K
17:Residue 15
V
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0


bottm of 3 at 20K
18:Residue 16
L
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0


bottm of 3 at 20K
19:Residue 17
X
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

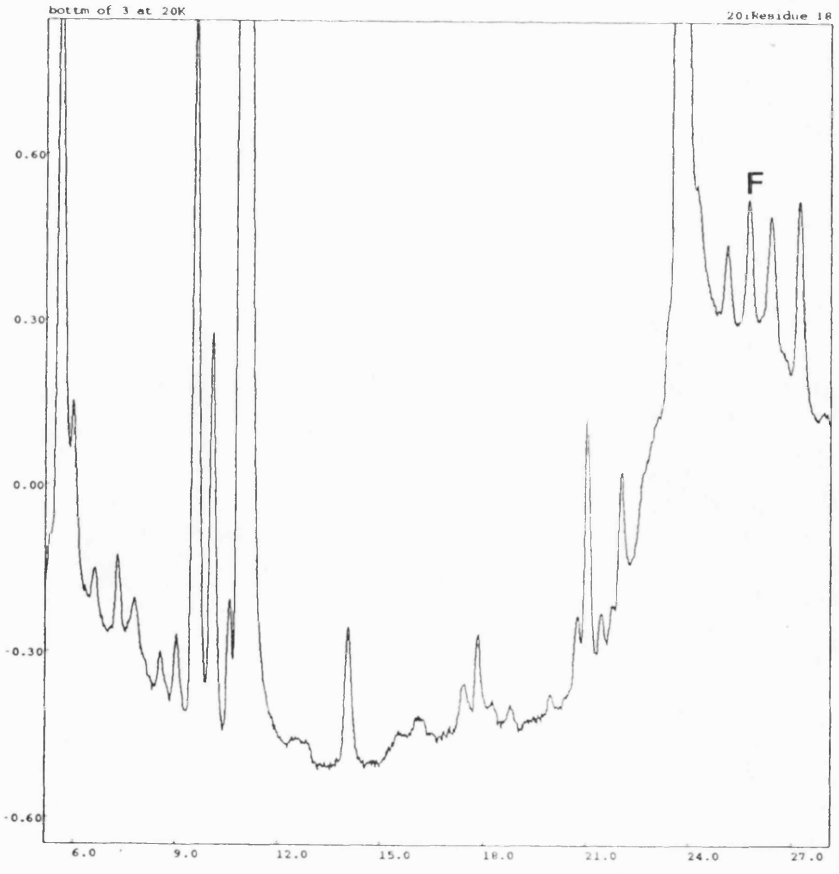

bottm of 3 at 20K
20:Residue 18
F
0.60
0.30
0.00
-0.30
-0.60
6.0
9.0
12.0
15.0
18.0
21.0
24.0
27.0

Figure 3.10 Comparison of the partial sequence of the peptide obtained from hsp104 digests with the published sequence of hsp104. I. Sequence of fragment D (see Fig. 3.8) obtained from peptides blotted onto PVDF (*), and from blotted peptides covalently attached to PVDF (♦), (see section 3.4.2). (+), combination of both sequences. **II.** Published sequence of hsp104 (from Parsell *et al.*, 1991). Amino acids are designated by their single letter codes (see page 17).

I

```
          1                                                  20
*   X X F L T N Q L Q Y K P Y X V L X F•

♦   X X F L T N Q L Q Y X P Y S V L X F D E⊗

+   X X F L T N Q L Q Y K P Y S V L X F D E
```

II

```
MNDQTQFTERALTILTLAQKLASDHQHPQLQPIHILAAFIETPEDGSVPYLQNLIEKGRYDYDLFKKVVNRNL   73
VRIPQQQPAPAEITPSYALGKVLQDAAKIQKQQKDSFIAQDHILFALFNDSSIQQIFKEAQVDIEAIKQQALE
LRGNTRIDSRGADTNTPLEYLSKYAIDMTEQARQGKLDPVIGREEEIRSTIRVLARRIKSNPCLIGEPGIGKT  719
AIIEGVAQRIIDDDVPIILQGAKLFSLDLAALTAGAKYKGDFEERFKGVLKEIEESKTLIVLFIDEIHMLMGN
GKDDAANILKPALSRGQLKVIGATTNNEYRSIVEKDGAFERRFQKIEVAEPSVRQTVAILRGLQPKYEIHHGV  365
RILDSALVTAAQLAKRYLPYRRLPDSALDLVDISCAGVAVARDSKPEELDSKEQSIAIDSSRDKSSRERVECR
LHTKRKLKLARQKEASLQEELEPLRQRYNEEKHGHEELTQAKKKLDELENKALVAERRYDTRTAADLRYFAIP  511
DIKKQIEKLEDQVAEEERRAGANSMIQNVVDSDTISETAARLTGIPVKKLSESENEKLIHMERDLSSEVVGQM
DAIKAVSNAVRLSRSGLANPRQPASFLFLGLSGSGKTELAKKVAGFLFNDEDMMIRVDCSELSEKYAVSKLLG  657
TTAGYVGYDEGGFLTNQLQYKPYSVLLFDEVEKAHPDVLTVMLQMLDDGRITSGQGKTIDCSNCIVIMTSNLG
AEFINSQQGSKIQESTKNLVMGAVRQHFRPEFLNRISSIVIFNKLSRKAIHKIVDIRLKEIEERFEQNDKHYK  803
LNLTQEAKDFLAKYGYSDDMGARPLNRLIQNEILNKLALRILKNEIKDKETVNVVLKKGKSRDENVPEEAEEC
LEVLPNHEATIGADTLGDDDNEDSMEIDDDLD  908
```

* Traces from on line phenylthiohydantoin analyser are presented in Fig. 3.9; ⊗ Traces not shown for this sequence.

Chapter 4 Effects of stress on the plasma membrane.

4.1 Introduction

The potentially cytotoxic effects of heat stress are thought to result from damage to protein assemblies and to membranes. Most heat-shock studies have focussed on the induced heat-shock proteins. To date there are only a few reports concerning the effects of stress on membrane structure *per se*. These have demonstrated temperature effects on membrane fluidity (Watson, 1984 and 1987) and have revealed that long term acclimation to high temperatures is correlated with an increased saturation of membrane lipids (Quinn and Williams, 1985). Given that the first component of the cell that encounters the environment is the cell membrane it is remarkable that there have been so few studies on stress inducible phenomena associated with this part of the cell.

Many of the inducers of thermotolerance such as ethanol and heat shock *per se* (see Table 1.1) are known to damage proteins. The increase in levels of aberrant protein has been implicated in the induction of the hsp synthesis (and could conceivably activate other responses). The question arises as to whether all proteins are equally likely to become initiators of stress inducible responses. Several pieces of evidence suggest that proteins embedded in lipid bilayers are of primary concern. Firstly, there is the finding that the concentration of aliphatic alcohols required for induction of thermotolerance is related to their water-lipid partitioning coefficient (Hahn *et al.*, 1985). The greater the partitioning of the alcohol into lipids (i.e the higher the coefficient) the lower the required concentration in the surrounding medium. Though total concentration of various alcohols in the medium can vary by as much as 100x, the concentration in the membrane required to induce thermotolerance remains constant, suggesting that membrane rather than cytosolic proteins are at risk. The energetics of thermotolerance are in agreement with this. The thermodynamics of the induction of thermotolerance involve a very sharp temperature transition in Chinese hamster ovary cells (see Fig. 4.1). The transition has a half width of $\leq 0.5^{\circ}C$ which is too narrow to reflect phase changes of proteins in aqueous solution, and is more likely to represent phase changes of proteins in lipid bilayers (Hahn and Li, 1990). The similarity of the graphs in Fig. 4.1 suggest that cell death, induction of thermotolerance and induction of hsps all have their origin in the critical target represented by this sharp temperature transition. Also, in mammalian cells the polyene antibiotic amphotericin B is capable of inducing thermotolerance (Basrur and Hahn, unpublished, cited in Hahn and Li, 1990). This drug does not penetrate into the cytosol, its only known action involving binding to cholesterol, which, in mammalian cells is found almost exclusively in the plasma membrane. Hahn and Li speculate that cells regard amphotericin B/cholesterol complexes as denatured proteins. Alternatively, these complexes may form ionophores in the membrane. Calderwood *et al.* (1988) have demonstrated that heat induces second

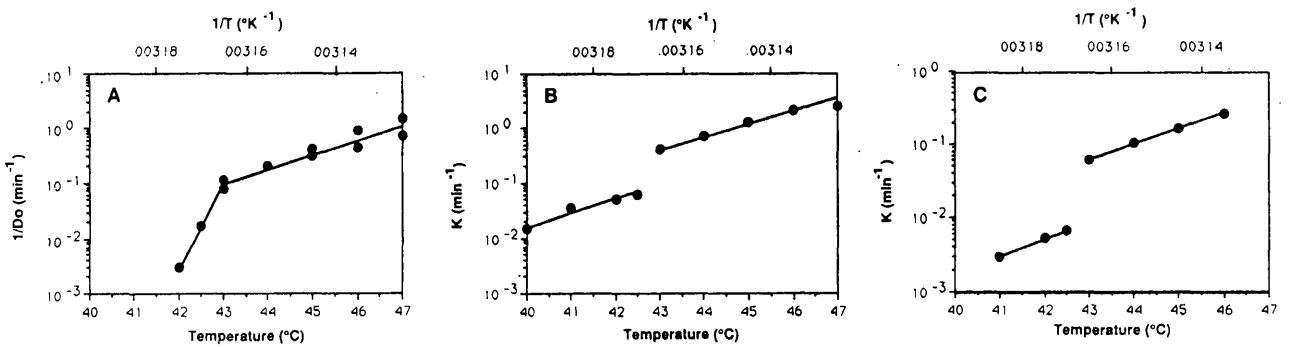


Figure 4.1 Arrhenius plots for heat-killing, induction of thermotolerance, and induction of heat shock proteins in Chinese hamster cells (HA-1). (*A*) Plot of the inverse of the log of the slope of the survival curves obtained at various temperatures against the inverse of the absolute temperature. (*B*) Rate at which thermotolerance is induced as a function of the duration of the triggering. This rate is plotted vs. the inverse of the absolute temperature of the appropriate treatment. (*C*) Similar to *B*, except that the time to induce a maximum amount of hsp70 was used to determine the ordinate. Thus, *C* indicates rate of induction of hsp70 synthesis. The last two Arrhenius plots are statistically indistinguishable, suggesting that the induction of either thermotolerance and synthesis of hsps involve the same or at least very similar mechanisms. The sharp break in all three Arrhenius plots suggests a cooperative process, perhaps involving a phase change either in proteins or in lipids. The slope of each line is a measure of the activation energy of the process; this is ~120 kcal/mole in *A* and *B*, consistent with the suggestion that protein denaturation is involved.

Figure from Hahn and Li (1990).

messenger cascades (see section 1.8.5 and Fig. 1.5). These have their initiating signals in the plasma membrane. In addition, heat shock disturbs transmembrane ion gradients; in yeast, the H^+ gradient created and maintained by the plasma-membrane-associated H^+-ATPase is particularly vulnerable (see section 3.1). Furthermore, the activity of this enzyme has been shown to influence stress tolerance (see section 3.2 and Coote *et al.*, 1991). Rates of H^+ efflux are known to increase at higher sub lethal temperatures known to induce thermotolerance (Coote *et al.*, 1991).

The above shows that evidence for the importance of plasma membrane integrity in stress survival is increasing. However there have been no reports to date of eukaryotic stress proteins associating with this membrane. It is nevertheless conceivable that cells target proteins with a damage limitation function to the plasma membrane as part of their inducible-stress responses. In addition, changes in levels of proteins which are present in the membrane prior to stress should also be examined. Accordingly, the changes in the protein composition of plasma membranes caused by heat shock and other types of stress were analysed.

Transcription of the gene coding for H^+-ATPase (*PMA1*) is heat inducible (Curran *et al.*, 1988). The question arises as to whether the *PMA1* mRNA is translated and the resulting polypeptide inserted into the membrane during stress (this is one of the questions the analysis described above will answer). An increase in the number of H^+-ATPase molecules in the cell membrane may explain the elevated levels of H^+ efflux during heat shock (Coote *et al.*, 1991). It should be noted that there is no precedent for changes in the level of this protein under any circumstances. Where studied, rates of H^+ efflux are solely regulated by altering the activity of H^+-ATPase, rather than by alteration of enzyme levels (see section 3.1 and references cited therein). The activated state of H^+-ATPase is preserved after purification of plasma membranes and probably corresponds to a covalent modification of the enzyme. Assaying the H^+-ATPase of isolated plasma membranes from unstressed and stressed cells will determine whether heat shock has a similar effect.

4.2 Plasma membrane isolation.

An initial review of the literature suggested that the current method of choice for isolating plasma membranes involves preparing yeast sphaeroplasts (for a review of plasma membrane isolation protocols see Rose and Veazey, 1988). This is followed by addition of cationic beads which coat the negatively charged surface of the sphaeroplasts, making the plasma membrane much denser than any other cellular organelle, enabling isolation of the membrane by centrifugation once the sphaeroplasts have been lysed (Schmidt *et al.*, 1983 and Cartwright *et al.*, 1987). This method is unsuitable for investigating changes occurring

Table 4.1 The H^+-ATPases of biological membranes.

Type*	Structure *	Catalytic intermediate *	H^+/ATP *	Inhibitors @				
				Vanadate	NaN_3	KNO_3	Des	DCCD
P-	Single polypeptide of M_r ca. 100kDa.	phosphorylated	1	+++	–	–	+++	+
F-	Complex, 8 types of sub unit. Forming two major complexes Fo (hydrophobic) & F1 (water soluble).	not phosphorylated	3	–	+++	–	–	+++
V-	Complex, as many as 7 different types of sub unit	not phosphorylated	2	–	–	++	++	++

% inhibition: +++ > 80%, ++ 40-80%, + 5-40% , - <5%.

*From Nelson and Taiz, 1989. @From Anraku *et al.*, 1989.

at the plasma membrane during stress, since an extended incubation with zymolyase (at least 30-45 minutes at 30°C) is required to sphaeroplast cells. This will almost certainly cause physiological changes which may be reflected in alterations to plasma membrane components. Indeed it has recently been found that sphaeroplasting cells induces transcription of genes coding for heat shock proteins (Adams and Gross, 1991). An attempt to eliminate this problem by using 1,10 phenanthroline to inhibit transcription would have been futile since the inhibitor enhances the mRNA abundance of certain heat shock genes (Adams and Gross, 1991). Also, cells cannot be sphaeroplasted in certain physiological states (e.g. at stationary phase). For these reasons only methods involving rapid disruption of cells were considered. After trying a number of different protocols for plasma membrane isolation, the best in terms of purity, yield and reproducibility was found to be a modification of the method of Serrano, (1978) (see sections 2.9-2.9.2).

Initial experiments were carried out using the prototrophic strain Σ1278b. It soon became apparent that proteins isolated from fractions derived from this strain after heat shock had been degraded to the extent that no proteins above 60kDa were seen on 1D SDS-PAGE gels. This occurred even though all steps were carried out at 4°C in the presence of protease inhibitors. Only slight degradation was noticeable in the fractions obtained from unstressed cells (not shown). This was not entirely unexpected since the expression of ubiquitin, a protein involved in protein degradation, is heat inducible (Finley *et al.*, 1987). Furthermore, in mammalian cells exposure to heat shock is followed by a rapid phase of proteolysis (Parag *et al.*, 1987). Such pronounced degradation may be the main reason why there are so few reports of hsps associating with eukaryotic cell membranes. The problem was overcome by the use of strain BJ2168 which is substantially deficient in vacuolar proteases (see section 2.2 for genotype).

4.3 Criteria used to confirm purity of the plasma membrane and mitochondrial fractions isolated.

The method used to isolate plasma membranes and mitochondria is well established and has been routinely used for many years by several different groups (e.g. Serrano, 1978; Portillo and Mazon, 1985; Eraso and Gancedo, 1987). Isolation of organelles by centrifugation through a discontinuous sucrose gradient is involved. Plasma membranes band at 1.223g/cm^3 on the preparative gradients whereas mitochondria band at 1.173g/cm^3. A number of tests were applied to show that membranes banding at 1.223g/cm^3 on the gradients were plasma membranes of high purity. Potentially the most important contaminants in plasma membrane fractions would be H^+-ATPases of the mitochondria and the vacuoles. Each of these H^+ pumps are members of a distinct class of enzyme (see section 3.1 and Table 4.1), distinguishable *in vitro* by use of orthovanadate,

NaN_3 (or oligomycin) and KNO_3 (see Table 4.1) as inhibitors. The H^+-ATPases of the mitochondria and the vacuole are not inhibited by orthovanadate, a specific inhibitor of the H^+-ATPase. It is therefore acknowledged that one of the best methods of assessing purity of yeast plasma-membrane fractions is to determine the extent of inhibition of the associated H^+-ATPase by orthovanadate (Rose and Veazey, 1988). Assaying H^+-ATPase activity as described in section 2.9.4, the H^+-ATPase activity of the $1.223 g/cm^3$ fraction showed greater than 90% inhibition by orthovanadate and less than 6% inhibition by NaN_3 (not shown). In contrast, the ATPase of the $1.173 g/cm^3$ fraction, a fraction which consists predominantly of mitochondria (Serrano, 1978; Serrano *et al.*, 1991), was inhibited ca. 80% by NaN_3 (not shown). Also the absence of cytochrome peaks in the absorption spectra of the $1.223 g/cm^3$ fraction (even in concentrated membrane suspensions) and the extremely low levels of the microsomal marker enzyme NADPH-cytochrome c reductase (Kobota *et al.*, 1977) in this fraction, were further indications of the lack of any major contamination by mitochondria and microsomal membranes (not shown).

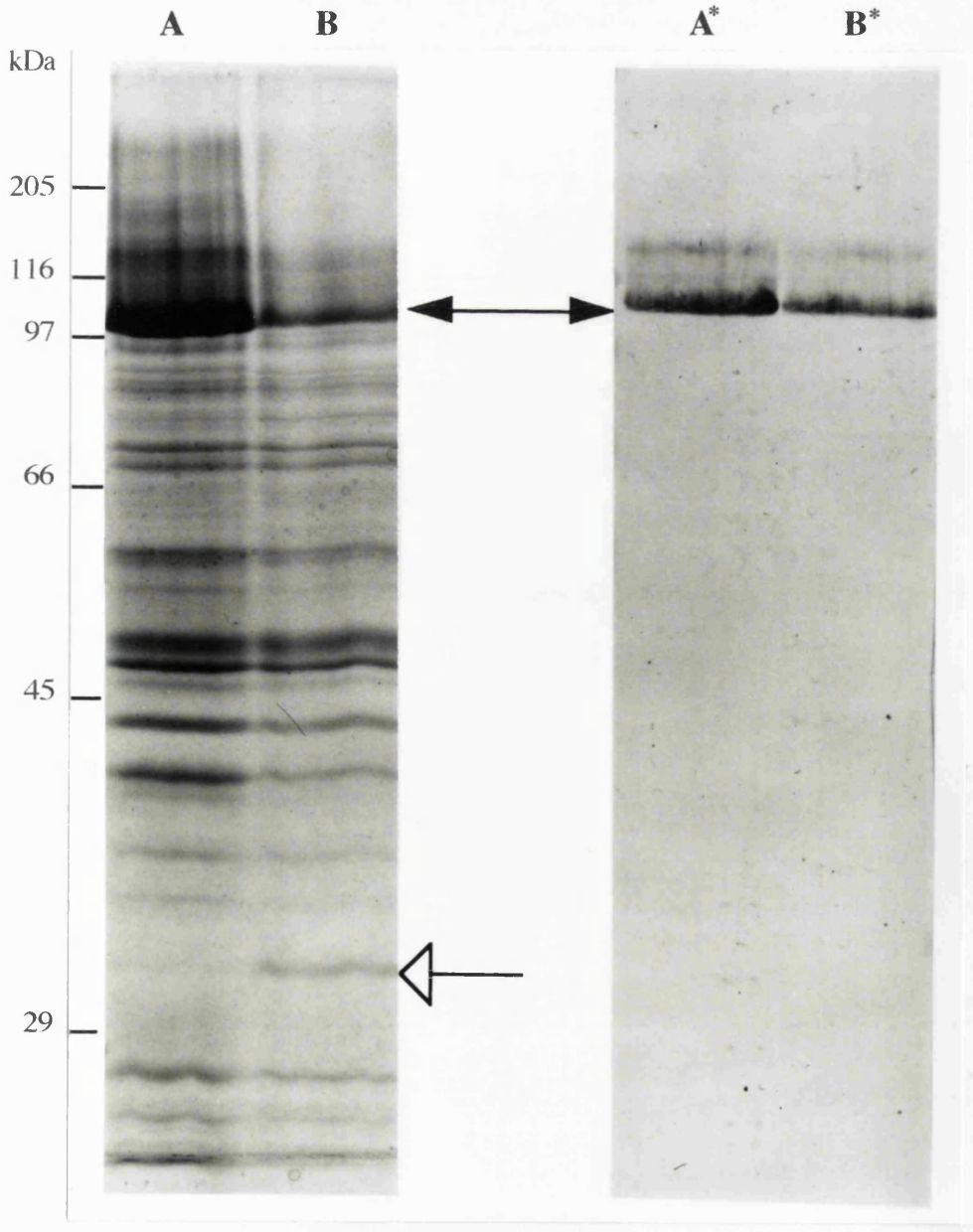
4.4 Results.

4.4.1 Heat shock causes prominent changes to the plasma-membrane-associated proteins of yeast.

Cultures of *S. cerevisiae* BJ2168 in exponential growth in rich media at 20^{0}C were harvested both in the unstressed state and following a heat shock to 40^{0}C. The heat shock employed (40 minutes at 40^{0}C, 1-2^{0}C above the maximum growth temperature of this *S. cerevisiae* strain on fermentative media) is relatively severe and promotes strong induction of heat shock proteins (Piper *et al.*, 1988). Membrane fractions were isolated from these cultures by banding on discontinuous sucrose gradients (see sections 2.9.2). The heavier banding membrane fraction of density 1.223g/cm^3 has been shown in a number of studies to consist almost entirely of plasma membranes (Serrano, 1978; Serrano, 1991). In this investigation this fraction was also assessed to be of high purity by application of a number of criteria (see section 4.3) and its proteins gave highly reproducible banding patterns on Coomassie blue stained 1D gels (see Fig. 4.2). These gels consistently revealed two prominent changes to plasma membrane protein composition with heat shock: (a) a reduction in the levels of the most abundant plasma-membrane protein, the 110kDa plasma – membrane-associated H$^+$-ATPase; and (b) the appearance of a new protein band with an approximate molecular mass of 30kDa. These changes are visible in Fig. 4.2, lanes A and B. Western blots of these membrane protein samples probed with a polyclonal antiserum raised against whole plasma-membrane H$^+$-ATPase (Serrano *et al.*, 1986) confirmed the decline in H$^+$-ATPase, with no appearance of lower molecular-mass bands with epitopes recognised by the antiserum that might have corresponded to H$^+$-ATPase degradation products (Fig. 4.2 lanes A* and B*).

Extensive precautions were taken to ensure that these changes in plasma membrane proteins do not reflect proteolytic artefacts. Most of the proteolysis in yeast cell extracts is due to the vacuolar proteases (Achstetter and Wolf, 1985). However this study was conducted using a strain essentially lacking such activities. Also protease inhibitors were included in all the solutions used in plasma membrane isolation, though their omission during the isolations was found not to result in any major alteration to the gel profiles of plasma membrane proteins.

Figure 4.2 Proteins of isolated plasma membranes of unstressed and heat shocked *S. cerevisiae*. Sub-cellular fractionation was carried out on cultures of strain BJ2168 which had been grown to exponential phase in rich media at 20^{0}C. (A, B) 12.5% gel stained with Coomassie blue of plasma membrane proteins of unstressed cells (A) and cells given a 40 minute 20-40^{0}C heat shock (B). Molecular masses of markers are indicated on the left. On the right are indicated the 110kDa H+-ATPase (solid arrow) and a 30kDa protein that is plasma membrane associated with the heat shocked cells only (open arrow). (A*, B*) western blot analysis of the same plasma membrane protein samples from unstressed (A*) and heat shocked (B*) cells. A polyclonal anti-serum to whole H +-ATPase was used to detect this protein. All lanes were loaded with 50μg of total membrane protein.



4.4.2 Plasma membrane proteins synthesised *de novo* during stress.

To determine whether any of the proteins from the membranes of heat shocked cells correspond to proteins synthesised *de novo* during heat shock, cells were pulse-labelled in the absence and presence of a heat shock (as described in section 2.9.3) and their membranes isolated. [^{35}S] methionine was used initially as isotope in these studies of plasma membrane proteins labelled *in vivo*. Figs. 4.3 .1, .2 and .3 are three different exposures of the same fluorogram (32, 64 and 10 day exposure times respectively). The results revealed very little, if any, incorporation of *de novo* synthesised H+-ATPase into the plasma membranes during the heat shock (Fig. 4.3 lane B). Immunogold labelling of yeast sections has previously shown that there is no significant pool of H+-ATPase in internal membranes (Serrano *et al.*, 1991). This, when considered together with the lack of any appreciable H+-ATPase-labelling in heat shocked *S. cerevisiae*, leads to the conclusion that the decline in the level of H+-ATPase caused by heat shock (Fig. 4.2 lane B) is due to turnover in the absence of synthesis. The question arises as to the fate of the *PMA1* mRNA that is produced during heat shock (see section 4.5.1).

4.4.3 The 30kDa protein is the only heat shock protein that substantially copurifies with plasma membranes.

A 30kDa heat-inducible protein was the major nascent protein associated with the plasma membrane in heat stressed cells (Fig. 4.3 lane B). This polypeptide was not labelled prior to the heat shock either in the plasma membrane fraction (Fig. 4.3 lane A) or the soluble fraction (Fig. 4.3 lane C). It is therefore a heat shock protein, designated hsp30, since it does not correspond to any previously reported heat shock protein.

[^{35}S] methionine labelling would not have detected any methionine-free plasma membrane-associating hsp. At least one hsp of yeast (hsp26 - see section 1.2.6) has previously been found to lack methionine (Bossier *et al.*, 1989). The pulse labellings of plasma membrane proteins in Fig. 4.3 were therefore repeated using [^{3}H] leucine as isotope in place of [^{35}S] methionine. Apart from hsp30, no previously unidentified heat shock protein was detected in the plasma membranes of heat shocked cells with the use of this more abundant amino acid for *in vivo* labelling (Fig. 4.6 lane B). Small amounts of major hsps substantially isolated in the postribosomal supernatant fraction from heat shocked cells (namely hsp104, hsp90, hsp70 and hsp35; see lane D in Figs. 4.3 and 4.6) were found to be associated with plasma membrane preparations from heat shocked cells. However incorporation of label into these bands on gels of plasma membrane proteins was consistently only a small percentage of the labelling of hsp30 (see lane B of Figs. 4.3 and 4.6) The exception here is hsp26 which seems to copurify with the plasma membrane in

significant quantities (see Fig. 4.6, lane B and Fig. 4.7).

hsp30 does not correspond to any previously reported yeast hsp. Staining of protein gels and *in vivo* pulse-labelling studies conducted in conjunction with subcellular fractionation of heat shocked yeast reveal that the cellular levels of most well characterised hsps are considerably in excess of total cell levels of hsp30. This is expected given that most hsps are found in the cytosol whereas hsp30 is associated with the plasma membrane. Most of these other hsps are isolated predominantly in the high-speed supernatant fraction during subcellular fractionation of heat shocked yeast (see lane D of Figs. 4.3 and 4.6). There are however exceptions to this rule, such as the mitochondrial hsp60 (Reading *et al.*, 1989). hsp30 is, however, the most abundant hsp in the plasma membrane fraction of density 1.223g/cm^3 even though its total levels in heat stressed cells are considerably less than total cellular levels of hsp104, hsp90 hsp70, hsp35 and hsp26. Barely noticeable amounts of hsp30 were seen in the lighter membrane fraction banding at density 1.173g/cm^3 on the preparative gradients (Fig. 4.4 lane B), a fraction which primarily consists of mitochondrial membranes with a small proportion of the plasma membranes (Serrano *et al.*, 1991). Therefore, the data presented so far indicate that hsp30 is unique in that it is the only protein induced by the heat shock response which substantially copurifies with the plasma membrane (in addition, data presented in Chapter 5 demonstrates that hsp30 is extremely hydrophobic and as such is likely to be an integral membrane protein).

Figure 4.3.1 *In vivo* pulse labelling of proteins of the plasma membrane and postribosomal (soluble) fractions of *S. cerevisiae* before and during heat shock using [^{35}S]-methionine. Strain BJ2168 was grown to exponential phase at 20^{0}C and pulse labelled with [^{35}S]-methionine (as described in section 2.9.3) either for 40 minutes at 20^{0}C, or 10-50 minutes after temperature upshift to 40^{0}C. Labelled proteins of the plasma membrane fractions from the unstressed (A) and heat shocked (B) cells; also, the postribosomal supernatant fractions from the same labellings (C and D respectively) were separated on a 12.5% gel and visualised by fluorography. Molecular masses of markers are indicated on the left, as are the positions of the 110kDa H$^+$-ATPase (solid arrow, samples A and B) and a 30kDa protein of heat-shocked plasma membranes (open arow, sample B only). The major heat-shocked proteins of the soluble fraction are indicated on the right. All lanes were loaded with 10,000 cpm acid precipitable material.

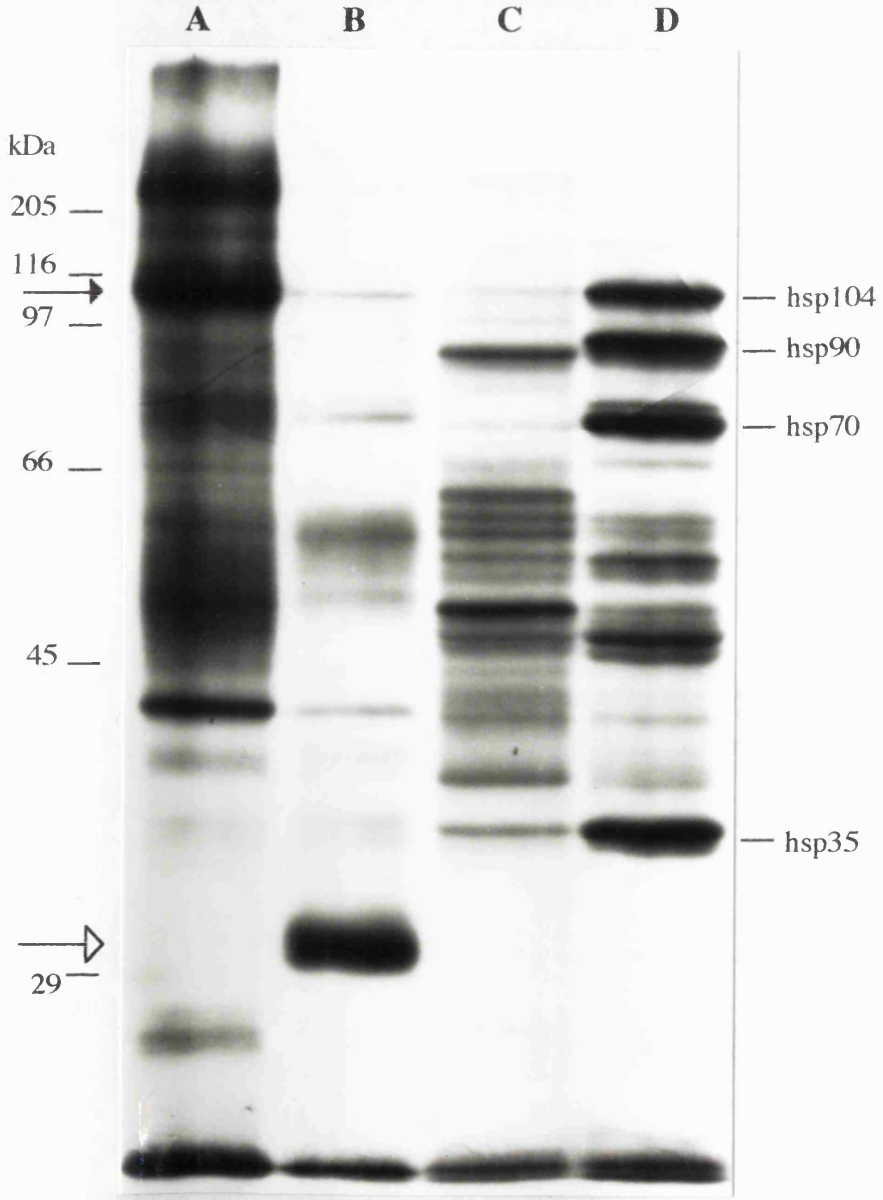


Figure 4.3.2 ***In vivo*** **pulse labelling of proteins of the plasma membrane and postribosomal (soluble) fractions of** ***S. cerevisiae*** **before and during heat shock using [^{35}S]-methionine.** A longer exposure of the gel shown in figure 4.3.1.

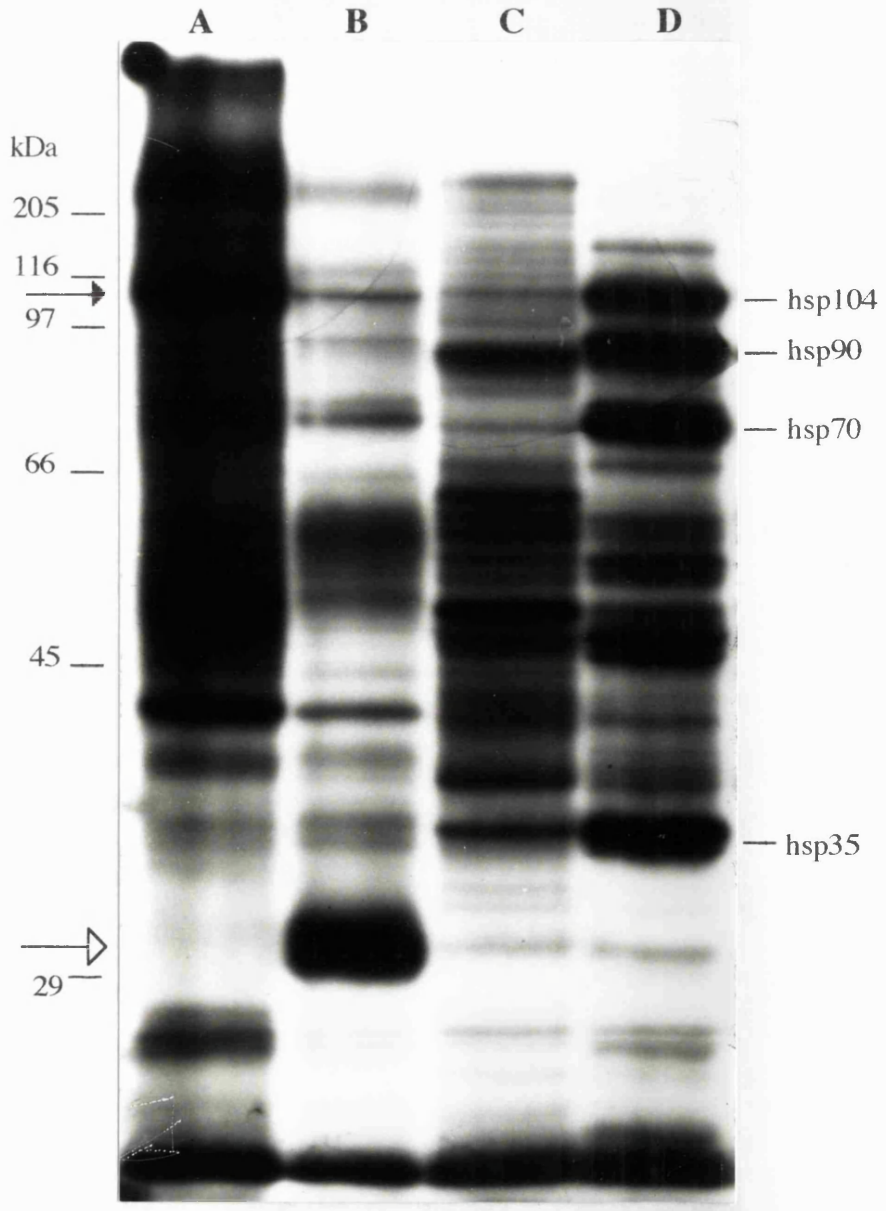
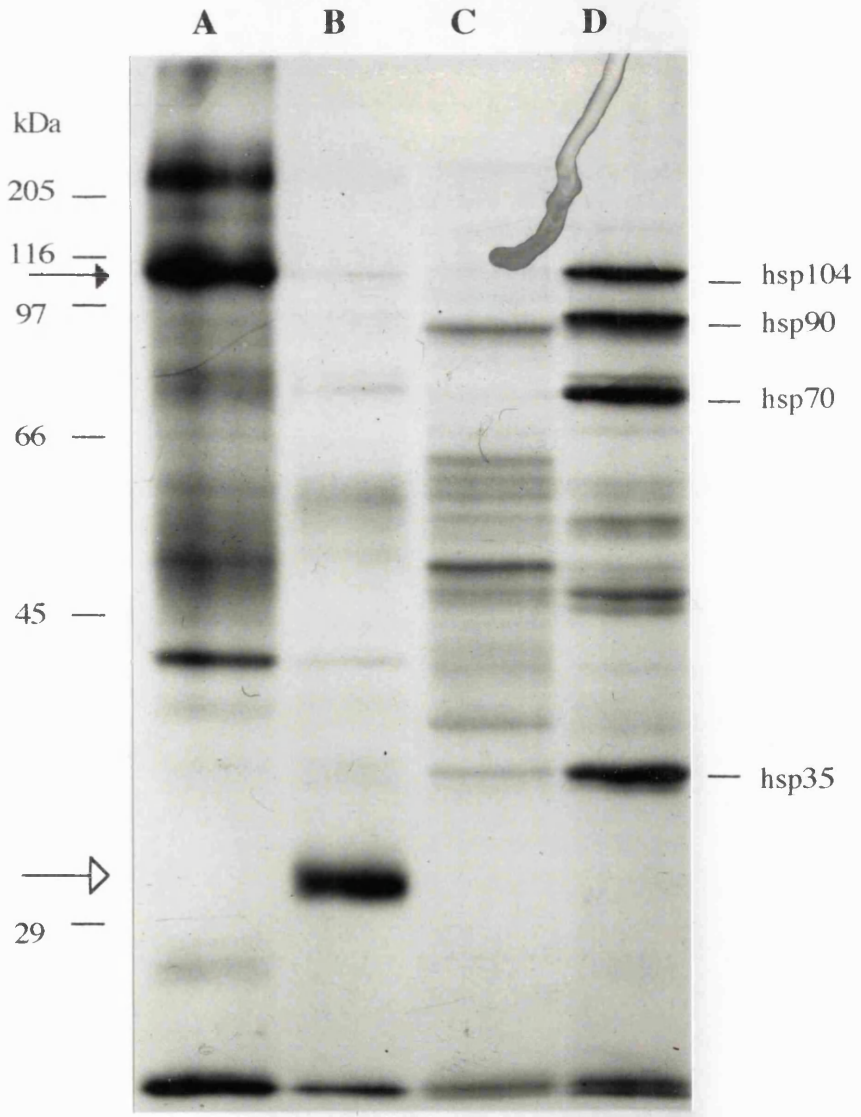


Figure 4.3.3 ***In vivo*** **pulse labelling of proteins of the plasma membrane and postribosomal (soluble) fractions of** ***S. cerevisiae*** **before and during heat shock using [^{35}S]-methionine.** A shorter exposure of the gel shown in figure 4.3.1.



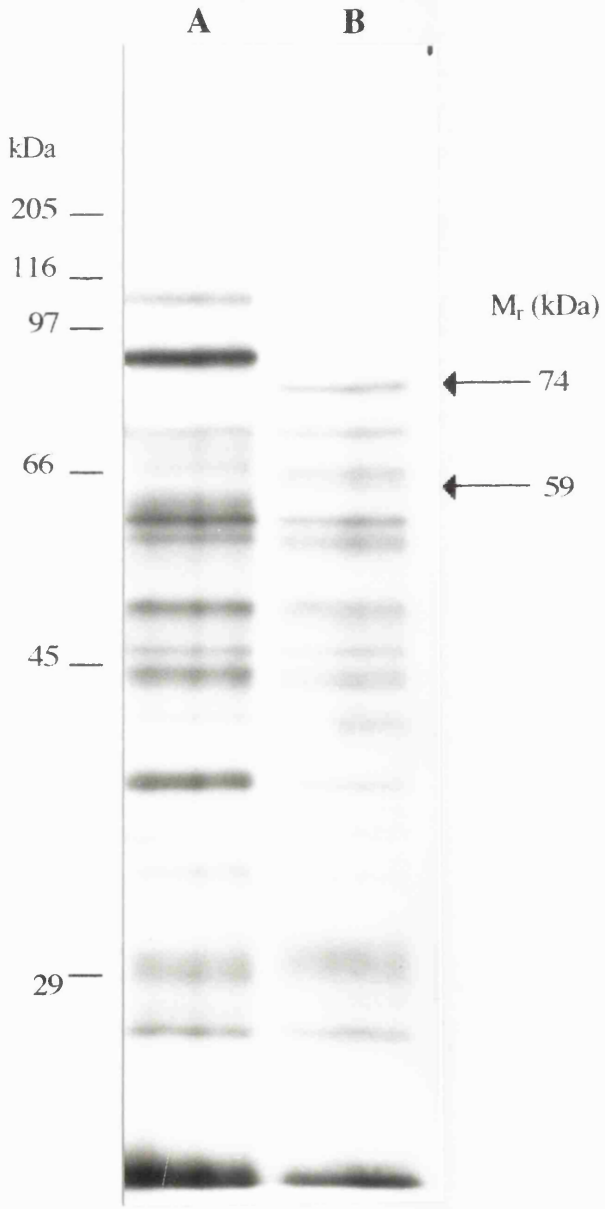
4.4.4 Mitochondrial proteins synthesised *de novo* during stress.

There is no account in the literature concerning changes in the protein composition of mitochondria brought about by stress. Though there is an account describing the analysis of proteins in mitochondria from heat shocked *Neurospora crassa*, it should be noted that identical experiments using unstressed cells were not carried out (Plesofsky-Vig and Brambl, 1990). This type of analysis could not have determined whether the proteins visualised were synthesised i) *de novo* during heat shock, or ii) both prior to *and* during stress.

To ascertain if the cell targets hsps to the mitochondrion during heat shock, proteins from unstressed and heat shocked mitochondria in the 1.173g/cm^3 (mitochondrial) fractions from the *same* pulse labelling experiment described in section 4.4.2 were resolved on gels and visualised by fluorography (Fig. 4.4). Lane B of Fig. 4.4 shows that mitochondria of *S. cerevisiae* acquire two new proteins of M_r 74 and 59kDa during heat shock. However, it is very likely that these proteins correspond to the mitochondrially located hsp70 homolog (mt-hsp70), the product of the *SSC1* gene (Craig *et al.*, 1989); and hsp60, the product of the *MIF4* gene (Reading *et al.*, 1989), respectively. Both genes are essential for growth at normal temperatures and are heat inducible. Also both possess an N terminal leader rich in basic and hydroxyl amino acids which is characteristic of mitochondrial targeting peptides. mt-hsp70 and hsp60 are involved in the import and assembly of mitochondrial proteins (see sections 1.21 and 1.22). Though it is well established that both proteins are heat shock inducible, there is no report in the literature showing that they are imported into the mitochondrion during stress. The presence of two extra proteins in heat shocked mitochondria (Fig. 4.4 lane B) with apparent molecular weights very close to 70 and 60kDa suggests that this is the case. Successful import of any polypeptide during heat shock is somewhat surprising when one considers the damage heat shock causes to the structure and function of mitochondria (see section 1.8.1).

These increased levels of mt-hsp70 and hsp60 in heat shocked mitochondria could act as damage limitation agents (acting in the same way as cytosolic hsp70, see section 1.2.1). Alternatively, these proteins could give mitochondria a "head start" during recovery from heat shock by rapidly importing/re-folding the influx of mitochondrial precursors needed to replace irreversibly damaged proteins.

Figure 4.4 *In vivo* pulse labelling of proteins of the mitochondrial fraction from *S. cerevisiae* before and during heat shock using [^{35}S]-methionine. Labelled proteins of the mitochondrial fractions from the unstressed (A) and heat shocked (B) cells, from the labellings described in section 4.4.2, were separated on a 12.5% gel and visualised by fluorography. Molecular masses of markers are indicated on the left. The positions of inducible proteins are indicated on the right. Both lanes were loaded with 2,500 cpm of acid precipitable material.



4.4.5 Resolution of high (M_r >205kDa) proteins.

The fluorograms presented in figures 4.3 and 4.4 were derived from 12.5% SDS-PAGE gels (15% in the case of Fig. 4.6) and as such provide poor resolution of proteins with an M_r greater than ca. 205kDa. For this reason the proteins from plasma membrane, mitochondrial and soluble extracts from the [^{35}S] methionine pulse labelling described in section 4.4.2 were resolved on a 7% gel (Fig. 4.5). This fluorogram does not reveal the existence of any novel high-molecular-weight hsps associating with any of the fractions.

It should be noted that polypeptides with molecular masses below ca. 14kDa are not resolved by the conventional SDS-PAGE system employed for all the gels shown thus far (see section 2.10.1). If time had permitted the possibility that hsps of M_r <14kDa associate with plasma membranes or mitochondria from heat shocked cells would have been investigated by using the Tricine SDS-discontinuous buffer system of Schagger and Von Jagow (1987) which can resolve proteins with an M_r as low as 2.5kDa.

Figure 4.5 The high molecular–weight proteins of the plasma membrane, soluble and mitochondrial fractions of unstressed and heat shocked *S. cerevisiae*. Proteins from various cellular fractions, obtained from the labellings described in section 4.4.2, were separated on a 7% gel and visualised by fluorography. The fractions from unstressed (U) and heat shocked (HS) cells were run alongside each other, and the amounts of acid precipitable radioactivity loaded were the same as those in figures 4.3 & 4.4. Molecular masses of markers are indicated on the left as is the position of the H^+-ATPase. Major heat-shock proteins of the soluble fraction are indicated on the right.

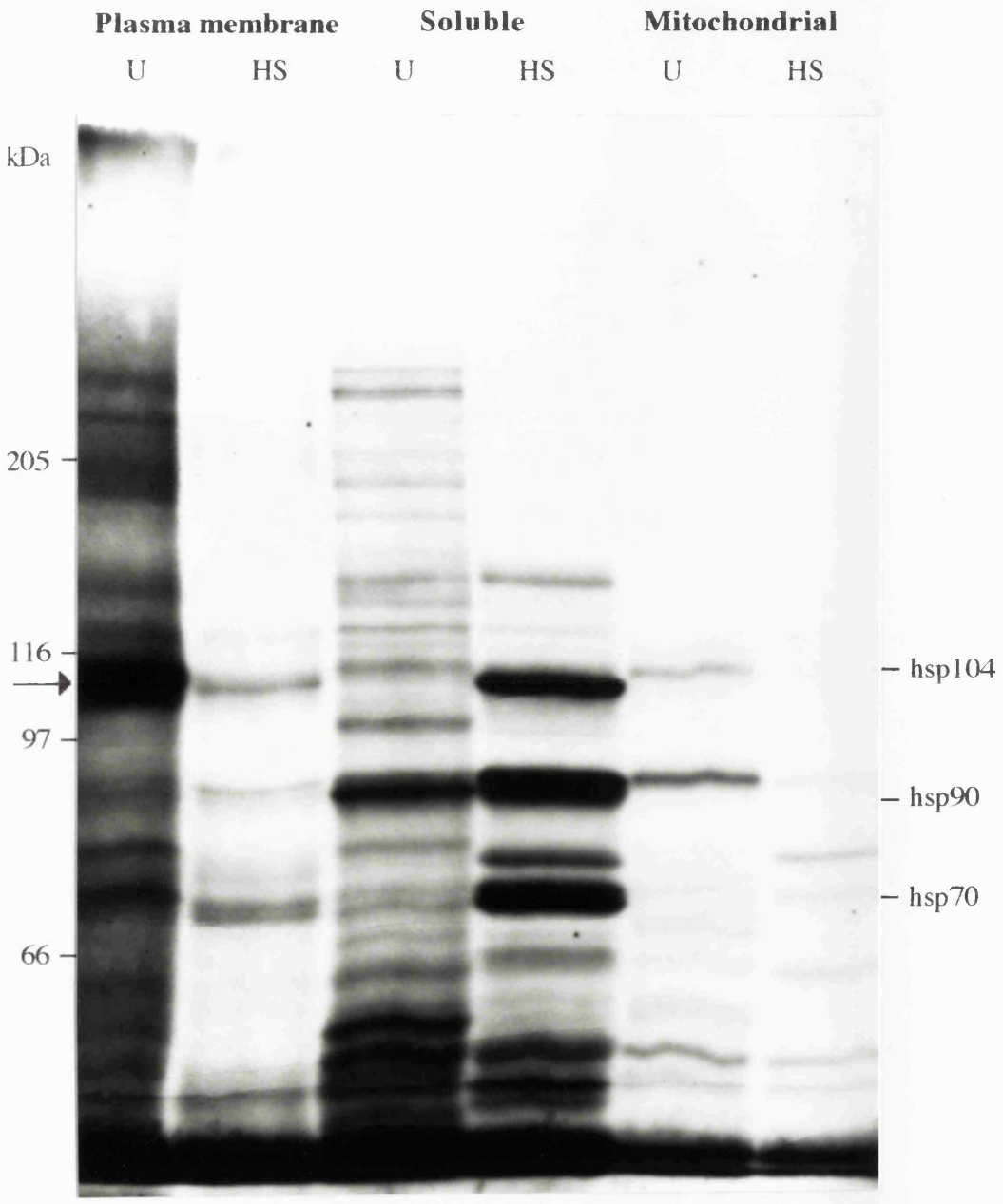


Figure 4.6.1 ***In vivo*** **pulse labelling of proteins of the plasma membrane and postribosomal (soluble) fractions of** ***S. cerevisiae*** **before and during heat shock using [^{3}H]-leucine as isotope.** The experiment was identical to that in Figure 4.3, except that [^{3}H]-leucine (see section 2.9.3) was used to label the proteins of unstressed and heat shocked cells, and a higher percentage (15%) gel was used for protein separation. The fluorogram shows labelled proteins of the plasma membrane fractions from the unstressed (A) and heat shocked (B) cells; also, the postribosomal supernatant fractions from the same labelings (C and D respectively). Molecular masses of markers and positions of the H^+-ATPase; the 30kDa protein of heat-shocked plasma membranes; and the major heat-shock proteins of the soluble fraction, are indicated as described in previous figures. All lanes were loaded with 10,000 cpm acid precipitable material.

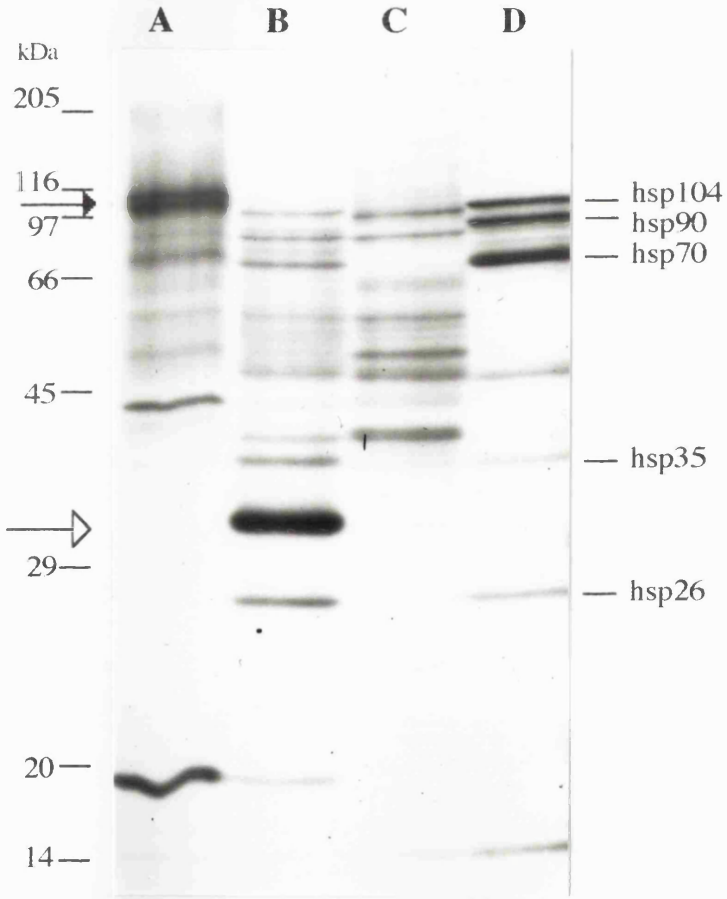
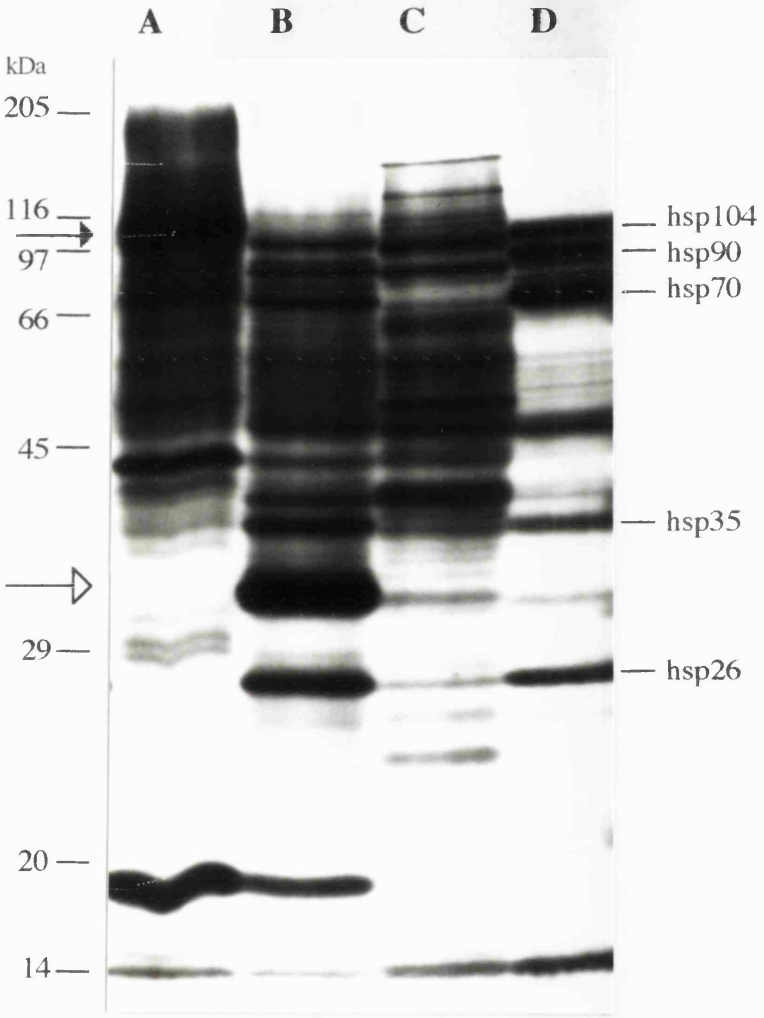


Figure 4.6.2 ***In vivo*** **pulse labelling of proteins of the plasma membrane and postribosomal (soluble) fractions of** ***S. cerevisiae*** **before and during heat shock using [^{3}H]-leucine as isotope.** A longer exposure of the gel shown in figure 4.6.1.



4.4.6 The 26kDa protein associating with plasma membranes from heat shocked cells is hsp26.

Figure 4.6 lane B reveals that a 26kDa protein associates with plasma membranes from heat shocked cells. This protein co-migrates with cytosolic hsp26 (Fig. 4.6 lane D). hsp26 of *S. cerevisiae* does not contain methionine, the N terminal methionine being removed post-translationally (Bossier *et al.*, 1989). Accordingly, hsp26 was not detected when cells were pulse labelled with [^{35}S] methionine (hence the absence of this protein in lane D of Fig. 4.3). Given that [^{35}S] methionine labelling also failed to detect the plasma membrane-associated 26kDa protein (compare lane B in both Figs. 4.3 and 4.6) it is likely that the membrane associated protein is also hsp26. To confirm this a western blot of a gel identical to that shown in Fig. 4.6 was probed with antiserum raised against hsp26 (as described in sections 2.11.3 and 2.11.4). As expected both the cytosolic and membrane associated proteins cross reacted with the antiserum (see Fig. 4.7.A). An identical blot, probed with antiserum raised against hsp90 (Fig. 4.7.B) is also presented.

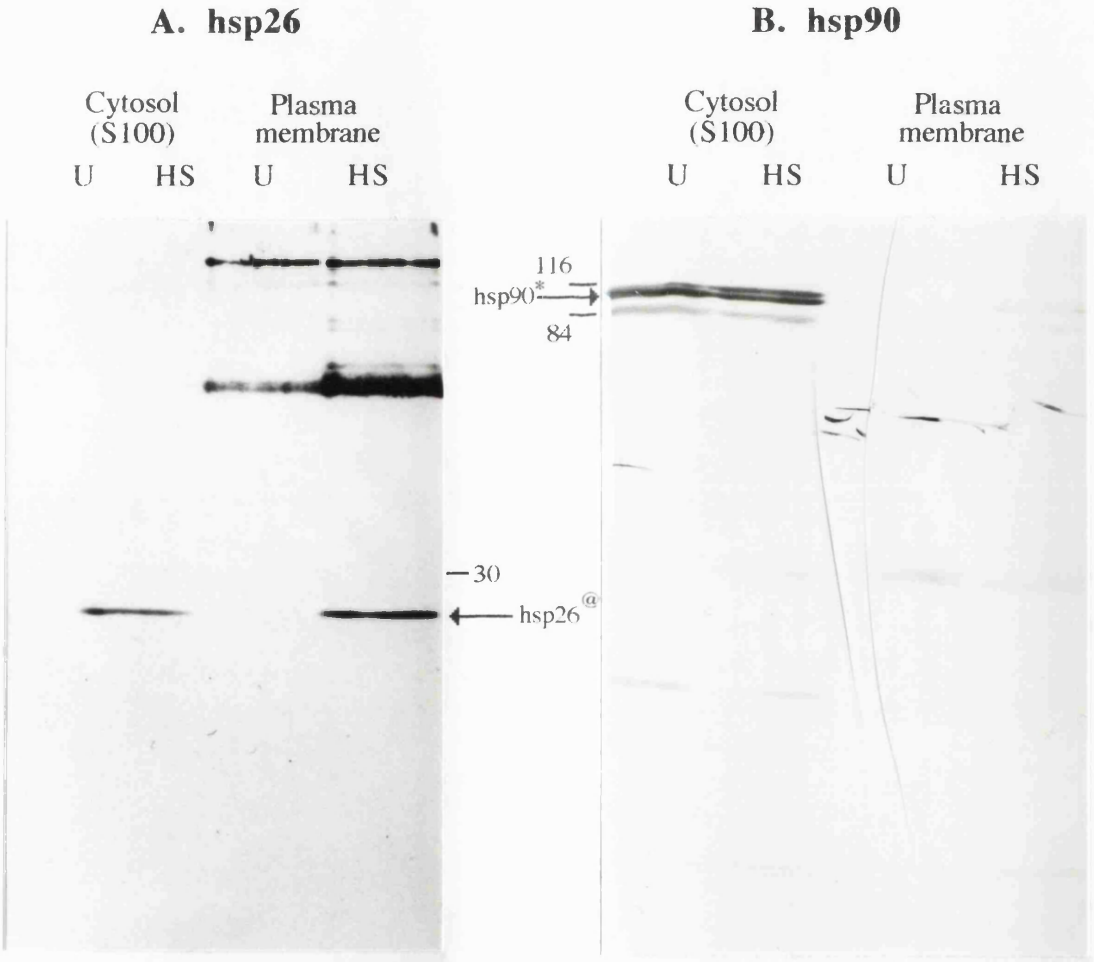
Pulse labelling illustrates that only small amounts of the major hsps predominantly found in the cytosol (hsp104, hsp90, hsp70, hsp35) are associated with the plasma membrane (Fig. 4.3). In the case of hsp90 this is also demonstrated immunologically (Fig. 4.7.B). The exception is hsp26; significant amounts of the polypeptide are plasma membrane associated. This is evident when one compares levels of hsp26 in lane D (cytosol) with levels in lane B (plasma membranes) of Fig. 4.6; comparison of the corresponding lanes of the immunoblot in Fig. 4.7.A leads to the same conclusion. All lanes of the gel from which the western blot shown in Fig. 4.7.A was derived, were loaded with equal amounts of protein. This, coupled with the fact that amounts of hsp26 associating with the plasma membrane approach the amounts found in the cytosol (Fig. 4.7.A), indicate that the concentration of hsp26 in the former is greater than the concentration of hsp26 in the latter. In addition, high levels of hsp26 are also associated with mitochondria from heat shocked cells; this too was shown by pulse labelling as well as by immunoblotting (not shown).

The finding that hsp26 seems to associate with membranous structures has also been noted in other organisms. In *Neurospora crassa* large quantities of the hsp26 homolog (designated as hsp30 in this organism) associate with mitochondria (Plesofsky-Vig and Brambl, 1990); in higher plants hsp22 is found at the thylakoid membranes (Adamska and Kloppstech, 1991). In both cases association of the corresponding protein was stable in the presence of high salt concentrations. In binding assays, the mitochondrial hsp26 homolog of *Neurospora crassa* synthesised *in vitro* bound strongly to mitochondria from heat shocked cells but not to mitochondria from unstressed cells (Plesofsky-Vig and Brambl, 1990). This suggests that it is a change in: i) membrane structure *per se*; or ii) a membrane associated component, that is responsible for tight binding of these low molecular weight

hsps to membranes. The role of hsp26 in this case may involve limitation of the damage that heat shock will cause to: i) membrane bound proteins; or ii) the membrane itself.

All organisms possess at least one homolog of hsp26 (Lindquist and Craig, 1988); in higher plants there is a prominent and heterogeneous group of these small hsps (14-27kDa) coded for by multi-gene families (Schoffl and Key, 1982). Most of the cellular complement of hsp26 is located in the cytosol and is incorporated into large heat-shock granules (see section 1.2.6). The function of hsp26 remains unclear. Yeast strains in which the corresponding gene has been deleted have no discernable phenotype (see section 1.2.6). Consequently, it is difficult to speculate on the role these hsps play at the plasma membrane (data presented here) the mitochondrion (Plesofsky-Vig and Brambl, 1990) or the thylakoid (Adamska and Kloppstech, 1991).

Figure 4.7 The association of cytosolic hsps with the plasma membranes of heat shocked cells. Proteins from the plasma membrane and soluble fractions from unstressed (U) and heat shocked (HS) cells, prepared as described in sections 2.9-2.9.2, were resolved on 15% gels. The proteins were transferred onto nylon membranes as described in section 2.11.3. Blots were probed with antisera raised against the two cytosolic hsps indicated, as described in section 2.11.4. Positions of pre-stained markers are indicated in the centre of the figure (only markers nearest to the protein of interest are shown). All tracks on the gels were loaded with 50μg of protein.



* Two proteins strongly cross-reacted with the antiserum since antibodies raised against hsp90 will also recognise the slightly smaller constitutively-synthesised homolog, hsc90 (Cheng *et al.*, 1992). These bands do not represent non-specific interactions since they did not appear when an identical blot was probed with pre-immune anti-serum (not shown).

@ A negative control could not be carried out since pre-immune antiserum was unavailiable. However, this band does not appear at the corresponding position in the unstressed fractions which is expected given that one would not expect to see hsp26 here. Accordingly, the strong cross-reaction with a protein of M_r 26kDa in the stressed fractions only, indicates that this band represents binding of antobodies specific for hsp26. Probing an identical blot with pre-immune antiserum would determine whether the two additional bands recognised in the plasma membrane isolates represent specific cross-reaction with anti-hsp26 antibodies.

4.4.7 hsp30 is induced during entry into stationary phase.

A number of heat shock proteins are induced as yeast cells undergo the transition from exponential growth to stationary phase, these becoming major cell proteins in stationary cultures. Among the inducible proteins are hsp26 (Petko and Lindquist, 1986); hsp12 (Praekelt and Meacock, 1990); hsp104 (Sanchez and Lindquist, 1990); polyubiquitin (Finley *et al.*, 1987); and some of the members of the hsp70 family, namely the products of the *SSA1/2* and *SSA3* genes (Werner-Washburne *et al.*, 1989; also see Table 1.2). To determine if hsp30 is similarly induced at stationary phase, plasma membranes were prepared from cells which had 12-18 hours previously entered stationary phase through carbon source (glucose) limitation. Analysis of the proteins of this membrane preparation clearly showed the acquisition of hsp30 and a decline in levels of the H^+-ATPase (Fig. 4.8, lane B), changes identical to the two major changes to plasma membrane protein composition seen with heat shock (Fig. 4.2, lane B). hsp30 therefore resembles other yeast hsps in that it is induced both by heat stress and during entry into stationary phase.

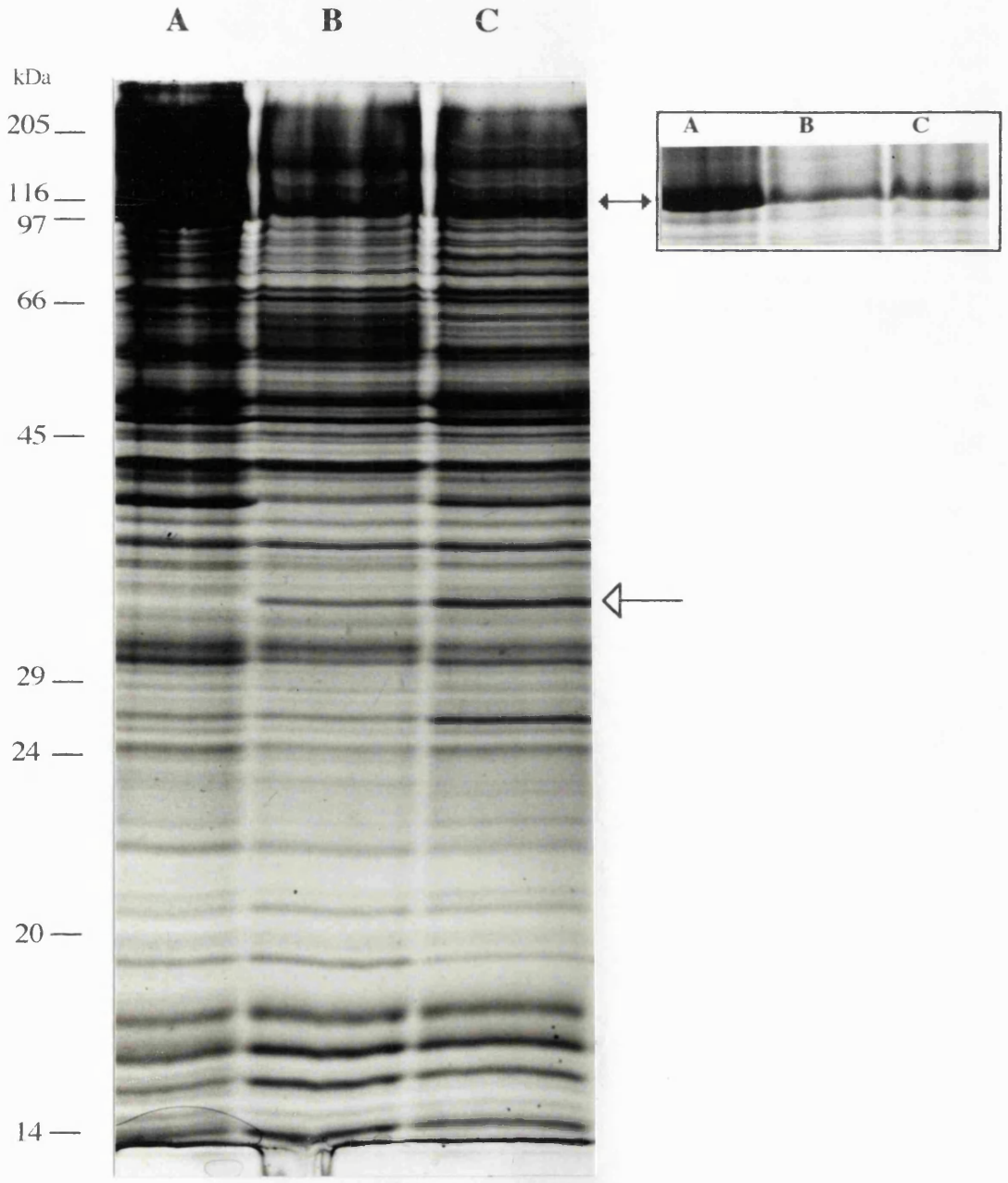
Figure 4.8 The protein composition of plasma membranes from stationary phase cultures of *S. cerevisiae*. A 15% gel stained with Coomassie blue, of plasma membrane proteins from the following cultures (grown in rich media) of strain BJ2168:

(**A**) **Unstressed** - cells were grown to exponential phase at 20^{0}C.

(**B**) **Stationary phase** - cells were harvested 18 hours after cultures had reached a density of 1x10^8 cells/ml. During this time cell density did not increase further.

(**C**) **Heat shocked** - cells from cultures described in A were given a 40 minute 20-40^{0}C heat shock.

100µg total membrane protein was fractionated in each lane. Molecular masses of markers are indicated on the left, the H$^+$-ATPase (solid arrow) and hsp30 (open arrow) are indicated on the right. The dramatic reduction in levels of the H$^+$-ATPase exhibited by heat shocked and stationary phase cells is illustrated more clearly by the inset on the right, which shows the appropriate section of a 10% gel loaded as described above.



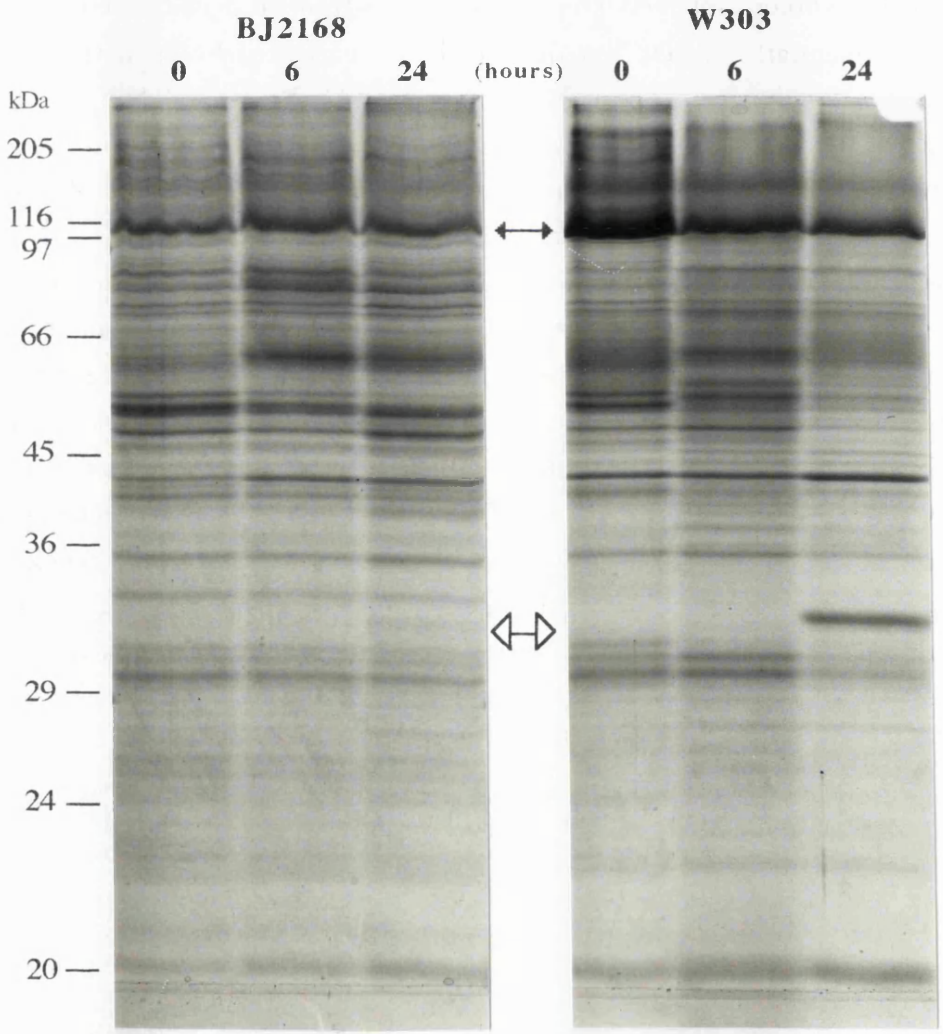
4.4.8 hsp30 is induced during itrogen starvation.

The gene coding for polyubiquitin (*UBI4*) is induced during starvation for nitrogen (Kirk, personal communication) as well as during heat shock and entry into stationary phase (Finley *et al.*, 1987). *UBI4* has been shown to be essential for survival during nitrogen starvation (Finley *et al.*, 1987). There are no reports concerning the induction of other hsp genes caused by nitrogen limitation.

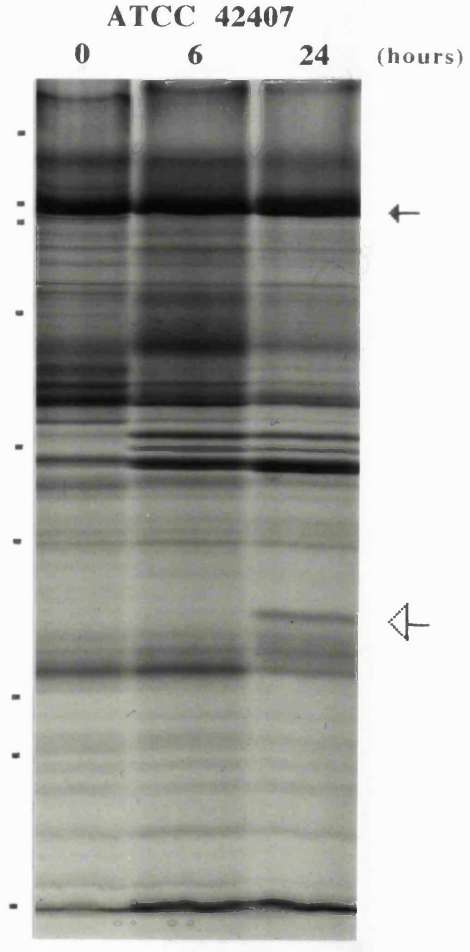
Deprivation of a nitrogen source to glucose growing cells is one of the ways by which protein half-life values are determined. The bulk of proteins in *S. cerevisiae* are quite stable having half life ($t_{1/2}$) values of ca.70 hours (Lopez and Gancedo 1979; Gancedo *et al.*, 1982). However, all membrane bound proteins so far examined show substantially lower $t_{1/2}$ values, ranging from 3 to 11 hours (Benito *et al.*, 1991). To study these proteins Lagunas and coworkers, routinely prepare membranes from nitrogen limited yeast. Cells growing exponentially at 30^{0}C on rich media are harvested by centrifugation, and resuspended in an ammonium free medium as described in Busturia and Lagunas (1986). Dr. Lagunas (Instituto de Investigaciones Biomedicas del CSIC, Madrid) kindly provided plasma membranes from cells which have been limited for nitrogen for up to 24 hours. The proteins from these samples were resolved by 1D SDS-PAGE and visualised by Coomassie blue staining (Fig. 4.9). Samples from strains BJ2168, (see section 2.2 for genotype), W303* and the wild type ATCC 42407 were provided. Levels of H+-ATPase did not undergo major changes. However, all three strains induced hsp30 between 6 and 24 hours after nitrogen was removed from the culture medium. There were also a number of changes in the levels of proteins which were present prior to nitrogen starvation (most evident with respect to proteins with an M_r >66kDa). In addition there is the dramatic induction of a 45kDa protein after 6 hours of nitrogen starvation in strain ATCC 42407 *only* (Fig. 4.9). This is peculiar given that all 3 yeasts used were strains of *S. cerevisiae* and as such would have been expected to respond to nitrogen limitation the same way..

*W303 (MATa *ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*)

Figure 4.9 The protein composition of plasma membranes from cultures of *S. cerevisiae* limited for nitrogen. Cells growing exponentially at 30°C in rich medium were harvested by centrifugation, washed with ammonium free medium, resusupended in this medium in the prescence of 2% (w/v) glucose, and incubated at 30°C (as described in section 4.4.8). Aliquots removed at the indicated times were used to prepare plasma membranes*. Changes occurring in the protein composition of the membranes from BJ2168, W303 and the wild type ATCC 42407 during nitrogen limitation, were examined by separating the proteins on 15% gels followed by staining with Coomassie blue. All tracks were loaded with 60μg of total membrane protein. Molecular masses of markers, together with the position of H+-ATPase and hsp30 are indicated as described in previous figures.



* All plasma membrane isolates were kindly provided by R. Lagunas (see section 4.4.8)


ATCC 42407
0
6
24
(hours)

4.4.9 Effects of osmotic shock.

It is well established that short term incubation with high (but sub lethal) concentrations of NaCl renders cultures tolerant to a more severe osmotic shock. The factors involved in acquisition of osmotolerance are poorly understood. Data presented in chapter 3 (Fig. 3.5) show that activity of the H^+-ATPase is one of the determinants of tolerance to high salt. This prompted an examination of the changes in H^+-ATPase activity that occur in parallel with acquisition of osmotolerance. Furthermore, a preliminary analysis of the changes in the protein composition of plasma membranes caused by osmotic shock was made.

Where studied, acquisition of osmotolerance by *S. cerevisiae* is diminished if protein synthesis is inhibited during an adaptive osmotic shock. Though there are a few examples in the literature where levels of particular *S. cerevisiae* proteins have been shown to increase during salt stress (see section 4.5.3), there is no report describing comparison of protein profiles derived from total cell extracts of osmotically shocked cells with those from unstressed cells. This is in sharp contrast to the situation in higher plants where there are many accounts describing changes in gene expression caused by salt stress (see section 4.5.3). The result of such an analysis carried out in *S. cerevisiae* is presented in Fig. 4.11.

Osmotolerance can be induced in exponentially growing yeast by incubating cultures in media with an NaCl concentration ranging from 0.25 to 0.75M (Blomberg and Adler, 1989). Typically, cultures subjected to intermediate stresses which induce tolerance to more severe stresses, do not result in any detectable cell death and exhibit appreciable rates of protein synthesis (as in the case of a pre-adaptive heat shock to 40^0C). Osmotic shock is no different (see Table 4.2 A and B for effects on protein synthesis and % survival figures for 50 minute incubations with 0.25M and 0.5M NaCl; both of which would induce osmotolerance).

Cultures of strain BJ2168 were grown to exponential phase in rich media at 20^0C. A volume of 4M NaCl was added to give desired final concentrations. Cells were harvested following a 40 minute incubation; plasma membranes were prepared as described in sections 2.9-2.9.2. Proteins from these isolates were resolved by 1D SDS-PAGE alongside samples from unstressed and heat shocked cells. Proteins were visualised by staining with Coomassie blue. Levels of the H^+-ATPase did not change significantly during osmotic shock, in contrast to the dramatic reduction in levels caused by heat shock (Fig. 4.10). Also, unlike heat shock (Fig. 4.2) entry into stationary phase (Fig. 4.8) and nitrogen starvation (Fig. 4.9), salt stress does not induce hsp30 (unless it is induced in levels too low to be detected by Coomassie blue staining). However, there is a discernable reduction in levels of various proteins that existed prior to salt stress (Fig. 4.10). This work could be continued by a more thorough analysis involving pulse labelling of cells

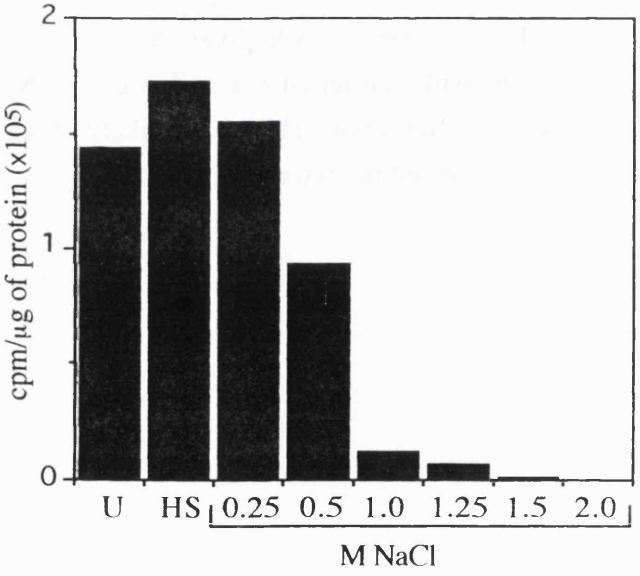
during osmotic shock, similar to the analysis carried out with respect to heat shock (see Figs. 4.3. and 4.6). Also, H^+-ATPase activity of membranes derived from osmotically shocked cells was assayed as described in section 2.9.4 (see section 4.4.10 and Fig. 4.12.1)

In addition to promoting acquired osmotolerance, incubation of cells with 0.25 or 0.5M NaCl is also associated with appreciable rates of protein synthesis (Table 4.2.A). Proteins (pulse labelled *in vivo*) of total cell extracts from osmotically shocked cultures of strain BJ2168 (prepared as described in the legend to Table 4.2.A) were resolved by 1D SDS-PAGE and visualised by fluorography. Protein profiles from osmotically shocked yeast were compared with those from unstressed and heat shocked cells (Fig. 4.11 A and B). It is noticeable that osmotic shock does not induce any of the hsps. This is somewhat surprising given the wide range of stresses that induce hsps (see Table 1.1) Instead, Fig. 4.11 A and B show that osmotic shock induces proteins of the following M_r : i) 20kDa (present at low levels in unstressed cells) ii) 31kDa and iii) 43kDa (plus another protein with a slightly higher M_r). None of these are strongly induced (unlike hsps during heat shock). However,they may play an important role given that inhibiting protein synthesis during pre-adaptive incubations with sub-lethal concentrations of NaCl diminishes the acquisition of osmotolerance (Trollmo *et al.*, 1988). The likely identities of at least the 31kDa and 43kDa proteins are discussed in section 4.5.3.

Table 4.2 A. The effect of osmotic shock on protein synthesis. Strain BJ2168 was grown to exponential phase at 20^{0}C and pulse labelled with [^{3}H]-leucine as described in section 2.6.1, under the following conditions: 40 minutes at 20^{0}C (U), 10-50 minutes after a temperature upshift to 40^{0}C (HS) and, 10-50 minutes after addition of 4M NaCl to give the final concentrations indicated. Total cellular protein was extracted and the amount of radioactivity incorporated per μg of protein was determined, as described in sections 2.6.2 and 2.7, respectively. Data represent mean values of 3 determinations, values obtained did not vary by more than +/- 5%.

B. Survival of osmotically shocked cells. The proportion of cells surviving the treatments described above was also determined, as described in section 2.5.3. The figures given represent the mean of 8 assays, the values obtained did not vary by more than +/- 6% in each case.

A.

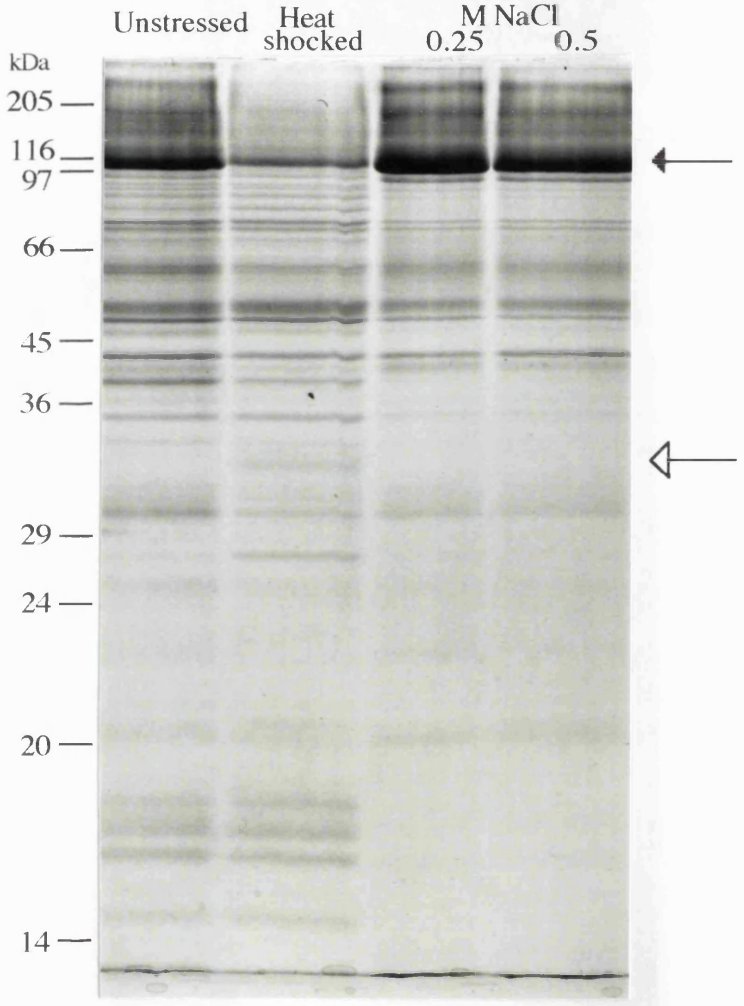


Conditions of pulse labelling

B.

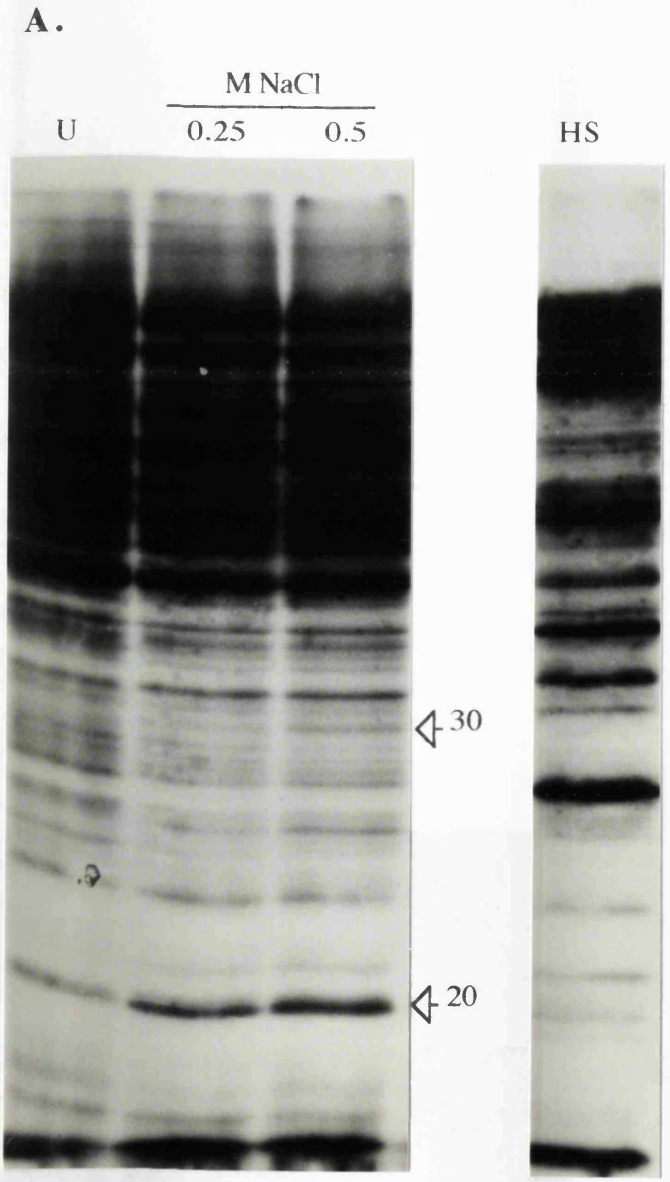
Treatment	U	HS	M NaCl 0.25	0.5	1.0	1.25	1.5	2.0
% survival after 50 minutes	100	101	103	100	62	18	7	2

Figure 4.10 Proteins of isolated plasma membranes from osmotically shocked yeast. Plasma membranes from osmotically shocked cells* were prepared as described in section 4.4.9. Total membrane proteins were resolved on a 15% gel and visualised by staining with Coomassie blue. Molecular mass markers and the positions of H^+-ATPase and hsp30 (heat shock lane only) are indicated as described in previous figures.

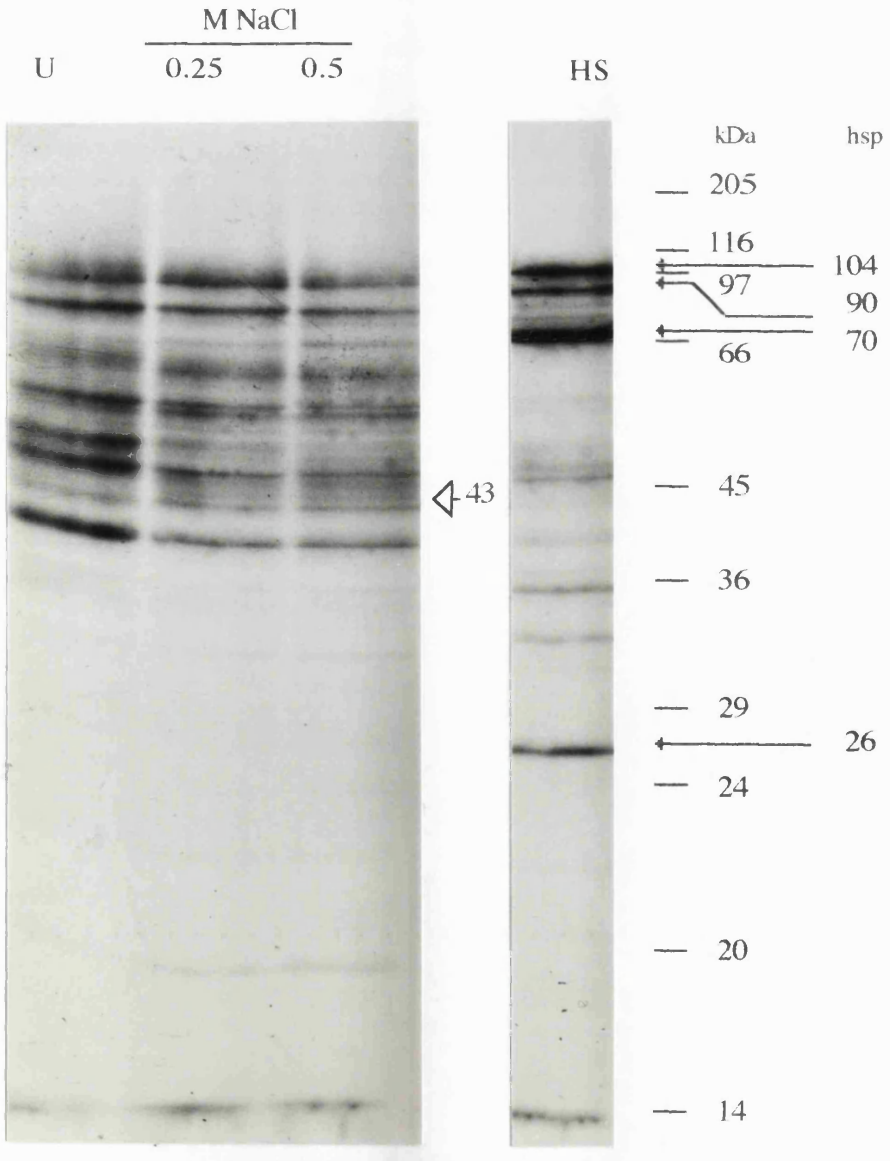


*Cells were maintained in rich media throughout and, unlike the pulse labelling of osmotically shocked cultures described in Fig. 4.11, were not transferred to minimal media.

Figure 4.11 Proteins synthesised during sub-lethal osmotic shock. Samples of total cellular protein extracted from the pulse-labellings described in Table 4.2.A were separated on 15% gels and visualised by fluorography. Each lane was loaded with 5μg of protein. Samples from unstressed (U) and heat shocked (HS) cells were run alongside samples from cells pulse labelled for 40 minutes in the presence of 0.25 and 0.5M NaCl. Positions of the major hsps (solid arrows); proteins induced by osmotic shock (open arrows); and molecular mass markers (solid lines) are indicated. A & B are different exposures of the same gel. [Samples from cells incubated with > 0.5M NaCl were not analysed because such stresses were found to be lethal and, not surprisingly, result in vastly diminished rates of protein synthesis (see Fig. 4.2 B & A, respectively)].



B.


M NaCl
U
0.25
0.5
HS
kDa
hsp
205
116
104
97
90
70
66
43
45
36
29
26
24
20
14

4.4.10 Activation of H+-ATPase by stress.

The crucial physiological role of H+-ATPase requires strict regulation; this always occurs at the level of specific activity which can vary by as much as 10 fold (Serrano, 1983). Though the molecular basis of regulation remains unclear it is well established that it does not involve an increase in levels of the enzyme. Where studied, the activated state of the enzyme is preserved after purification of plasma membranes and probably corresponds to a stable covalent modification of the polypeptide (Serrano,1983; Eraso and Gancedo, 1987; Rosa and Sa-Correia, 1991; Viegas and Sa-Correia, 1991).

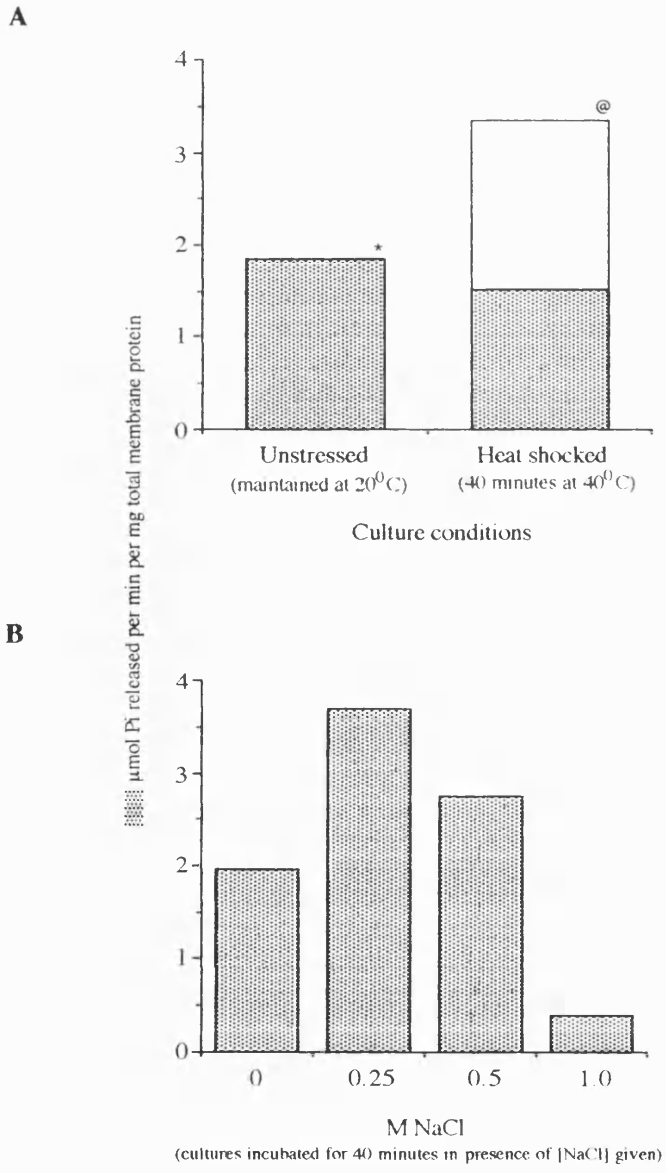
Since activity of H+-ATPase is one of the determinants of stress tolerance, there are grounds to suspect that activity of this enzyme is also stress regulated. Consequently, the activity of purified plasma membranes derived from heat shocked and salt stressed cells was determined as described in section 2.9.4. It should be noted that activity is usually expressed per mg of membrane protein. In all circumstances so far examined levels of the enzyme do not change, so values from different isolates can be compared. However, heat shock causes a dramatic reduction in the levels of H+-ATPase (Fig. 4.2, lane B). Expressing activity of the enzyme from unstressed and heat shocked cells per mg of membrane protein therefore represents the activity of different amounts of enzyme. To enable comparison of H+-ATPase *per se*, the amount of enzyme remaining in plasma membranes after heat shock was quantified by densitometric scanning of the band representing H+-ATPase (on the immunoblot presented in Fig. 4.2) as described in section 2.11.4.2. From these scans (Fig. 4.12.2) it was found that a 40 minute 20-40^{0}C heat shock resulted in a decrease in H+-ATPase levels of 55%. This problem was not encountered when cells were osmotically shocked. Incubating cells in 0.25, 0.5M (see Fig. 4.10) and 1.0M (not shown) NaCl, did not cause significant changes in levels of the H+-ATPase polypeptide.

A 40 minute 40^{0}C heat shock results in activation of H+-ATPase activity of purified plasma membranes by 1.8x (Fig. 4.12.A); activity of the enzyme from cells incubated with 0.25 and 0.5M NaCl for 40 minutes was respectively 1.9 and 1.4x greater than the enzyme from unstressed cells (Fig. 4.12.1). Implications of this are discussed in sections 4.5.1 and 4.5.3). Subjecting cultures to the intermediate stresses described above, enable cells to survive when challenged with a more severe stress. In contrast, H+-ATPase from cells which underwent a more severe osmotic shock (1M NaCl; outside the range of concentrations which confer osmotolerance) was 4.9x *less* active than enzyme from unstressed cells. This was not surprising given that the 40 minute incubation with 1M NaCl caused a 38% loss in the number of viable cells (Table 4.2.B); such a treatment is obviously damaging various components of the cell (in addition to the H+-ATPase) and

constitutes a lethal stress. These results suggest that conditions which lead to acquisition of both osmotolerance and thermotolerance result in activation of H^+-ATPase.

Figure 4.12.1 Effect of stress on H+-ATPase activity Plasma membranes were purified from heat shocked (A) and salt stressed (B) cultures of BJ2168, as described in sections 4.4.1 and 4.4.9, respectively. H+-ATPase activity from these isolates was determined as described in section 2.9.4, and is compared with the activity associated with membranes from unstressed cells.

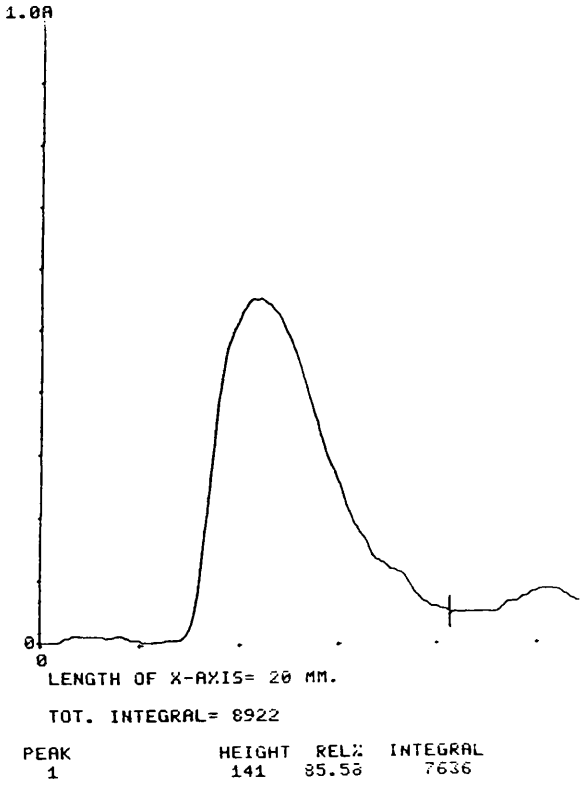
(Data represent mean values of 6 determinations; values obtained not varying by more than +/- 5% for each set.)



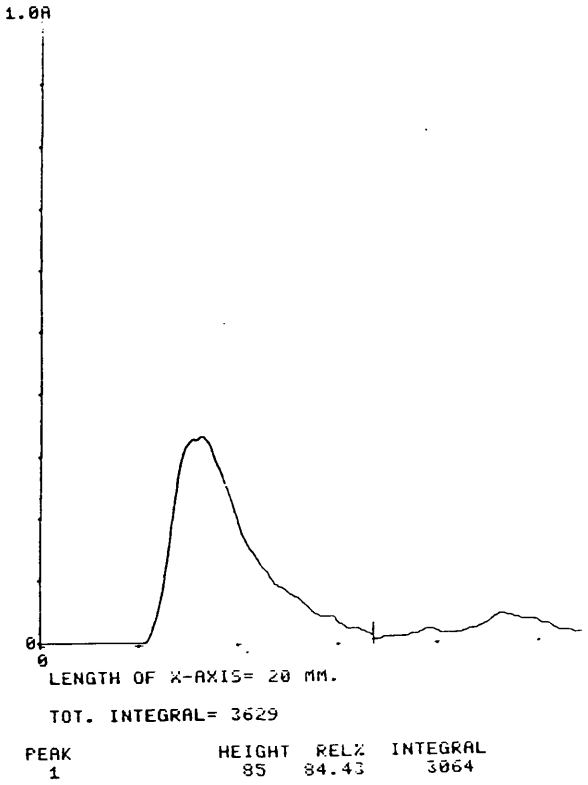
[@]Heat shock causes a dramatic reduction in levels of H+-ATPase (see Fig. 4.2.) and prohibits comparison of H+-ATPase activity *per se* before and after heat shock, since activity is usually expressed per mg of total membrane protein. This was overcome by quantifying the drop in levels of the enzyme. Fig. 4.12.2 shows that 55% of the H+-ATPase polypeptide is lost during a 40 minute 40⁰C heat shock. Accordingly, the activity from heat shock isolates was adjusted to the level shown by [@] (* and [@] represent activity of the same amount of H+-ATPase).

Figure 4.12.2 Quantifying the decline of H+-ATPase levels during heat shock. The bands indicated on the immunoblot shown in Fig. 4.2 represent H+-ATPase. They were scanned by reflective densitometry as described in section 2.11.4.2. Integration of the curves representing H+-ATPase was carried out automatically. Representative scans of immunodecorated H+-ATPase from unstressed (A) and heat shocked (B) isolates are provided. The figures given beneath each scan represent mean integrals of 6 scans carried out along the length of each band; values did not vary by more than +/-10% for each set.

A



B



Mean integral: 6981

Mean integral: 3141

(mean integral of B is 45% of that for A)

4.4.11 *In vitro* phosphorylation of plasma membrane proteins from stressed cells.

Activity of the H+-ATPase from purified plasma membranes increases when cells are exposed to elevated temperatures or osmotic shock (Fig. 4.12.1). Furthermore, activity of this enzyme is one of the determinants of tolerance to these two types of stress (Figs. 3.1, 3.2 and 3.5). Accordingly, the mechanisms activating H+-ATPase during stress are of interest. The activated state of the enzyme is preserved after purification of plasma membranes and probably corresponds to covalent modification of the polypeptide. This is true of all known agents that activate H+-ATPase such as glucose (Serrano, 1983) and acidification of media during growth (Eraso and Gancedo, 1987). Covalent modification by phosphorylation is the most common mechanism involved in biological regulation (Kennelly and Krebs, 1991). Phosphorylation of other membrane bound polypeptides could also play a role in the response to stress. For these reasons the *in vitro* phosphorylation activities associated with membrane proteins from unstressed and heat shocked cells were examined as described in section 2.15

There are various reports of *in vitro* phosphorylation of plasma membrane proteins from both *S. cerevisiae* (McDonough and Mahler, 1982; Kolarov *et al.*, 1988) and *Sch. pombe* (Amory *et al.*, 1980; Amory and Goffeau, 1982). The reaction conditions used vary with respect to reaction temperature as well as concentrations of ATP and $MgCl_2$ used. Also, certain groups carry out such experiments on Triton-washed plasma membranes (Ulaszewski *et al.*, 1987 [a]). Reaction conditions used in section 2.15 are based on those described by Kolarov *et al.*, (1988). Kolarov *et al.* maintain that most of the phosphorylation reactions detected were attributable to a particular seryl kinase. For work described here plasma membranes were not washed in detergent prior to use since activity of many membrane bound enzymes is dependent on lipids (Sanderman, 1978). Also, lipid specificity may be important for physiological regulation as indicated by control of protein kinase C by diacylglycerol (Nishizuka, 1986).

The presence of the aspartyl-phosphate catalytic intermediates can obscure detection of phosphorylation of polypeptides by membrane kinases. However, phosphoproteins were resolved on Laemmli gels, the alkali pH of which causes hydrolysis of aspartyl phosphates (Amory *et al.*, 1980). Any incorporation of ^{32}P detected therefore represents the regulatory action of kinases (or autophosphorylation events). All of the O-linked phosphates (seryl, threonyl and tyrosyl phosphate) and the much rarer N-linked phosphates (histidinyl lysyl and arginyl phosphate) are stable on Laemmli gels (Castellanos and Mazon, 1985 and Wei and Mathews, 1991, respectively). The acidic methanol solutions used to stain gels had no effect on the phosphoprotein profiles or the extent to which each phosphorylated protein exposed the X-ray film (not shown). Therefore N-linked phosphoamino acids constitute a negligible proportion of the phosphoamino acids detected

by the autoradiograms presented in Figs. 4.13 and 4.14, since they are hydrolysed under acidic conditions (Duclos *et al.*, 1991). This is the first report comparing phosphoprotein profiles of plasma membranes from stressed cells with those from unstressed cells.

Figure 4.13 reveals that under conditions of assay many of the membrane bound polypeptides from unstressed, heat shocked and stationary phase yeast are phosphorylated. The most prominent is the ca.110kDa polypeptide which co-migrates with H^+-ATPase. This is not unexpected given the many reports in the literature describing the phosphorylation of H^+-ATPase when plasma membranes are treated with γ-[^{32}P] ATP (e.g. McDonough and Mahler, 1982; Yanagita *et al.*, 1982; Kolarov *et al.*, 1988). The most noticeable features of the phosphorylation profiles derived from the 3 membrane isolates are as follows:

i) Extent to which H^+-ATPase is phosphorylated. All lanes in the gel shown in Fig. 4.13 were loaded with the same amount of protein. Accordingly, only half as much H^+-ATPase from heat shocked cells was subjected to *in vitro* phosphorylation relative to the amount from unstressed cells (since heat shock results in at least a 50% reduction in levels of this enzyme). However, the bands representing H^+-ATPase in Fig. 4.13 from these two isolates are equally as intense (this is most noticeable in the lowest exposure). This indicates that the polypeptide from heat shocked cells is phosphorylated to a greater extent than the polypeptide from unstressed cells. This could be due to a) H^+-ATPase from heat shocked cells being more susceptible to phosphorylation b) increased activity of the appropriate kinase or c) activity of a kinase distinct from that acting in membranes from unstressed cells. The last two possibilities could be distinguished simply by subjecting the phosphorylated H^+-ATPase bands from the unstressed and heat shocked cells to limited proteolysis by protease V8 as described in section 2.12; followed by comparing autoradiograms of the resulting peptide maps (as demonstrated by Fig. 3.7). The greater activity of H^+-ATPase from heat shocked cells could be due in part to this greater degree of phosphorylation.

ii) Extent to which the polypeptide of M_r 50kDa is phosphorylated. The second most prominent phosphoprotein in all 3 isolates migrates as a diffuse band of M_r of 50kDa. and is phosphorylated to a greater extent in membranes from stationary phase cells (Fig. 4.13). Any account concerning experiments involving *in vitro* phosphorylation of membrane proteins invariably reports the existence of a prominent phosphoprotein of this molecular weight (e.g McDonough and Mahler, 1982, Amory and Goffeau, 1982). One report identifies it as the regulatory sub-unit of cAMP-dependent protein kinase (Behrens and Mazon, 1988).

iii) Phosphorylation of hsp30. Both entry into stationary phase and heat shock induce hsp30 (Fig. 4.8). However, only hsp30 in heat shock fractions is phosphorylated *in vitro* (Fig. 4.13). Speculation on implications of this must await determination of the role

of hsp30.

The 12.5% gel shown in Fig. 4.13 poorly resolves proteins with an M_r lower than ca. 30kDa. Identical samples were run on a 15% gel. Fig.4.14 shows that stationary phase membranes have an extra phosphoprotein of M_r29kDa. This is either due to i) phosphorylation during stationary phase of a protein present both prior to and during entry into stationary phase or ii) phosphorylation of a protein that is only present in stationary phase cells. Closely scrutinising lane B of the Coomassie stained gel presented in Fig. 4.8 suggests the latter (an extra protein at very low levels is discernable, although it could simply be a degradative product rather than a protein induced during stationary phase). Resolving this problem by the use of radiolabelled amino acids is not possible due to the inherent difficulties in pulse labelling cells as they enter stationary phase. There is no record of phosphorylation of this protein in the literature. Whether or not synthesis of this protein is induced on entry into stationary phase could be elucidated by probing northern blots of mRNA isolated from cells harvested at different stages of growth with the corresponding gene (once it becomes available). Finally, this gel also included phosphoproteins from cells which had been limited for nitrogen over 24 hours. Again there are differences in the extent of phosphorylation of the H+-ATPase and the 50kDa polypeptide. Also, though hsp30 is present in this sample it is not phosphorylated (unlike hsp30 from heat shocked cells).

One could argue as to the physiological relevance of such *in vitro* studies. It should be noted, however, that autoradiograms of peptide maps derived from *in vivo* and *in vitro* phosphorylated H+-ATPase are identical (McDonough and Mahler, 1982). Consequently, it is likely that purified plasma membranes possess an identical kinase activity that operates both *in vivo* and *in vitro*. Also, where studied, hydrolysis of phosphorylated H+-ATPase (phosphorylated *in vivo* or *in vitro*) only yields phosphoserine (McDonough and Mahler, 1982; Kolarov *et al.*, 1988).

Figure 4.13 ***In vitro*** **phosphorylation of plasma membrane proteins from** ***S. cerevisiae*****.** The plasma membranes from the unstressed, heat shocked and stationary phase cultures described previously were phosphorylated (as described in section 2.15), and the resultant phosphoproteins were separated on a 12.5% gel and visualised by autoradiography. Three different exposures are shown. The 30μl reaction mixture consisted of purified plasma membranes (50μg of protein), 50mM MES-KOH (pH 6.0), 6mM $MgCl_2$ and 15μM ATP (composed of 15μM Na_2ATP mixed with 1μCi of 3,000 Ci/mmol [γ-^{32}P] ATP). The reactions, carried out at 30^{0}C, were started by the addition of ATP and stopped 10 minutes later by adding 30μl of SDS gel sample buffer. Molecular mass markers and the positions of H^+-ATPase and hsp30 are indicated as described in previous figures.

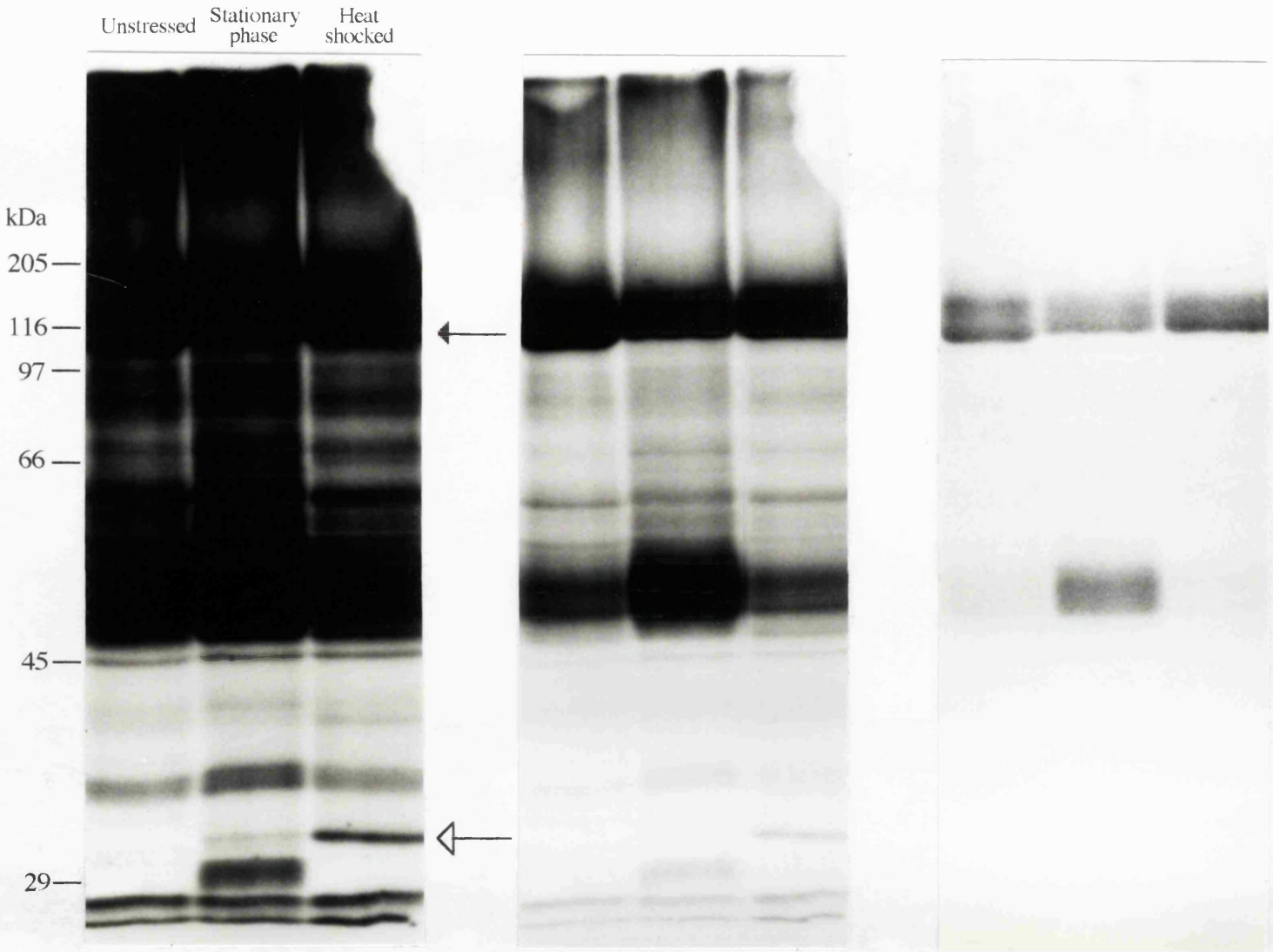
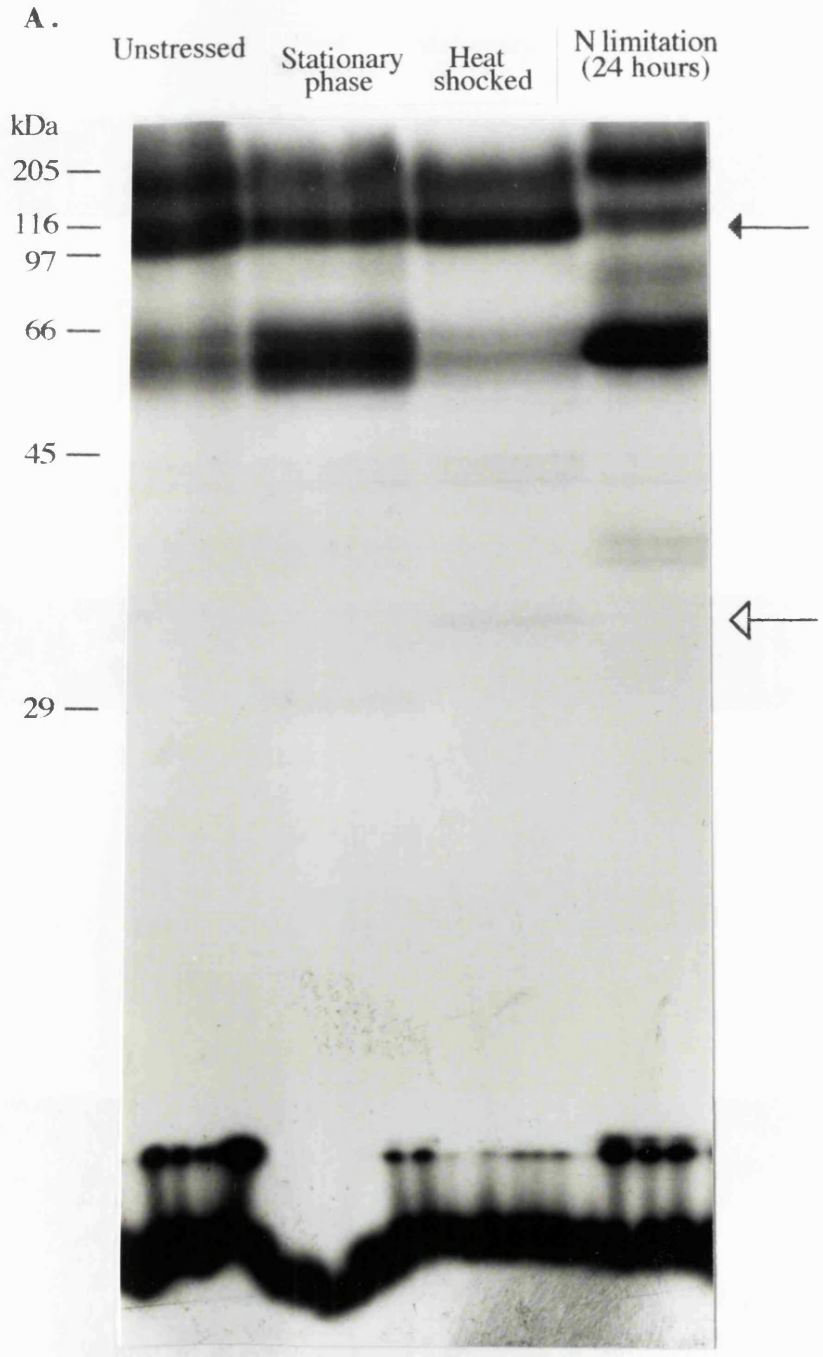
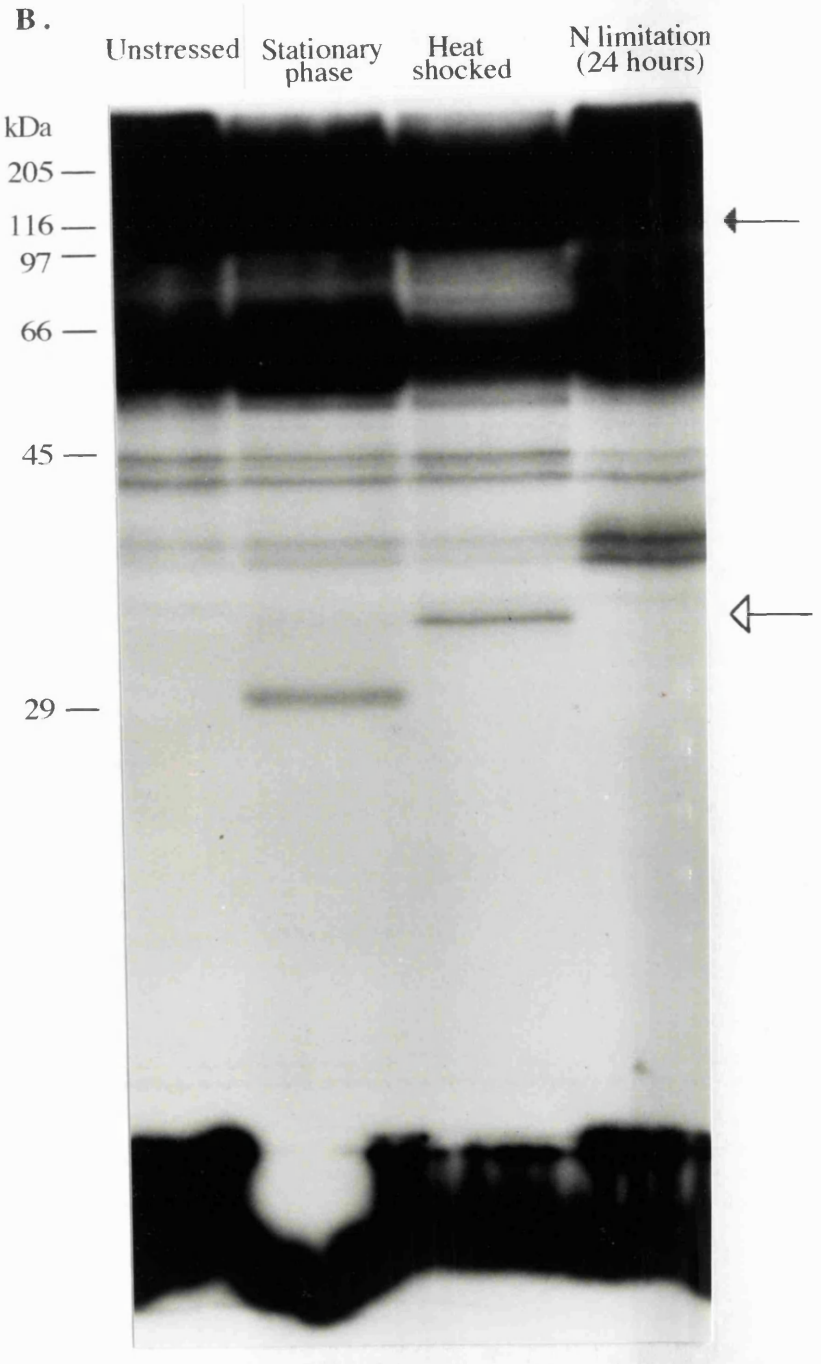

Unstressed
Stationary phase
Heat shocked
kDa
205
116
97
66
45
29

Figure 4.14 Resolving low molecular-weight phosphoproteins derived from *In vitro* phosphorylation of plasma membrane proteins. Phosphorylated membrane proteins prepared as described in Fig. 4.13 were resolved on 15% gels and visualised by fluorography. Two different exposures are shown (**A** and **B**). In addition to the unstressed, heat shock and stationary phase samples, phosphorylated membrane proteins from cells (of the same strain) which had been maintained for 24 hours under nitrogen starvation conditions (as described in section 4.4.8) were also included. Molecular mass markers and the positions of H+-ATPase and hsp30 are indicated as described in previous figures.




B.
Unstressed
Stationary phase
Heat shocked
N limitation (24 hours)
kDa
205
116
97
66
45
29

4.5 Discussion

4.5.1 Reduction of H+-ATPase levels and induction of hsp30 caused by heat shock.

The integrity of the plasma membrane is probably an important factor determining survival of a number of different stresses, yet events that occur at this membrane during stress are still poorly understood. The stimulation of plasma-membrane H+-ATPase activity in yeast by several inducers of the heat-shock response almost certainly helps to restore intracellular pH in response to its decline consequent upon increases in membrane permeability. In the fermentative bacterium *Streptococcus faecalis*, cytoplasmic acidification causes a similar increase in H^+ extrusion through enhanced activity of a H^+ translocating ATPase (Kobayashi *et al.*, 1986). Work presented here identifies a reduction in levels of the most abundant plasma-membrane protein, the H+-ATPase, during both heat shock and entry into stationary phase. Around 55% of the enzyme is lost during a 40 minute 20-40^{0}C heat shock. It follows that this turn-over of H+-ATPase in heat stressed cells, at a time when it is not being replenished by *de novo* synthesis, might progressively reduce the capacity for sustained H+-ATPase activity *in vivo* at 40^{0}C. This in turn would steadily reduce the ability of the cell to counteract the intracellular acidification that is one of the consequences of heat shock. However, H+-ATPase activity of purified plasma membranes from heat shocked cells is almost twice that from unstressed cells (Fig. 4.12.1). Since heat shock results in a 55% decline in H+-ATPase levels this implies that rates of H^+ efflux (*in vivo*) would be sustained during heat shock. However, rates of H^+ efflux determined *in vivo increase* by 50% when cells are incubated at higher (but sub lethal) temperatures (Coote *et al.*, 1991). This suggests that H+-ATPase activation caused by heat shock is due to i) a type of activation that is preserved during membrane purification (Fig. 4.12.1) as well as ii) 'additional' activation only detectable *in vivo* (Coote *et al.*, 1991).

It is surprising that the cell degrades more than half of the H+-ATPase in the cell membrane during a 40 minute 20-40^{0}C heat shock. This may be due to a more rapid rate of denaturation of the enzyme during heat shock, necessitating its removal from the membrane. Alternatively, H+-ATPase may be removed from the membrane so that the cell can maintain an adequate energy charge during stress. In the unstressed state, this enzyme usually uses ca. 40% of the ATP made by the cell. A ca. 2 fold activation of H+-ATPase during heat shock (Fig. 4.12.1) would surely compromise the cellular energy charge. It is conceivable that maintaining i) rates of H^+ efflux and ii) acceptable levels of ATP in the cell, is achievable by a combination of a 2 fold activation of the H+-ATPase and a ca. 50% reduction in levels of the enzyme.

The $t_{1/2}$ of the enzyme at 40°C is less than 40 minutes (Fig. 4.12.2); this is remarkable given that the $t_{1/2}$ of the enzyme in the unstressed cell is about 11 hours (Benito *et al.*, 1991). Furthermore, the degradation seems to be complete, in that low molecular weight fragments of the enzyme are not found in the membranes of heat shocked cells (see Fig. 4.2). Heat shock therefore activates a system that removes H+-ATPase from the cell membrane. hsps may play a role here since various components on the proteolytic degradation pathway based on ubiquitin are induced by heat shock (see section 1.2.7). To ascertain whether this is true, cells could be heat shocked both in the presence and absence of inhibitors of protein synthesis. The reduction in levels of H+-ATPase in the plasma membranes of these cells could then be compared. Alternatively, mutants lacking one or more of the components of the ubiquitin pathway could be used.

Remarkably, the major gene encoding the H+-ATPase (*PMA1*) is still actively transcribed in *S. cerevisiae* during heat shock, even though very little of its product is incorporated into the plasma membrane at these temperatures. It seems likely that, during recovery from heat shock, the resulting pool of *PMA1* mRNA will be used to restore levels of H+-ATPase. If this were true, *PMA1* mRNA would be unique, as the only heat inducible mRNA that is not translated during heat shock. Alternatively, *PMA1* mRNA may be translated at high temperatures but i) retained in the endoplasmic reticulum until temperatures return to normal levels or ii) retained in the cytosol since, with its multiple transmembrane domains, it may be too complex a structure to assemble in cells that are in a state of severe stress. However, it is doubtful that the latter is true, since hsp30 is an integral membrane protein (Fig. 5.5; and H. Boucherie, personal communication) with several membrane spanning domains, and is capable of accumulating to high levels in the plasma membrane of heat shocked cells.

With the induction of the heat shock response a single heat-shock protein (hsp30) becomes associated with the plasma membrane. It is not found in other cell fractions, either before or after a heat shock, and is therefore an hsp that is targeted to the plasma membrane. hsp30 is highly hydrophobic, tightly associated with the plasma membrane and therefore almost certainly an integral membrane protein (Fig. 5.5). Also, hsp30 accumulates in the membrane at a comparatively high level, after only 40 minutes at 40°C (Fig. 4.8, lane C). It is therefore a candidate for a heat shock protein which might exert a damage limitation role specifically at the plasma membrane. Conceptually, hsp30 might influence membrane order or might act to protect key membrane proteins. Targets for such protection could include plasma membrane H+-ATPase, an enzyme the cell cannot replenish in the stressed state. This is a plausible proposition given the stress protective role of other hsps (e.g hsp70, section 1.2.1). Heat shock proteins that promote folding and assembly of proteins are specifically targeted to mitochondria, chloroplasts, the endoplasmic reticulum, and the cytosol (Gething and Sambrook, 1991). As yet there has been no report in the

literature describing a protein that plays a similar role with respect to plasma membrane proteins. It would not be surprising if such a protein were to be targeted to the plasma membrane; and if so, hsp30, as the only hsp to be targeted to the plasma membrane, might fulfill this role."Protective" hsps such as hsp70 interact with many proteins and not surprisingly they exhibit extremely heterogeneous elution profiles during gel filtration. If hsp30 does have a similar "protective" role nearest neighbour analysis would reveal hsp30 associating with many membrane proteins. This would involve treating plasma membranes with agents that cross link proteins, followed by SDS-PAGE to resolve these protein complexes. Antisera raised against hsp30 could be used to probe Western blots of these gels. Interaction of hsp30 with other proteins would be revealed by a ladder of bands on the blot. Identical blots could be probed with antisera raised against other membrane proteins; this would determine which proteins interact with hsp30. It must be appreciated that the effective concentration of protein in plasma membranes is high. The frequency of random collisions is therefore that much greater. Detection of non-specific interactions by this analysis could be achieved by reducing i) the concentration of the cross linking reagent, ii) incubation time and iii) incubation temperature to levels below the transition temperature of the phospholipid, as this effectively freezes the steady state (Findlay, 1990).

Determination of whether hsp30 has any specific function must await detailed biochemical and physiological analysis of a strain disrupted in the gene for this protein. Whether or not it has a specific role, or perhaps resembles hsp26 of yeast in having no easily identifiable function, the strong evolutionary conservation of the heat shock response makes it probable that structural (and possibly functional) homologs of hsp30 will be found in organisms other than yeast.

Some of the results from this chapter namely those illustrating loss of H+-ATPase and acquisition of hsp30 by the plasma membrane due to heat shock, have been published (Panaretou and Piper, 1992; a copy of this article has been provided, see Appendix I).

4.5.2 Activation of H+-ATPase during heat shock.

To the list of factors that activate H+-ATPase can be added heat shock and osmotic shock (the latter is discussed in section 4.5.3). The mechanisms which control activity of this enzyme and which are capable of causing a 10 fold increase in specific activity remain poorly understood. The best studied activator of H+-ATPase is glucose (Serrano, 1983; Portillo *et al.*, 1989; and Portillo *et al.*, 1991). Various reports suggest that the C-terminal domain has an important role to play in regulation of activity (Portillo *et al.*, 1989; and Portillo *et al.*, 1991). A mutant lacking the last 11 amino acids produces an enzyme which, in cells starved for glucose, has the kinetic parameters of the wild type enzyme activated by glucose fermentation (Portillo *et al.*, 1989). It is possible that heat shock may also exert its

regulatory effect on the enzyme via the C-terminus. This is a valid argument given the similarity of the responses of the cell to i) heat shock and ii) agents that cause cell proliferation (such as glucose). These 'shared' responses include increased turn over of inositol phospholipids and increases in levels of intracellular calcium (see below). The various *S. cerevisiae* H^+-ATPase mutants available provide an opportunity to test this. For instance, the H^+-ATPase activity of the mutant lacking part of the C-terminus of the polypeptide is constitutively active (Portillo *et al.*, 1989). Activity of the H^+-ATPase of purified membranes from heat shocked cells of this mutant (or even rates of H^+ efflux *in vivo*) during heat shock could be determined. A stimulation of H^+ efflux rates would indicate regulation occurring at a region other than the C-terminus, whereas the absence of stimulation would indicate that, as for glucose, regulation occurs via the C-terminus.

The activated state of H^+-ATPase is preserved after purification of plasma membranes and may correspond to covalent modification of the polypeptide. The most common type of covalent modification is phosphorylation. There are numerous reports describing phosphorylation of the H^+-ATPase (see section 4.4.11). One report describes a seryl kinase, the activity of which causes a 5 fold activation of H^+-ATPase (Kolarov, 1988). Work presented here indicates that enzyme in membranes from heat shocked cells, compared to that from unstressed cells, is phosphorylated to a greater extent on incubation with [γ^{32}P] ATP (Fig. 4.13). A number of the cellular responses caused by heat shock are known to activate various kinases. The action of some of these could activate H^+-ATPase. These are reviewed below.

One of the ways by which extracellular signals are transduced into intracellular events is by activation of phospholipase C. There are numerous examples of this in mammalian cells (Nishizuka, 1988) and yeast (Kaibuchi *et al.*, 1986). Phospholipase C hydrolyses plasma membrane inositolphospholipids, the initial products being diacylglycerol (Dag) and inositol triphosphate (IP_3). Water soluble IP_3 diffuses into the cytosol and releases Ca^{2+} from internal stores. Dag remains in the membrane and activates protein kinase C. In mammalian cells heat shock is one of the signals that causes this (see section 1.8.5 and Fig. 1.5). Given the evolutionary conservation of heat inducible phenomena and the fact that there are reports describing the existence of inositolphospholipids (Patton and Lester, 1992) and protein kinase C in yeast (Hunter, 1990), it is likely that release of Dag and IP_3 occurs in yeast during heat shock. Protein kinase C could phosphorylate H^+-ATPase thereby activating it. In agreement with this is the finding that phorbol esters which are well known activators of protein kinase C, activate yeast H^+-ATPase (by an order of magnitude similar to that determined for heat shock in work described here). Also, upon incubating cells with ^{32}P, the presence of phorbol esters increases the extent to which the enzyme is phosphorylated (Portillo and Mazon, 1985). In

addition to this, the potential increase in intracellular Ca^{2+} could also activate H^+-ATPase, by modulating the activity of a Ca^{2+}/calmodulin kinase (Londesborough and Nuutinen, 1987). Consistent with this is the finding that glucose (which activates H^+-ATPase) elicits Ca^{2+} influx in yeast (Kaibuchi *et al.*, 1986) and, the C- terminal domain (which mediates glucose activation) contains a potential phosphorylation site for the calmodulin-dependent protein kinase. Site directed mutagenesis of this site dramatically reduces glucose mediated activation of the enzyme (Portillo *et al.*, 1991). In addition, Ca^{2+} ionophores activate H^+-ATPase of *Neurospora crassa* (Lew, 1989). Finally, one of the effects of heat shock is an increase in the levels of cAMP (see section 1.6). This would increase the activity of cAMP-dependant protein kinase (protein kinase A). Some of the protein kinase A of the cell is known to be anchored to the cell membrane, via its regulatory sub-unit (Behrens and Mazon, 1988; Mitts *et al.*, 1990). Evidence for the activation of H^+-ATPase mediated by cAMP is controversial. Glucose, the best studied activator of H^+-ATPase, also elicits an increase in levels of cAMP. Exogenously added cAMP can activate H^+-ATPase in a strain where the enzyme responsible for synthesis of cAMP is defective (Ulaszewski *et al.*, 1989). In contrast, activation of H^+-ATPase by glucose still occurs in mutants with constitutively active protein kinase A (Mazon *et al.*, 1989).

There are various ways by which the above propositions can be tested. For example, the activity of protein kinase A in membranes derived from unstressed and heat shocked cells can be determined, using the kinase-A-specific acceptor Kemptide as substrate (Behrens and Maia, 1986). Similar experiments can be carried out with respect to protein kinase C (as described by Castagna *et al.*, 1982). It should be noted that the only account in the literature which describes effects of heat shock on protein kinase C describes a slight *decrease* in activity associated with particulate fractions from heat shocked cells (Bagi and Hidvegi, 1990). However this was the result from a long term heat shock. It should be noted that the heat induced increase in levels of Dag (the activator of protein kinase C) occurs within 2 minutes and levels then return to control values by 8 minutes (Calderwood *et al.*, 1987). Accordingly, isolates from cells given short-term heat shocks must be used.

4.5.3 The response to osmotic shock.

It is well established that incubating exponentially growing yeast cells with between 0.25 and 0.7M NaCl causes a preconditioning that allows survival during a subsequent challenge with a higher concentration of NaCl which would normally be lethal (Blomberg and Adler, 1989). These cells are also thermotolerant, though the physiological overlap between osmotolerance and thermotolerance is unidirectional i.e thermotolerant cells are *not* osmotolerant (Trollmo *et al.*, 1988). Few studies have been carried out on the molecular

basis of acquired osmotolerance in yeast, in contrast to the vast amount of literature that exists concerning this subject with respect to higher plants. This is due to water stress imparted by drought or high salinity being the most widespread abiotic stress and constitutes the most stringent factor in limiting plant distribution and productivity (Downton, 1984). However, the molecular basis of acquired osmotolerance is still poorly understood.

H^+-ATPase activity of purified plasma membranes increases almost 2 fold when cells are incubated with concentrations of NaCl which would promote acquired osmotolerance (Fig. 4.12.1 and section 4.4.10). Activity of this enzyme may be expected to be higher than usual as part of a Na^+ extrusion mechanism to control the concentration of this toxic ion in the cytoplasm. A Na^+/H^+ antiporter driven by proton motive force is a likely candidate for such a mechanism since existence of such an antiporter is well substantiated for bacterial cells (Padan *et al.*, 1981) and its presence has been implied in plant cells (Braun *et al.*, 1986 and references therein). Yeast membranes may also possess such an enzyme though there is no such report in the literature as yet. The rate at which a Na^+/K^+ antiporter operates may also be involved, given that H^+-ATPase indirectly drives K^+ uptake in yeast (Serrano, 1988). Levels of K^+ could subsequently regulate the action of a Na^+/K^+ antiporter, thereby regulating the rate of Na^+ efflux. The increase in K^+ uptake by *S. cerevisiae* after salt transfer is in agreement with this (Singh and Norton, 1991). Again, there is no report of such an antiporter in the cell membranes of yeast as yet. Activity of H^+-ATPase has previously been implicated in the salt tolerance of higher plants (see section 7.1).

Unlike cells undergoing heat shock and many other forms of stress (see section 1.1) osmotically shocked cells i) do not seem to repress the synthesis of most of the proteins synthesised prior to the stress (Fig. 4.11) and ii) do not induce hsps. However Fig. 4.11 reveals that salt stress induces 3 polypeptides of M_r : i) 20 kDa ii) 31kDa and ii) 43kDa (plus another protein just larger than 43kDa). Though this induction seems to be nowhere near as strong as the induction of hsps with heat stress it may be significant because where studied, acquisition of osmotolerance is dependent on protein synthesis, and, prolonged incubation with intermediate concentrations of salt may result in accumulation of large amounts of these proteins (Fig. 4.11 shows amounts synthesised during only 40 minutes of salt stress). It is likely that the 43kDa polypeptide is glycerol-3-phosphate dehydrogenase (GPD) (Andre *et al.*, 1991) . It is well established that *S. cerevisiae* accumulates glycerol in response to osmotic shock (Blomberg and Adler, 1989). This is primarily due to induction of GPD, one of the enzymes in the glycerol production pathway; GPD has an M_r of 43kDa as determined by Andre *et al.*, (1991). The 31kDa product may be the product of the *HAL1* gene recently isolated by Serrano*. Transcription of *HAL1* is induced by osmotic shock and it encodes a protein of 32kDa. The region of the

promoter responsible for salt induction has been delineated and is the first "osmotic shock induction element" to be isolated from yeast. When over-expressed, *HAL1* allows *S. cerevisiae* to grow in the presence of high salt concentrations while disrupting the gene diminishes salt tolerance. The *HAL1* gene is present in higher plants making it an excellent candidate for genetic engineering of improved salt tolerance. Antisera raised against both GPD and the *HAL1* product are available, therefore immunoblotting could determine whether the 43 and 31kDa proteins in Fig. 4.11 are GPD and the *HAL1* product, respectively. Finally, there is no record in the literature of yeast inducing a 20kDa polypeptide during osmotic shock (Fig. 4.11). Growing yeast in the presence of 0.5M NaCl may allow a substantial accumulation of this protein which would facilitate its purification. The corresponding gene could be cloned using antisera raised against this protein or a degenerate oligonucleotide based on a limited N terminal sequence. Subsequent studies could involve the effects on osmotolerance of over expressing (or deleting) this gene.

In contrast to the situation in yeast, there are many reports describing the induction of proteins in salt stressed plants yet the roles of most of these proteins are unclear. Among these proteins are enzymes involved in the production of osmolytes (e.g. myoinositol o-methyl transferase, Vernon and Bohnert, 1992; betaine aldehyde dehydrogenase, Weretilnyk and Hanson, 1990); and osmotin, a 26kDa protein made in large quantities during salt stress (Singh *et al.*, 1989). Also, there are many other salt inducible proteins, which seem to share the same biochemical characteristics such as high hydrophobicity. It is thought that they preserve structural integrity of cells during the desiccation process (Skriver and Mundy, 1990). An attempt to find such proteins associating with the yeast cell membrane revealed that no new proteins associate with this membrane during salt stress in *S. cerevisiae* (see Fig. 4.10).

*Unpublished. Isolation of *HAL1* was announced at the *Workshop on salt tolerance in microorganisms and plants: physiological and molecular aspects* held at the Fundacion Juan March, 11-13 November 1991, Madrid (Spain).

Chapter 5 Characteristics intrinsic to hsp30 preclude raising antisera and generation of a limited N-terminal sequence.

5.1 Introduction

At elevated temperatures a single heat-shock protein (hsp30) becomes associated with the plasma membrane. It is not found in other cell fractions either before or after a heat shock, and is therefore an hsp that is targeted to the plasma membrane. Entry into stationary phase and nitrogen limitation also induce the synthesis of this protein. The possible function of hsp30 is discussed in section 4.5.1. One of the more credible possibilities involves the protection of various membrane proteins by hsp30 during stress. To investigate this, nearest neighbour analysis could be carried out as suggested in section 4.5.1. This necessitates raising antisera against hsp30. Antisera could be used for many other purposes as it is now common for immunological methods to be used for the intracellular localisation, quantitation, topographical analysis and purification of membrane components (Bailyes *et al.*, 1990). Antibodies could also be used to modulate biological function and as probes in the molecular cloning of the corresponding gene from expression libraries. However, expression systems are constructed using cDNA. Therefore, for the purpose of isolating the gene encoding hsp30, a conventional expression library cannot be used as it is would be derived from mRNA synthesised under normal temperatures (hsp30 mRNA would not be present). This would necessitate the construction of an expression library derived from mRNA synthesised during heat shock. Alternatively, the gene could be cloned by screening a genomic library with a degenerate oligonucleotide derived from a partial N-terminal sequence of hsp30. Also, entering a partial N-terminal sequence into the protein databanks may reveal homology with other proteins, thereby providing a clue as to the function of hsp30 (as was carried out for the partial sequence of hsp104, see section 3.4.3).

Cloning the corresponding gene would allow i) production of the protein in large quantities for structural studies such as X-ray crystallography; ii) use of the cloned DNA itself to probe the genomes of other organisms in order to ascertain whether the gene is conserved in evolutioniii) rapid determination of the primary structure of the corresponding protein, as sequencing DNA of the cloned gene is much faster than sequencing the protein. Also the DNA sequence may even shed light on the function of the protein by virtue of homology with the sequences of genes with known functions in the databanks; about one third of sequences present in genomes are related to entries in the current databank (Chothia, 1992). In addition, the quantity of protein within cells can be modulated. In most organisms this can be achieved by introducing extra copies of the gene (Skou, 1988) or by modifying gene expression using anti-sense methodologies (Green *et al.*, 1988). In yeast, due to the high frequency of homologous recombination, there is the additional prospect of

deleting genes of interest (Struhl, 1983). Determination of the function of hsp30 could be revealed by detailed biochemical and physiological analysis of a strain disrupted in the gene for hsp30. There is also the possibility of replacing the wild type gene with *in vitro* mutagenised alleles which have been modified at a region of interest.

Ultimately, one may need to purify the polypeptide from yeast membranes. As yet the function of this protein remains unknown which precludes the use of various techniques such as affinity chromatography involving, for example, the use of a non-metabolisable substrate as specific ligand. However, certain physical properties of the polypeptide may be of use. A common post-translational modification of eukaryotic plasma membrane proteins is limited glycosylation. Initial fractionation of membrane proteins is often achieved very successfully with carbohydrate affinity resins (Findlay, 1990). In order to determine whether hsp30 is glycosylated Western blots were probed with the appropriate reagents, as described in section 2.11.5 (see section 5.2.4 and Fig. 5.4)

hsp30 seems to be specifically targeted to the plasma membrane. Another strategy which could be used as an initial step in a purification scheme could involve the separation of integral from peripheral membrane proteins. Therefore, to determine the degree to which hsp30 is bound to the plasma membrane, phase partitioning by temperature induced separation in Triton X-114 was carried out as described in section 2.14 (see section 5.2.5 and Fig. 5.5)

5.2 Results

5.2.1 hsp30 injected into rabbit did not provoke a strong immune response.

hsp30 was not available in pure form. However, it could be separated from other proteins on SDS-polyacrylamide gels (see lane C of Fig. 4.8). Resolving plasma membrane proteins from unstressed and heat shocked cells reveals that i) hsp30 is relatively abundant in heat shock fractions and ii) there were no bands at the corresponding position in the unstressed fraction. Therefore, reasonable quantities of hsp30 in relatively pure form, could be obtained by simple excision of the appropriate band from SDS gels. Antigens purified in this way often induce good antibody responses (Harlow and Lane, 1988 [c]). The antigen is denatured by this route of preparation, so resulting antibodies would be particularly good for techniques requiring denaturation-specific antibodies as well as techniques involving native hsp30 (though it would be expected that some of the antibodies would not bind to the native antigen).

Gel fragments were processed for immunisation as described in section 2.11.4.1. The particulate nature of material from gel slices precludes the use of mice or rats as hosts and necessitates the use of larger animals such as rabbits (Harlow and Lane, 1988). However, this was not a disadvantage as rabbits are the best choice for polyclonal antibody production, even when antigen is limiting. Deciding on the dose of antigen is difficult since effective dose delivered to the immune system may bear little relationship to the introduced dose. In general, however, the minimum dose of antigen for the rabbit is 10μg per injection (Harlow and Lane, 1988). For work described here at least 10 times this amount was used. Producing hsp30 in large quantities was a laborious and time consuming process involving isolation of plasma membranes from many litres of cells and running numerous gels. Therefore it would have been useful to know in advance whether the immune system of the rabbit would recognise hsp30. Unfortunately, there are no tests to determine whether a given molecule will be immunogenic.

The method employed to both "purify" and administer hsp30 as antigen, had previously been used in the same laboratory to raise high titre antisera against both hsp26 and hsp90 from *S. cerevisiae* (J. Coleman, unpublished results, and Cheng *et al.*, 1992, respectively). For this reason the chances of successfully raising antisera against hsp30 seemed to be high.

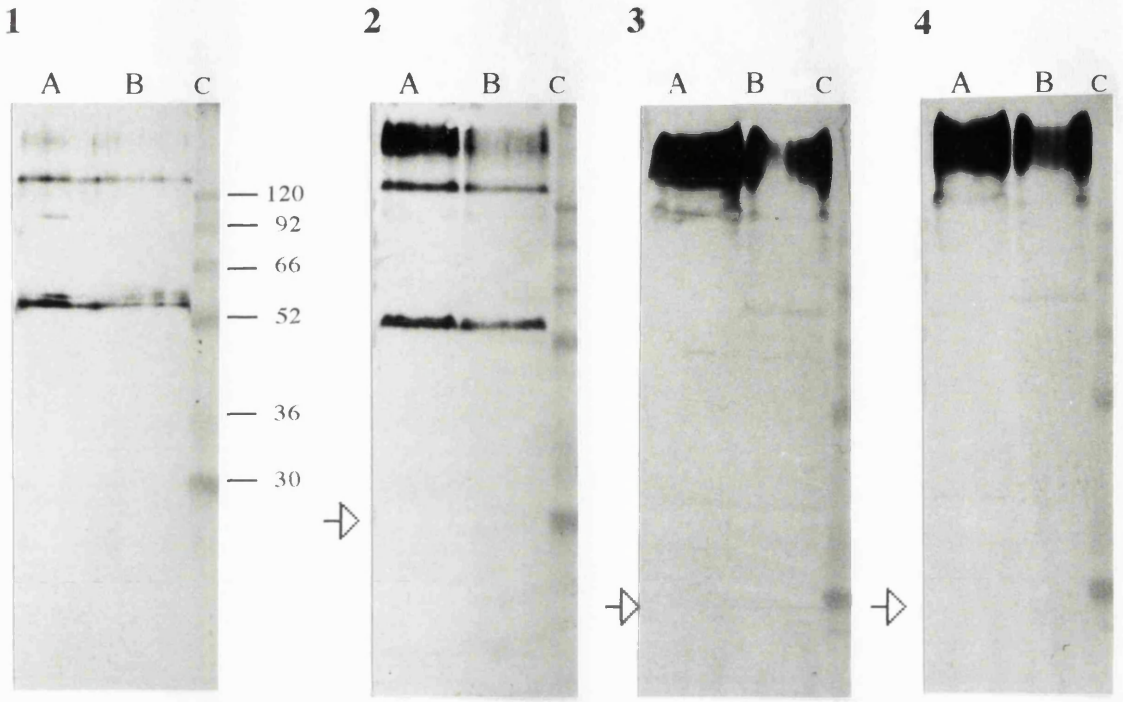
Fig. 4.8 shows that when 100μg of total plasma membrane protein from heat shocked cells is resolved by SDS-PAGE and stained with Coomassie blue, hsp30 appears in reasonably high quantities. Given that staining with Coomassie blue has a sensitivity of ca. 1μg /band, the hsp30 band must represent at least 2μg of protein. Plasma membranes isolated from 1L of cells typically yield 1.5 mg of protein; resolving all of this protein on numerous gels would result in isolation of at least 30μg of hsp30. The primary injection

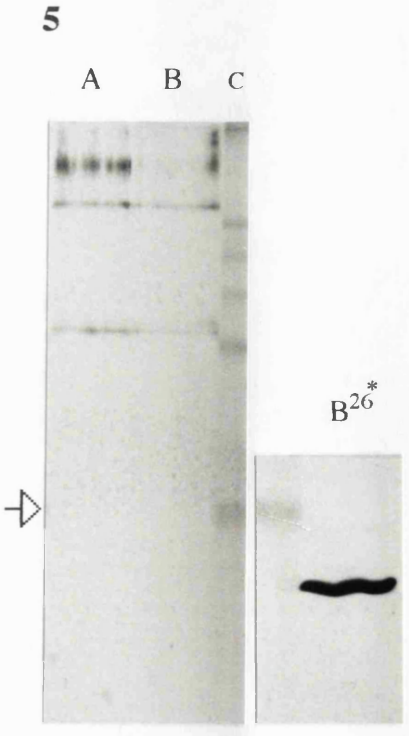
included 120μg of hsp30 in the presence of a complete Freund's adjuvant (a preparation that includes killed *M. tuberculosis* which non-specifically stimulates the immune system and renders antibody responses more persistent). Boosts, each incorporating 100μg of hsp30, were also administered at two week intervals, in the presence of incomplete (i.e. without *M. tuberculosis*) Freund's adjuvant (as described in section 2.11.4.1). In total, ca 520μg of hsp30 was administered; this represents the isolation of plasma membranes from ca.17L of heat shocked cells. The rabbit was bled 10 days after each boost. Serum prepared from the blood was used to probe Western blots of total membrane protein from unstressed and heat shocked cells.

A standard immune response would involve B cells bearing surface antibodies specific for the antigen appearing 5-6 days after the primary injection. The second injection should result in the number of these B cells increasing exponentially. The production of antibodies (of class IgG) in the serum should reach its peak 5-10 days after this second injection (Harlow and Lane, 1988 [c]). However, probing Western blots of plasma membrane proteins with antisera generated from blood taken at this stage did not reveal the presence of hsp30 antibodies (Fig. 5.1). There was the possibility that insufficient hsp30 had been presented to the immune system, therefore 3 more doses of antigen were administered as described above. None of the antisera derived from blood taken after each of these injections contained antibodies to hsp30. Possible reasons for this are given in section 5.3.

Figure 5.1 Probing western blots with antisera derived from a rabbit injected with hsp30. Plasma membrane proteins (100μg) from unstressed (A) and heat shocked (B) cells were resolved by 15% SDS-PAGE and subsequently blotted onto nylon membranes. The blots were probed with pre-immune (control) antiserum (1) and antiserum prepared 10 days after second (2), third (3), fourth (4) and fifth (5) injections of polyacrylamide gel fragments containing hsp30, as described in section 2.11.4.1 (minimum dose of hsp30 per injection was 100μg). Lane B^{26} represents visualisation of hsp26 using the appropriate antiserum. Lane C shows the positions of blotted pre-stained markers, the molecular masses of which are indicated to the right of frame 1 only. Antibodies against hsp30 would have been visualised by bands at the position marked by the open arrow.

* Anti hsp26 antiserum was diluted by 1/250 prior to use. Antiserum derived from rabbits injected with hsp30 was diluted by 1/50 prior to use (this usually constitutes a vast excess of antibodies, with respect to antisera of reasonably high titres).


1
A
B
C
120
92
66
52
36
30
2
A
B
C
3
A
B
C
4
A
B
C


5
A
B
C
B26*

5.2.2 hsp30 is retained in gels during western blotting.

Proteins which are to be sequenced in a gas phase sequencer may be most easily prepared without losses by blotting onto polyvinylidene difluoride (PVDF) membrane (Flannery *et al.*, 1989). Nylon or nitrocellulose membranes are unsuitable since they do not withstand the reagents used in automated sequencing machines (Matsudaira, 1990). The modifications to the usual SDS-PAGE method needed to minimise N-terminal blocking during electrophoresis are detailed in section 2.13. Most sequencing facilities require 10-100pmol of material for N-terminal sequence analysis (Matsudaira, 1990). This limit corresponds to 0.3-3μg of a 30kDa protein. Fig 4.8, lane C shows that separating 100μg of plasma membrane proteins from stressed cells by SDS-PAGE, results in clear resolution of ca. 2μg of hsp30. hsp30 should be amenable to N-terminal sequencing once blotted onto PVDF.

The most commonly used buffer for electroblotting of SDS gels is that developed by Towbin and consists of 25mM Tris-192mM glycine (pH 8.3) and 20% methanol (Towbin *et al.*, 1979). This buffer results in excellent electrophoretic transfer of soluble proteins. However, proteins from the *S. cerevisiae* plasma membrane were transferred so poorly that they were hardly detected when the PVDF membranes were stained with Coomassie blue. A review of the literature revealed that many groups develop their own modifications of electrophoretic transfer buffer to suit each protein of interest (Bailyes *et al.*, 1990). The variables which influence protein transfer, and how they were altered to improve blotting of membrane proteins are given below:

i) Transfer buffer pH. When electroblotting SDS gels, neutral or slightly alkaline buffers are used so polypeptides behave as anions. Inducing further de-protonation would be expected to increase the extent of elution towards the anode. Therefore, pH was raised from the conventional value of 8.3 to 11 by replacing Tris-glycine with 3-(cyclohexylamino)-1-propanesulphonic acid (CAPS).

ii) Methanol content. Methanol (20% v/v) is usually included since it minimises swelling of the gel during blotting (Hames, 1990). However, methanol also reduces the efficiency of electrophoretic elution by stripping proteins of SDS. Partial removal of SDS is not so significant in the case of blotting soluble proteins. However, loss of SDS will have serious consequences with respect to membrane proteins as their solubility relies upon the presence of the detergent. For this reason, methanol content was reduced to 5% which lead to a dramatic improvement in the electrophoretic elution of membrane proteins (see Fig. 5.2 A).

iii) Presence or absence of SDS. Various groups maintain that including 0.1-0.01% (w/v) SDS in the transfer buffer will improve electrophoretic elution of proteins (Flannery *et al.*,

1989; Hames, 1990). This is surprising since though SDS may improve elution *per se*, the detergent will compete with protein for binding sites on the blotting membrane. In addition, the manufacturers of PVDF specifically recommend the omission of SDS or any other detergent from transfer buffers. Not surprisingly, when SDS was included at concentrations as low as .01% (w/v), there was a total absence of protein on blots as judged by staining with Coomassie blue. As a compromise, electroblotting was carried out from gels that had been equilibrated with transfer buffer for 5 minutes (equilibration times are usually 15-20 minutes), the SDS in the gel being sufficient for elution of most proteins (as recommended by Matsudaira, 1990).

iv) The amount of current used and the length of the time the current is applied. This depends on the type of blotting apparatus used. Electrophoretic elution can be achieved by complete immersion of a gel membrane sandwich in a buffer tank with platinum wire or steel plate electrodes (wet transfer). Alternatively, the gel membrane assembly surrounded by absorbent paper soaked in transfer buffer, can be placed between graphite plate electrodes (semi-dry transfer). Of the two, wet transfer was found to be the best. Proteins were transferred at 50 volts for a minimum of 5 hours (Fig. 5.2.A, frame 1). Blotting for longer periods of time (upto 18 hours) did not improve significantly on the extent of electroelution (compare frames 1 and 2 of Fig. 5.2.A).

The optimal conditions for blotting membrane proteins were found to be wet transfer for 5 hours in a buffer composed of 35mM CAPS-NaOH (pH 11) and 5% methanol. The SDS gel was equilibrated for only 5 minutes in the transfer buffer prior to blotting. PVDF membranes were stained with Coomassie blue as described in section 2.13. To aid resolution of hsp30, plasma membrane proteins were resolved on SDS gels that were twice as long as usual. Of the numerous blots carried out, two are presented representing the best results obtained (Fig. 5.2.A). Though efficient electroelution was obtained for the majority of plasma membrane proteins, hsp30 was not eluted at all as judged by Coomassie staining of the blot. To determine if any hsp30 had been transferred, 100μg of plasma membrane proteins, phosphorylated *in vitro* with [γ^{32}P]-ATP (as described in section 2.15) were blotted. The appropriate regions of the blot and the gel (dried down as described in section 2.11.2) were autoradiographed. Fig. 5.2.B shows that very little hsp30 was transferred; cerenkov counting of these bands revealed that this represented only ca. 1/10 of the hsp30 loaded on the gel. Given that there is ca. 2μg of hsp30 per 100μg of plasma membrane protein, this represents elution of ca. 20ng. At least 15 times more protein is necessary for N-terminal sequencing.

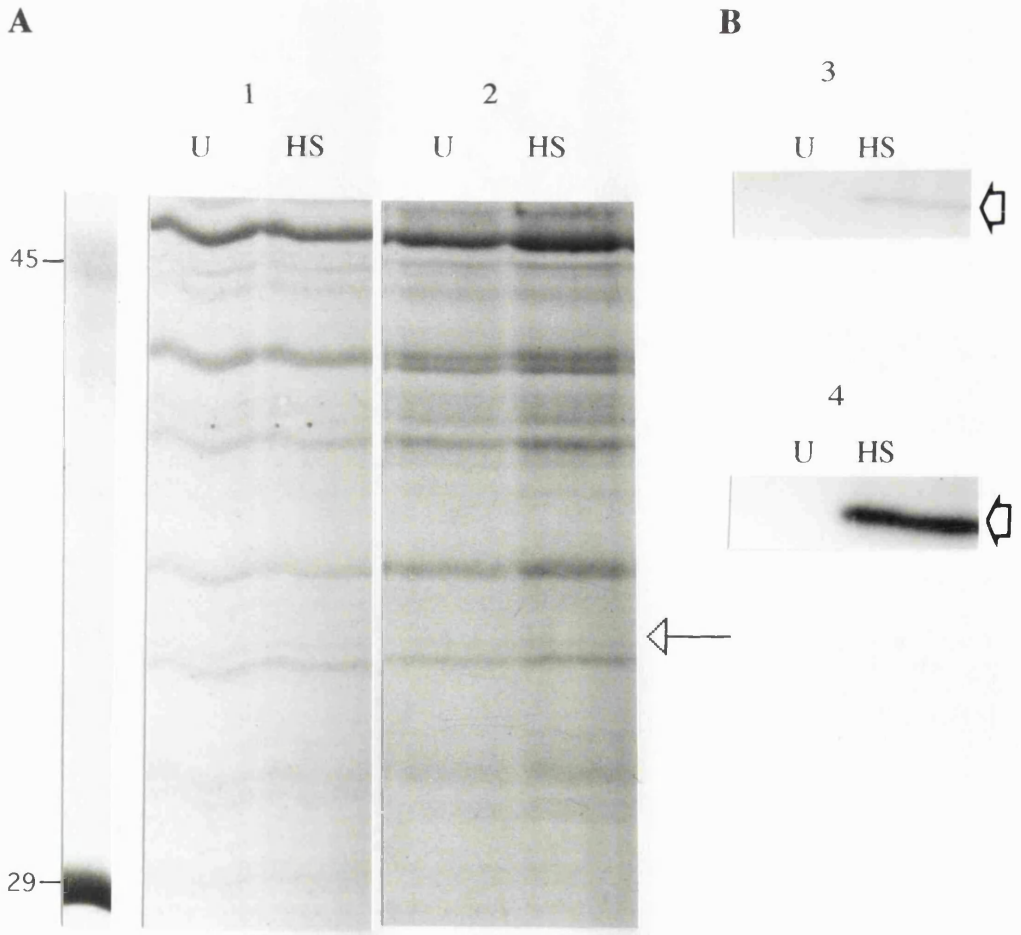
Bearing in mind that proteins are electroblotted as anions, the possibility exists that hsp30 elutes poorly because it is extremely basic. However, histones (which are highly basic) can be blotted (Hames, 1990). It is more likely that weak elution is due to extreme

hydrophobicity of the polypeptide. Accordingly, hsp30 may be composed mostly of membrane spanning domains with very small hydrophilic regions. It should be noted that H+-ATPase,with its 8-10 membrane spanning domains, blots efficiently (see Fig. 4.2). However, this polypeptide also has extensive hydrophilic stretches (Serrano *et al.*, 1986) which may be responsible for its efficient elution from gels. Phase partitioning with Triton X-114 confirms that hsp30 is indeed intimately associated with the plasma membrane and as such is likely to possess several membrane-spanning domains (see Fig. 5.5 and section 5.2.5).

The fact that hsp30 electroelutes so poorly could be why antibodies raised against hsp30 could not be detected by probing Western blots with anti-sera from rabbits injected with this protein (see Fig. 5.1 and section 5.2.1). However, a high titre antiserum would be expected to detect less than 1ng of protein (Bailyes *et al.*, 1990). Therefore, since blotting 100μg of plasma membrane protein would result in transfer of ca. 20ng of hsp30 to blotting membranes (see previous page), the low efficiency of elution was not considered to be a problem in this case.

Figure 5.2 hsp30 elutes poorly from gels during electrophoretic transfer. **A** Plasma membrane proteins (100μg) from unstressed (U) and heat shocked (HS) cells were resolved by 12.5% SDS-PAGE. Gels twice as long as usual were used to provide the best possible resolution of hsp30. Proteins were blotted onto PVDF for 5 hours (1) or 18 hours (2) as described in section 2.11.3. Eluted proteins were visualised by staining blots with Coomassie blue as described in section 2.13. Position of molecular mass markers are indicated on the left. The open arrow indicates where one would have expected to see hsp30.

B A blot identical to the one described in A (1) was performed using phosphorylated plasma-membrane proteins prepared as described in section 2.15. After electrophoretic transfer the appropriate regions of the blot (3) and the gel (4) were autoradiographed. The gel fragment was dried down as described in section 2.11.2, prior to autoradiography. hsp30 is indicated by an open arrow (in HS lanes only).



5.2.3 hsp30 is resistant to protease digestion.

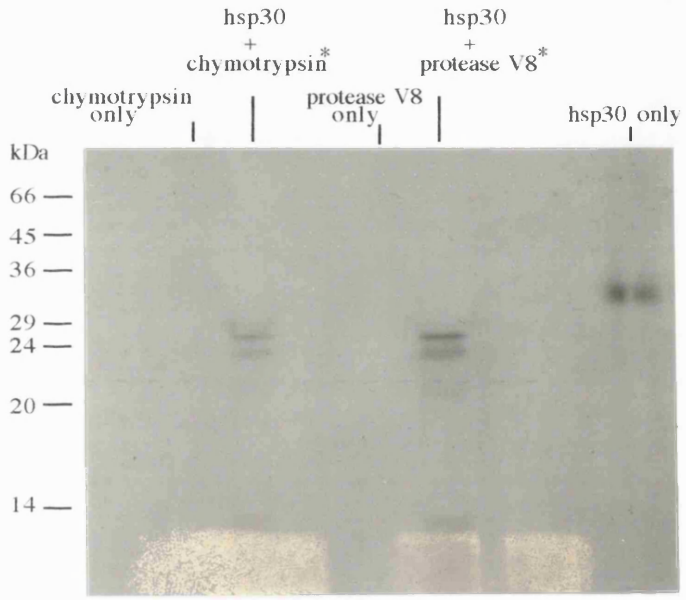
The extreme hydrophobicity of hsp30 may explain its poor electroelution from gels. However, some of the fragments from a limited digest of hsp30 may be predominantly hydrophilic. These could be blotted onto PVDF and sequenced. This can achieved by excising hsp30 from a gel like that in Fig. 4.8, subjecting it to limited proteolysis, followed by separation of the cleavage products by 15% SDS-PAGE (as described in section 2.12). Typically, the substrate:protease ratio would be ca. 100:1 (Flannery *et al.*, 1989). Because of the additional steps involved, one usually needs 5x more starting material than is required for simple N-terminal sequencing of intact proteins (Matsudaira, 1990). For this reason all digests carried out involved the use of 3 bands of hsp30 like the one shown in Fig. 4.8, lane C (representing a total of ca. 6μg of protein).

Using endoprotease V8 at the substrate:protease ratio given above did not cleave hsp30 (not shown). This was in sharp contrast to a similar experiment involving the H+-ATPase as substrate, which gave a series of fragments (see Fig. 3.6). Protease V8 specifically cleaves the peptide bonds on the C-terminal side of glutamate residues; the protease hydrolyses all 17 different glutamoyl bonds that have been studied. Bonds involving hydrophobic amino acid residues with bulky side chains are known to be cleaved at a lower rate (Houmard and Drapeau, 1972). This is probably the most common type of glutamoyl bond present in hsp30, given the extreme hydrophobicity of the polypeptide (see Fig. 5.5). It would be expected that this could be overcome by incubating hsp 30 for extended periods in the presence of larger amounts of protease. Alternatively, a protease with a wider bond specificity could be used. Chymotrypsin was chosen since it is known to cleave bonds adjacent to bulky hydrophobic side-chains (Bond, 1989).

The above was repeated but this time i) a digest with chymotrypsin was also carried out ii) the substrate:protease ratio for both proteases was 6:1 and, iii) the incubation with protease (see section 2.12) was extended from 30 minutes to 90 minutes. Both incubations resulted in very limited digests yielding 5 fragments (Fig. 5.3), none of which could be blotted onto PVDF (not shown). The two most abundant fragments had apparent molecular masses near to that of the intact protein (ca. 29 and 24 kDa). The resistance hsp30 exhibited to digestion by high amounts of protease V8 and chymotrypsin is even more remarkable when one considers that the digests were carried out on denatured rather than native hsp30.

hsp30 only (i.e not treated with protease) was loaded into one of the lanes in Fig. 5.3. The polypeptide seemed to be present at low levels as evidenced by its poor staining by Coomassie blue.This was surprising given that the source of hsp30 was a number of gel slices containing ca. 6μg of protein in total. This was not surprising given that difficulties in successfully eluting integral membrane proteins from one gel to another are not uncommon (Kennedy *et al.*, 1988).

Figure 5.3 hsp30 is resistant to proteolytic cleavage. Plasma membrane proteins (100μg) were resolved by SDS-PAGE (as shown in Fig. 4.8). Gel slices containing hsp30 were applied to a second SDS gel in the presence of 1μg of the indicated protease, as described in section 2.12. Incubation in the presence of protease was raised from 30 to 90 minutes. Peptide fragments generated were visualised by staining with Coomassie blue. Each lane was loaded with 3 gel slices containing hsp30 (ca. 6μg of protein). Position of molecular mass markers is indicated on the left.



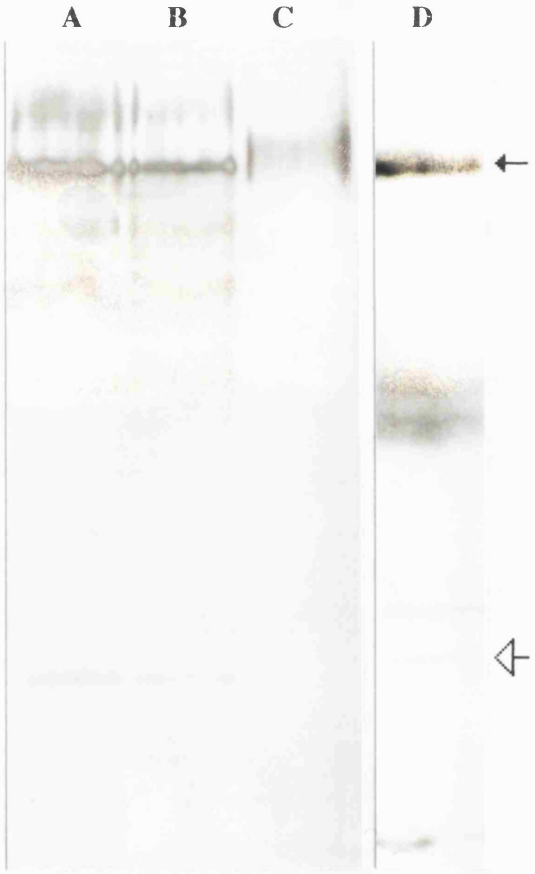
* Substrate:protease ratios used (ca. 6:1) were far in excess of those usually used for limited digests (ca. 100:1). Even so, hsp30 exhibited a high degree of resistance to proteolytic attack. This may have been due to inactivation of the proteases. This was considered unlikely as i) protease V8 gave satisfactory limited digests of hsp104 and H^+-ATPase when used at substrate:protease ratios as low as ca. 300:1 (see Fig. 3.6.B lane 7) and ii) chymotrypsin , when used in ratios of ca. 6:1, gave an extensive digestion of H^+-ATPase (See below). A single band containing H^+-ATPase, excised from a gel like that shown in Fig. 4.8 lane C, was used. The H^+-ATPase seems to have been extensively degraded in this case. This was not surprising given i) the very high substrate:protease ratio used and ii) the broad specificity of chymotrypsin.

5.2.4 Comparing the profile of glycosylated proteins of plasma membrane proteins from unstressed and heat shocked cells.

A common post-translational modification of eukaryotic plasma membrane proteins is limited glycosylation. *S. cerevisiae* is no different to other eukaryotes in this respect (Tanner and Lehle, 1987). Initial fractionation of membrane proteins is often achieved very successfully with carbohydrate binding proteins (lectins) which attach to supports easily and which retain activity in detergents (Findlay, 1990). Binding in the presence of detergents is of particular importance since purification protocols for membrane proteins invariably involve the use of detergents. The carbohydrate chains on *S. cerevisiae* proteins consist almost exclusively of mannose residues (Tanner and Lehle, 1987). The lectin most commonly used to detect such carbohydrate chains is concanavalin A from *Canavalia ensiformis* (Sutton, 1989). In order to determine whether hsp30 is glycosylated western blots were probed with this lectin, as described in section 2.11.5.

A glycosylated protein did not co-migrate with hsp30 (Fig. 5.4 lane B). Overall, the profile of glycosylated plasma-membrane proteins of the unstressed fraction was the same as that of the heat shocked fraction (Fig. 5.4).

Figure 5.4 Glycoprotein profiles of plasma membranes from unstressed and heat shocked cells. Plasma membrane proteins (100μg) from unstressed (A) and heat shocked (B) cells were resolved on a 12.5% SDS gel, alongside 1μg of the highly glycosylated protein invertase (C). The proteins were transferred to a nylon membrane as described in section 2.11.3. Glycosylated polypeptides were visualised by probing the blot with the concanavalin A/peroxidase system described in section 2.11.5. Nylon membranes cannot be stained by conventional procedures. Therefore, to visualise hsp30 on the blot the plasma membrane proteins were phosphorylated prior to electrophoresis as described in section 2.15. The position of hsp30 (open arrow) is indicated on an autoradiogram (D) of lane B; H^+-ATPase is indicated also (solid arrow).



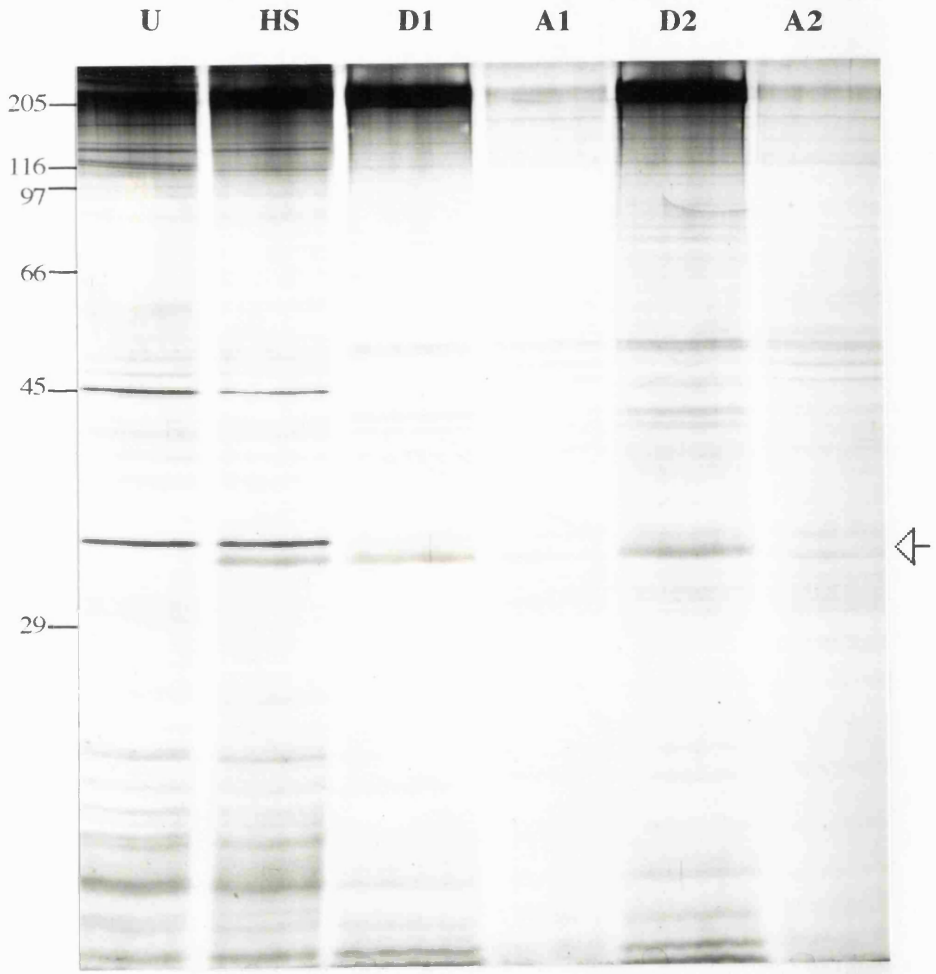
5.2.5 hsp30 is tightly associated with the plasma membrane.

Elevated temperatures tend to reduce the solubility of detergents in water and induce phase separation. The temperature at which this occurs is characteristic of the detergent used e.g. Triton X-100, 64°C; Triton X-114, 20°C (Thomas and McNamee, 1990). When phase separation occurs, hydrophilic proteins partition into the aqueous phase and hydrophobic proteins partition into the detergent phase. Consequently, phase separation after solubilisation of proteins with Triton X-114 has been proposed as a practical way to separate peripheral from integral membrane proteins (Bordier, 1981; Pryde, 1986).

Phase separation of plasma membrane proteins from heat shocked cells (as described in section 2.14) leads to hsp30 partitioning almost exclusively in the detergent phase (see Fig. 5.5). This suggests that the polypeptide is intimately associated with the plasma membrane and, as such, is likely to be an integral membrane protein with several transmembrane domains.

Since this technique was first used (Bordier, 1981), recent experience has indicated that not all hydrophobic proteins will behave in this way. Integral membrane proteins with extensive hydrophilic regions such as the nicotinic acetyl-choline receptor (Thomas and McNamee, 1990), or species with a single transmembrane domain and/or significant carbohydrate content (Findlay, 1990) partition in the aqueous phase. In addition, certain polypeptides such as nuclear lamins and all ras proteins (Schafer *et al.*, 1990) are associated with the plasma membrane by virtue of covalently-attached polyisoprenoid chains. The avidity of this attachment is sometimes increased by palmitoylation (Hancock *et al.*, 1989). Phase separation of these proteins, however, invariably results in their partitioning in both aqueous and detergent phases (Hancock *et al.*, 1989). In the light of such recent developments the almost exclusive partitioning of hsp30 in the detergent phase (Fig. 5.5) indicates that i) the polypeptide is tightly associated with the plasma membrane by virtue of several transmembrane helices and ii) there is an absence of extensive hydrophilic stretches and/or hydrophilic post-translational modifications such as glycosylation (in agreement with section 5.2.4) and iii) it is unlikely that association to the cell membrane involves covalent attachment of isoprenoids or fatty acids. The latter possibility could be tested in yeast using *ram1*, a mutant which lacks C-terminal isoprenylation of membrane proteins (Schafer *et al.*, 1990). In view of the evidence already obtained about hsp30, this was not considered worthwhile.

Figure 5.5. Phase separation of plasma membrane proteins of unstressed and heat shocked cells. Plasma membrane isolates from heat shocked cells were mixed with Triton X-114 and submitted to phase separation at 30°C as described in section 2.14. Aliquots of the input sample and of the aqueous and detergent phases after separation were analysed by 15% SDS-PAGE. Proteins were visualised by silver staining. (U), membrane proteins (10μg) from unstressed cells. (HS), input sample of membrane proteins (10μg) from heat shocked cells prior to phase separation. (D1) and (D2), detergent phases after phase separation using 0.5% and 1% Triton X-114 respectively. Tracks (A1) and (A2), corresponding aqueous phases. Molecular mass markers are indicated on the left, position of hsp30 is indicated on the right (open arrow, lanes HS, D1 and D2 only).



5.3 Discussion.

Attempts to raise polyclonal antiserum against hsp30 were unsuccessful. This could be due to one of several reasons. For a molecule to elicit a primary antibody response and a strong secondary response the host organism must possess B cell surface antibodies, T cell receptors and class II antigens that can bind processed fragments of the antigen (Roitt *et al.*, 1986). There is a theoretical limit to the total number of different antigen binding sites, so molecules must exist that have structures that cannot be recognised by the complement of available antigen-binding sites. Another reason why an animal might fail to respond to a given molecule is that appropriate B and T cells have been eliminated during development of cell tolerance (so that host molecules are not recognised, Roitt *et al.*, 1986). However, denatured hsp30 was used as antigen. This would give rise to epitopes not present in the putative host homolog. Indeed, hsp26 and hsp90 from *S. cerevisiae* induce potent immunogenic responses (see Fig. 4.7 A and B), even though mammals possess homologs of both proteins. As for hsp30, denatured hsp26 and hsp90 was used as antigen in both cases. Also, immunogens must promote cell to cell communication between T helper cells and B cells. When antigens enter B cells they are degraded; the resulting fragments are used to physically link B cells with T cells (Harlow and Lane, 1988 [c]). This physical link is essential for a potent immunogenic response. hsp30, however, exhibits a high degree of resistance to proteolytic attack, even in the presence of high amounts of a broad specificity protease (Fig. 5.3).

hsp30 is extremely hydrophobic (see Fig. 5.5). It is almost certain that this led to the formation of aggregates of the polypeptide within the rabbit used for preparation of antisera. If anything, this should have rendered the polypeptide more immunogenic. It is well established that monomeric fractions of protein induce poor immunogenic responses whereas aggregates induce potent ones (Harlow and Lane, 1988 [c]). There are few places at which the researcher can intervene in an attempt to induce a strong response to a particular antigen. Such opportunities are limited to modification of the antigen *per se* or alteration of injection conditions (Harlow and Lane, 1988 [c]). It should be noted that injection conditions used were those expected to induce an optimal response. However, there are several ways in which antigens can be modified such as conjugation to i) haptens (low molecular weight molecules with known B cell binding sites) or ii) carrier proteins (with known class II protein/T cell binding sites). Such modifications of the antigen would necessitate purification of hsp30 (preliminary steps for a purification scheme are given below). This would be a difficult task due to i) the laborious and time consuming preparation of plasma membranes from heat shocked cells and ii) the lack of knowledge concerning biochemical and physical properties of hsp30.

The few known characteristics of hsp30 could be exploited for purposes of purification. Phase partitioning reveals that hsp30 is among the most hydrophobic proteins

in the yeast plasma membrane. As its name suggests, hydrophobic interaction chromatography (HIC) separates proteins on the basis of their different strengths of interaction with a matrix-bearing hydrophobic groups such as phenyl sepharose (Roe, 1989). However, HIC has limited value for purifying integral membrane proteins like hsp30, as the essential presence of detergents will effectively abolish any meaningful hydrophobic interaction (Carson and Konigsberg, 1981). Instead, a scaled up version of phase extraction in Triton X-114 could be performed. This would result in detergent phase enriched in hsp30. hsp30, unlike many plasma membrane proteins, does not bind to concanavalin A. Accordingly, a subsequent step in an hsp30 purification scheme could involve lectin chromatography (Roe, 1989).

Purification of hsp30 could be facilitated by the use of strains containing mutations that block the pathway by which proteins are transported to the plasma membrane. The best mutant to use would be *sec1*, which is defective in the last step of the secretion and membrane assembly process (Holcomb *et al.*, 1988). At the restrictive temperature (37°C), a temperature at which *S. cerevisiae* will synthesise hsps, *sec1* cells accumulate a large pool of membrane bound vesicles. These vesicles should be rich in hsp30 as this is the only plasma membrane protein to be synthesised in reasonable quantities at elevated temperatures (see Fig. 4.3). These secretory vesicles can be isolated relatively free from contamination by other membranous structures such as the golgi, mitochondria or the vacuole (Holcomb *et al.*, 1988).

As the work described in this chapter progressed, it became obvious that cloning the gene coding for hsp30 could not involve manipulation of the polypeptide itself. In brief i) hsp30 did not induce an immune response (Fig. 5.1); ii) retention of hsp30 by gels during western blots did not allow limited N-terminal sequencing of the polypeptide (Fig. 5.2); and iii) attempts to sequence a hydrophobic segment of hsp30 failed due to poor elution of the protein from one gel to another as well as intrinsic resistance of the protein to proteolytic attack (Fig. 5.3).

If time had permitted attempts to clone the gene coding for hsp30 could have involved exploiting certain factors involved in expression of the gene. The protein only appears in membranes during heat shock, entry into stationary phase through carbon limitation and during nitrogen limitation. Differential hybridisation to a genomic library between mRNA made during i) exponential growth and ii) any one of the stresses previously listed, could enable cloning of the gene. Obviously, this may succeed due to the absence of hsp30 mRNA in exponentially growing (unstressed) cells. This scheme will also detect mRNAs of heat shock genes like those coding for hsp26, hsp12 or certain members of the hsp70 family, for example. These messages will be abundant in stressed cells. The presence of such 'contaminants' could be eliminated by preparing mRNA from membrane bound polysomes (Swida *et al.*, 1982), thereby taking advantage of the fact that hsp30 is the only stress protein specifically 'directed' to the cell membrane.

The gene coding for a hsp30 has since been cloned (H. Boucherie, personal communication). Not surprisingly, this was accomplished by differential hybridisation involving cDNAs derived from exponential and stationary phase cells, rather than by a scheme involving manipulation of the polypeptide *per se*. The gene sequence of *HSP30* indicates that the corresponding polypeptide is very hydrophobic, possessing 5 membrane spanning domains (H. Boucherie personal communication).

Chapter 6 Protein phosphorylation catalysed by S100 extracts from stressed cells.

6.1 Introduction

All organisms are capable of initiating a series of responses to heat shock. Though details of individual systems may differ, most of the following stress responses have been frequently observed:

1) Dramatic changes in the pattern of gene transcription by RNA polymerase II; i.e repression of most genes and induction of heat shock genes (see section 1.1).
2) Breakdown of existing polysomes and re-formation of new ones which in many organisms largely translate heat shock messages, resulting in the accumulation of high quantities of hsps (Lindquist, 1980).
3) Arrest of synthesis or processing (or both) of rRNA and consequently of ribosomes (Arrigo, 1980).
4) Arrest of the cell cycle (see section 1.7.1).
5) Activation of various enzymes including the plasma-membrane-associated H+-ATPase (work presented here and Coote *et al.*, 1991) and enzymes involved in synthesis of trehalose (see section 1.8.2).
6) Induction of various processes (as yet poorly understood) which lead to the acquisition of thermotolerance (see sections 1.7 and 1.7.1).
7) Various morphological changes or re-arrangements (see section 1.8.1).

Though considerable effort has been applied towards understanding cellular and biochemical responses to heat, the underlying processes occurring at the molecular level are still poorly understood. It is well established that protein phosphorylation is a major mechanism in the regulation of cellular functions (for reviews see Hunter, 1987; Kennelly and Krebs, 1991). Involvement of such processes in the heat inducible alteration of cell metabolism has been studied, but not in great detail. A review of his work is provided below. Most reports concern changes in the phosphorylation state of ribosomal, nuclear, and nucleolar proteins, caused by heat shock. There are no reports in the literature concerning changes in the phosphorylation state of plasma membrane proteins. For this reason plasma membrane isolates from unstressed and heat shocked cells were phosphorylated *in vitro*, and the resulting phosphoprotein profiles were compared (see Chapter 4). This chapter presents the results from similar experiments involving the soluble fraction (the post ribosomal supernatant).

Most work concerning the effect of heat shock on protein phosphorylation has been carried out in mammalian systems. There are few reports dealing with similar phenomena in yeast, and even fewer with respect to prokaryotes. The sole *E. coli* member of the highly

conserved hsp70 family (DnaK) and its homolog in *Mycobacterium bovis* autophosphorylate when incubated with ATP *in vitro* (McCarty and Walker, 1991; Peake *et al.*, 1991). This activity increases dramatically over the range of temperatures physiologically relevant for bacterial growth (McCarty and Walker, 1991). This temperature dependence could be of significance with respect to the role of DnaK as a molecular chaperone in helping cells counteract the deleterious effects of heat shock. Alternatively, the extent of phosphorylation of the protein may be involved in the mechanism by which DnaK controls the prokaryotic heat shock response (see section 1.3.1). It has even been proposed that both DnaK and its eukaryotic homolog hsp70 serve as a cellular thermometer directly sensing the temperature of the environment (Craig and Gross, 1991).

In eukaryotes, several groups have investigated the phosphorylation state of ribosomal proteins, by labelling *in vivo* with [^{32}P] phosphoric acid ($^{32}P_i$). Heat shock results in a rapid and reversible decline of the phosphorylation level of a single basic ribosomal protein (S6) of the small sub-unit. This has been detected in fungi (Pekkala and Silver, 1987), HeLa cells (Kennedy *et al.*, 1984), human fibroblasts (Richter *et al.*, 1983), *Drosophila* (Glover, 1982; Olsen *et al.*, 1983) and plant cells (Scharf and Nover, 1982). These studies did not determine whether this was due to inactivation of S6 kinase activities or activation of phosphoprotein phosphatases. In addition, concomitant with the dephosphorylation of S6 during heat shock, phosphate labelling of several ribosomal proteins of the large sub-unit is enhanced (Scharf and Nover, 1982; Kennedy *et al.*, 1984). A protein kinase from *S. cerevisiae* which specifically phosphorylates proteins of the large sub-unit has been isolated recently (Pilecki *et al.*, 1992). It remains to be seen whether heat shock stimulates its activity. It has been proposed that these events are connected with the selectivity of synthesis of heat shock proteins by the translational apparatus. However, prolonged incubation at high non-lethal temperatures will result in accumulation of hsps and an eventual restoration of a protein synthesis pattern similar to that prior to heat shock. Phosphorylation of S6 does not return to its normal unphosphorylated state under these conditions, only a shift back down to normal temperatures will achieve this. Accordingly, changes in phosphorylation state of S6 cannot be the main reason for the predominant translation of heat shock mRNAs (Scharf and Nover, 1982). Alternatively, S6 de-phosphorylation may play a role in heat-shock inducible cell-cycle-arrest. In metabolically inactive cells S6 phosphorylation is low; entry into the cell cycle is preceded by phosphorylation of S6 (Scharf and Nover, 1982 and references therein). Similarly, recovery from heat shock is characterised by re-phosphorylation of S6, which may subsequently lead to re-establishment of cell growth. Finally, It should be noted that contrary to the work described above, a recent report describes an *increase* in S6 phosphorylation caused by activation of S6 kinases during heat shock (Jurivich *et al.*, 1991). The difference may be due to growth phase since Jurivich *et al.* used cells in the G_0

(quiescent) state whereas the studies cited previously involved the use of exponentially growing cells (Jurivich *et al.*, 1991).

At high temperatures preferential translation of mRNAs coding for heat shock proteins may not be caused by changes in phosphorylation state of ribosomal proteins. Instead, phosphorylation of other components of the translational apparatus may be involved. The initiation factors eIF4B and eIF4F are dephosphorylated during heat shock (Legagneux *et al.*, 1990, and references therein). In contrast to this, heat shock causes the hyperphosphorylation of initiation factor eIF2α in both intact cells *and* cell lysates (Matts *et al.*, 1992, and references therein). This is due to the activation of an eIF2α kinase (DeBenedetti and Baglioni, 1986).

Similar experiments involving nuclear and nucleolar proteins of Chinese hamster ovary cells have also been reported. Heat shock induces the phosphorylation of a nucleolar protein with a molecular weight of 95kDa. In the non-nucleolar fraction of the nucleus, phosphorylation of a 54kDa protein is induced while a 35kDa protein is rapidly de-phosphorylated. These phenomena are proposed to play a role in regulation of transcription and in RNA processing during heat shock (Caizergues-Ferrer *et al.*, 1980).

Finally, it is well established that in eukaryotes, phosphorylation of heat shock transcription factor (HSF) is an essential step in the induction of heat-shock gene expression (see section 1.3.2). Surprisingly, there have been very few reports concerning how this occurs. The mechanism involved, whether it involves autophosphorylation or a protein kinase, is obviously modulated by heat. Accordingly, analysis of the HSF activating system will reveal the nature of the cellular component(s) that detect environmental stress, i.e. the so called "cellular thermometer" in the case of heat shock. Recent data suggest that HSF is phosphorylated by a protein kinase. Experiments using a permeabilised cell system revealed that genistein, a non-specific inhibitor of protein kinases, blocks transcription of at least one hsp gene in NIH 3T3 cells (Price and Calderwood, 1991). The way in which HSF enables transcription of heat shock genes is unknown. Gene specificity of RNA polymerase II may be altered by heat shock. It has been proposed that one way by which this can be achieved is to change the phosphorylation state of its carboxy-terminal repetitive domain (CTD) by CTD kinases. Enzymes responsible for CTD phosphorylation have been described, and lysates from heat shocked cells possess much higher CTD kinase activities than lysates from unstressed cells (Legagneux *et al.*, 1990). Stimulation of the kinase is protein synthesis independent. However, stress induced kinase activity has not yet been demonstrated to phosphorylate RNA polymerase II *in vivo.*

The above shows that many workers are attempting to explain how cells preferentially transcribe heat shock genes and preferentially translate the resulting messages, during heat shock. Not surprisingly, most of the literature pertaining to stress inducible changes in phosphorylation state concerns proteins associated with the ribosome,

the nucleolus and the non-nucleolar regions of the nucleus. Changes in the phosphorylation state of proteins in other regions of the cell could regulate other types of heat inducible phenomena. The plasma membrane is an obvious candidate for such a study. Firstly, it is well established that components of the cell membrane are involved in detecting and initiating responses to changes in the environment. Secondly, the activity of H+-ATPase, a known determinant of stress tolerance (see Chapters 3 and 4; Coote *et al.*, 1991), increases during heat shock. Protein phosphorylation may play a role in both of these phenomena. Surprisingly, there are no reports in the literature describing changes in the phosphorylation state of membrane associated proteins caused by heat shock. Results of a preliminary study designed to detect such changes are presented in Chapter 4.

Similar reports in the literature concerning modification of the phosphorylation state of cytosolic proteins are sparse and seem to be limited to studies on hsps. In higher eukaryotes phosphorylation of hsp26 increases during heat shock (Landry *et al.*, 1988). The yeast homolog does not appear to be phosphorylated (M. Tuite, personal communication). Also, human hsp90 is phosphorylated *in vivo*, and can be phosphorylated *in vitro* by casein kinase II (Lees-Miller and Anderson, 1989 [a]) and by a double stranded DNA activated kinase (Lees-Miller and Anderson, 1989 [b]). Human hsp90 also possesses an autophosphorylation activity (Csermely and Kahn, 1991). Again, similar activities have not been reported for the yeast homologs.Though the proteins from yeast and mammals are highly homologous, the motifs involved (with respect to the kinases) are found in humans and other mammals but not in yeast (see section 1.2.3). The physiological significance of the phosphorylation of hsps is unknown. Changes in the phosphorylation state of cytosolic proteins that exist both prior to and during heat shock could also be of interest. Such events could regulate heat-shock inducible events.

6.2 Rationale behind the conditions used for phosphorylation of cytosolic proteins.

Changes in the phosphorylation state of proteins can be studied by *in vivo* labelling of cell extracts with $^{32}P_i$ or *in vitro* labelling with [γ^{32}P]-ATP. The obvious advantage of *in vivo* labelling is that results obtained are likely to be physiologically relevant. However, the nature and concentration of components of a given system cannot be altered, thereby limiting the usefulness of experiments involving whole cells. This is overcome by using cell free extracts even though the physiological relevance of *in vitro* studies is frequently questioned. However, for ribosomes (Pilecki *et al.*, 1992) and the plasma membrane of yeast (see section 4.4.11), it has been found that the majority of proteins labelled with $^{32}P_i$ *in vivo* correspond to the proteins phosphorylated *in vitro* with [γ^{32}P]-ATP. The consensus is probably due to reaction conditions used for *in vitro* studies approximating physiological conditions. This especially applies to the concentration of ATP used which

rarely exceeds 100μM, the approximate physiological concentration of this metabolite in yeast (Weitzel *et al.*, 1987). Indeed, most *in vitro* studies use between 10 and 30μM ATP. (e.g. Kolarov *et al.*, 1988; Londesborough and Nuutinen, 1987).

Surprisingly, there are very few reports in the literature concerning modification of the phosphorylation state of cytosolic proteins caused by stress. This chapter presents the results of a preliminary study involving *in vitro* phosphorylation of cytosolic extracts from unstressed and heat shocked cells. Cultures of the protease deficient strain BJ2168 (see Table 2.1A for genotype) were grown to mid exponential phase at 20^{0}C in rich media. Cells were harvested immediately (unstressed), or, shifted to 40^{0}C for 40 minutes (heat shocked) prior to harvesting. Cytosolic extracts were prepared from both (see sections 2.9.1 and 2.16). As described in section 2.9.1, a crude cell extract is subjected to a number of centrifugation steps designed to remove cell walls, nuclei, mitochondria and other membranous structures. The final step involves a 2 hour 100,000xg spin which sediments ribosomes. The pelleted ribosomes are in the form of polysomes and the resulting supernatant is commonly termed as the S100 fraction or the post ribosomal supernatant. However, this S100 extract will contain some 40S and 60S ribosomal subunits as well as some monosomes (Feinberg and McLaughlin, 1988). Attempts to sediment monosomes by the final spin being carried out at significantly higher g force than 100,000xg would be counter productive. This would increase the risk of damaging membrane bound polysomes, fragments of which might remain in the supernatant (Lambert, 1989).

Endogenous ATP in the cytosolic extracts was removed by dialysis against a buffer which was at near-physiological pH and contained i) the most important physiological cations (K^+ and Mg^{2+}) at concentrations similar to those in intact yeast cells, ii) 1mM DTT, since the action of some kinases is dependent on free sulphydryl groups (Pilecki *et al.*, 1992) and iii) 10% glycerol as a stabilising agent to limit proteolysis (North, 1989). Extracts were phosphorylated by incubating 10μg of protein with [γ^{32}P]-ATP at 30^{0}C, as described in section 2.16. Reactions were terminated by the addition of an equal volume of SDS gel sample buffer, a standard method of stopping *in vitro* phosphorylation reactions. Proteins were resolved by SDS-PAGE and phosphoproteins visualised by autoradiography as described in sections 2.10.1 and 2.11.2, respectively. Both short term (5 seconds) and long term (10 minute) incubations with ATP were carried out. In addition concentrations of ATP varied between ultralow (10nM) to nearer physiological values (20μM). This type of manipulation can only be carried out with *in vitro* systems. Such dramatic variations in concentration of ATP and incubation time were performed since these studies were the first of their kind to be done with respect to cytosolic extracts from stressed cells. Also, it may be possible to distinguish between autophosphorylation and phosphorylation catalysed by kinases, by taking advantage of the fact that autophosphorylation reactions can occur very quickly and in the presence of extremely low levels of ATP (M. Mazon, personal communication). Future experiments involving modification of other elements of this

system are discussed in section 6.4.

6.3 Results.

6.3.1 Phosphoprotein profiles of S100 extracts reacted with [γ-^{32}P] ATP.

Autoradiograms of gels presenting the results of *in vitro* phosphorylation experiments on extracts from stressed cells are presented in Figs. 6.1 and 6.2. Autoradiograms of both long and short exposures to film of each gel are shown. The S100 extracts were phosphorylated with 20μM (Fig. 6.1) and 10nM ATP (Fig. 6.2). It should be noted that the higher concentration of ATP was prepared by mixing unlabelled ATP with [γ-^{32}P] ATP (3,000 Ci/mmol), as described in section 2.16. *In vitro* phosphorylation with an ultra-low concentration of ATP was achieved by incubation in the presence of [γ-^{32}P] ATP only, at a final concentration of 10nM. In both cases *only* the S100 extracts from heat shocked cells extensively phosphorylated a protein of M_r 66kDa (referred to as p66 from now on). This was noted after incubations with 20μM ATP lasting both 5 seconds and 10 minutes. In the presence of 10nM ATP this was noted after a 10 minute incubation only. However, the way in which the S100 extract is prepared influences the incubation time needed to phosphorylate p66 in the presence of 10nM ATP (see Fig. 6.4 and section 6.3.3).

Phosphorylation of p66 in heat shock extracts only is due either to i) phosphorylation of a protein which is only present during heat shock (i.e it is an hsp), or, ii) phosphorylation of a protein that is present both prior to and during a heat shock, but is only phosphorylated during heat shock. The second possibility seemed likely as p66 does not co-migrate with any of the major hsps (see Figs. 6.3.1 and 6.3.2, B). Also, there is no report describing a minor hsp of this M_r in yeast. Even though p66 is not an hsp, the possibility remained that its phosphorylation in heat shock extracts required the presence of hsps. This was found to be the case since the protein was not phosphorylated in an S100 extract prepared from cells heat shocked in the presence of cycloheximide (Fig. 6.2).

Some of the responses induced by heat shock are also induced on entry into stationary phase e.g. induction of hsps and an increase in the levels of trehalose (see section 1.6 and Wiemken, 1990, respectively). However, *in vitro* phosphorylation of p66 does not occur in extracts from stationary cells (Fig. 6.2).

The short term (5 seconds) and long term (10 minutes) incubations revealed the existence of a phenomenon that is not frequently reported in the literature, namely the apparent loss of phosphoproteins during incubation with [γ-^{32}P] ATP. Incubation with 10nM ATP resulted in the rapid (within 5 seconds) and extensive phosphorylation of proteins with an M_r of 31 and 32kDa, see Fig. 6.2. They are referred to as p31 and p32 from now on. This is true for extracts from both heat shocked and unstressed cells. These proteins were not visualised after a 10 minute incubation. This may be due to degradation

of the proteins during the longer incubation, or, the removal of ^{32}P from the proteins by phosphoprotein phosphatases. The former is unlikely given that the extracts were derived from a protease deficient strain. Furthermore, protease inhibitors, some of which lead to irreversible inactivation of proteases, were present throughout the cell fractionation procedure. Also, p31 and p32 can be visualised by staining with Coomassie blue, and both are still present after a 10 minute incubation with ATP (see Fig. 6.3.2 A).

This "dephosphorylation" was not seen when extracts were incubated for 10 minutes with 20μM ATP. As already indicated, this concentration of ATP was prepared by mixing unlabelled ATP with [γ-^{32}P] ATP (3,000 Ci/mmol). Accordingly, if p31 and p32 experience cycles of alternating kinase and phosphatase reactions, then it is likely that at any given point during the 10 minute incubation a given proportion of the p31 and p32 molecules will be phosphorylated with ^{32}P. In contrast, *In vitro* phosphorylation with an ultra-low concentration of ATP (10nM) was achieved by incubation in the presence of an ATP preparation in which the vast majority of ATP molecules were radiolabelled. Cycles of alternating kinase and phosphatase reactions might occur, but the concentration of ATP is so low that all of the ATP will have been hydrolysed during the long term incubation. Since there is no ATP with which to re-phosphorylate p31 and p32, the corresponding phosphoproteins are not visualised after a 10 minute incubation. To determine whether or not this is the correct interpretation of the results presented in Figs 6.1 and 6.2 one would have to prove the existence of alternating kinase and phosphatase reactions. This could be achieved simply by incubating S100 extracts with 10nM [γ-^{32}P] ATP (as described previously) for 10 minutes. By this time de-phosphorylation will have occurred, and a subsequent addition of a further 10nM [γ-^{32}P] ATP could be carried out followed by termination of the reaction a few seconds later. Resolution of the resulting phosphoproteins by SDS-PAGE will reveal if p31 and p32 have been re-phosphorylated.

Figure 6.1 Protein phosphorylation in S100 extracts using a near physiological concentration of [γ^{32}P]-ATP. Cultures of the protease deficient strain BJ2168 were grown at 20^{0}C to exponential phase in rich media. Cells were harvested either at 20^{0}C (**Unstressed**) or 40 minutes after a 20-40^{0}C upshift (**Heat shock**). S100 extracts were prepared and reacted with 20μM [γ^{32}P]-ATP, for the length of time indicated, as described in section 2.16. Each reaction was carried out using 10μg of protein. Following 15% SDS-PAGE, gels were dried and exposed to film for visualisation of phosphorylated proteins. **A** and **B** are short and long exposure times respectively. Molecular masses of markers are indicated on the left. Some of the **p**hosphoproteins are designated by their M_r in kDa e.g. **p66**.

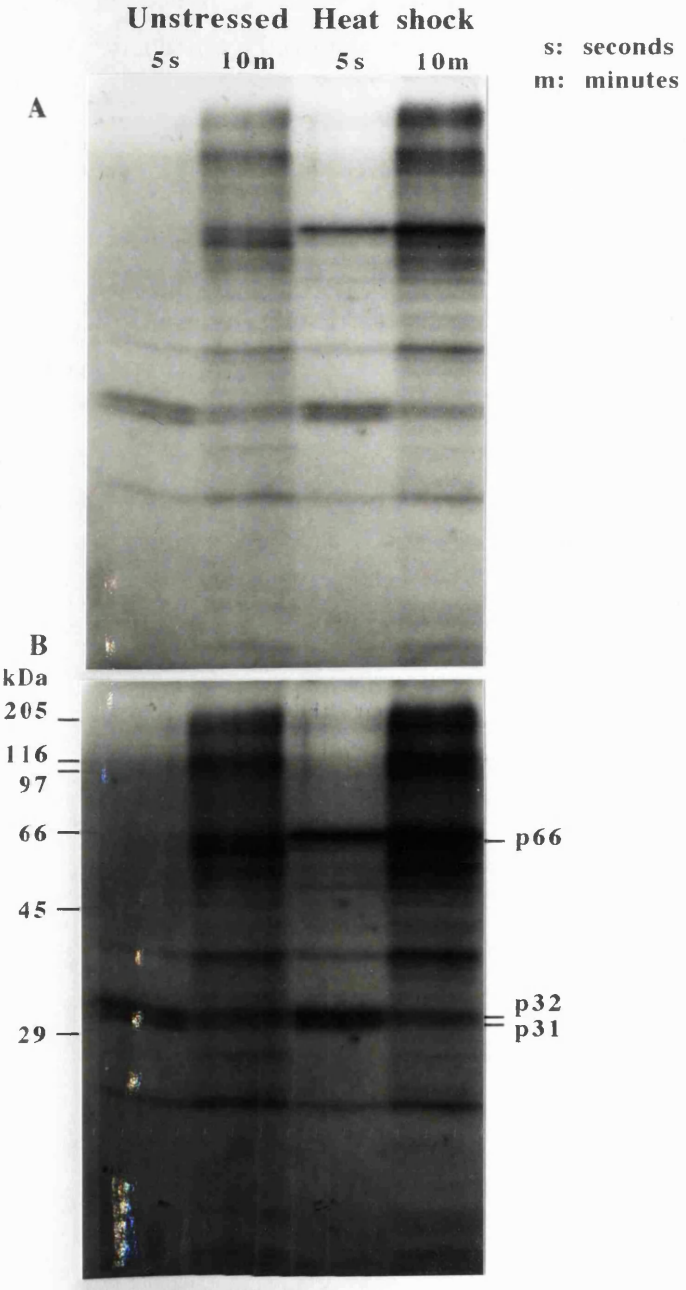
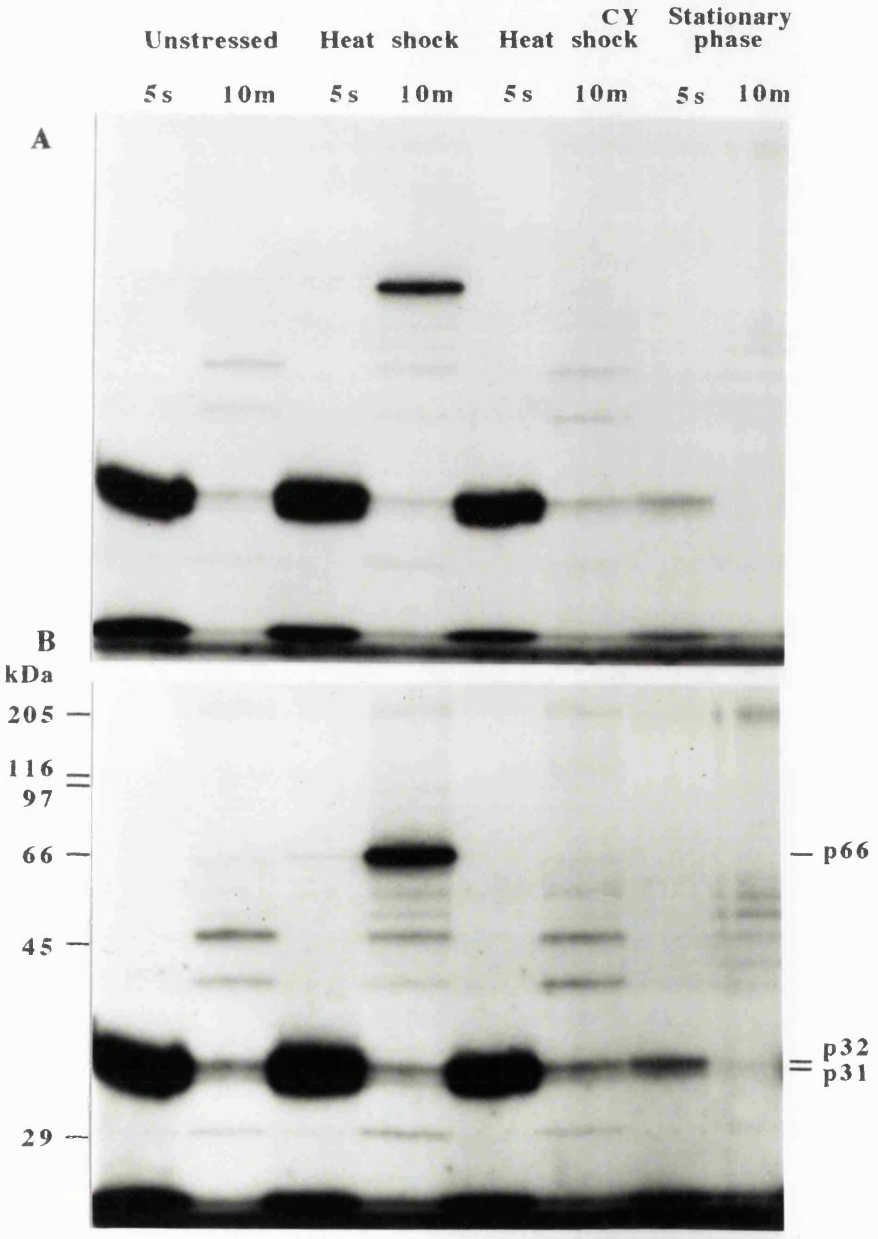


Figure 6.2 Protein phosphorylation of S100 extracts using an ultra-low concentration of [γ^{32}P]-ATP. S100 extracts from unstressed and heat shocked cells were subjected to *in vitro* phosphorylation as described in Fig. 6.1, except that 10nM [γ^{32}P]-ATP was used in this case. In addition extracts from cells heat shocked in the presence of cycloheximide at 50μg/ml of culture (**Heat shock^{CY}**), and, cells that were harvested 18 hours after cultures had reached a density of 1x10^8 cells/ml (**Stationary phase**) were also phosphorylated. Phosphoproteins are visualised as described in Fig. 6.1.



6.3.2 p66 is not an hsp.

Incubating S100 extracts from unstressed and heat shocked cells with [γ-^{32}P] ATP results in the phosphorylation of several proteins (Fig 6.1 and 6.2). None of them, however, co-migrate with the major hsps (Fig. 6.3.1). hsps from yeast, unlike those from higher eukaryotes, might be non-phosphorylatable (*in vitro* or *in vivo*). In agreement with this, there are no reports in the literature describing the modification by phosphorylation for any of the yeast hsps (the exception being the phosphorylation of plasma-membrane - associated hsp30 reported in Chapter 4). Alternatively, many kinases in the cytosolic extracts may have been inactive under the reaction conditions used here. Kinases that phosphorylate hsps may have been among them. This point is discussed in section 6.4.

Soluble extracts from unstressed and heat shocked cells were phosphorylated with 10nM [γ-^{32}P] ATP as described previously. Resulting phosphoproteins were resolved by SDS-PAGE and gels were stained with Coomassie blue prior to autoradiography. The stained polypeptides co-migrating with p31, p32 and p66 are indicated in Figs. 6.3.2 A and B. The phosphoprotein profiles were not altered during staining and de-staining procedures. However, the amount of radioactivity associated with these proteins as measured by a geiger counter decreased dramatically; from easily detectable levels prior to staining, to barely detectable levels after staining. The visualisation of proteins by Coomassie blue involves incubating gels in acidic solutions (see section 2.11.1). Loss of radioactivity during staining is not due to diffusion of proteins, since staining procedures immobilise proteins into gels. This suggests that the loss of radioactivity associated with these gels caused by staining is due to acid hydrolysis of the bonds linking phosphoryl groups to amino acids. The implications of this are described below. It should be noted that this is a qualitative observation. A quantitative analysis can be undertaken simply by blotting phosphoproteins onto PVDF, excising the bands of interest and subsequently determining how much radioactivity is lost during treatment with acid.

Phosphorylated amino acid residues in proteins are commonly classified into 3 main groups:

i) O-phosphates or O-phosphomonoesters (O-P). Formed by phosphorylation of hydroxy amino acids serine, threonine and tyrosine.

ii) N-phosphates or phosphoramidates (N-P). Formed by phosphorylation of basic amino acids arginine, histidine and lysine. The acceptor amino acid has most commonly been histidine.

iii) Acyl phosphates or phosphate anhydrides (A-P). Most commonly formed by phosphorylation of aspartate.

A fourth group, by far the least abundant, includes S-phosphates of the sulphydryl amino acid cysteine (S-P) (Hunter, 1991).

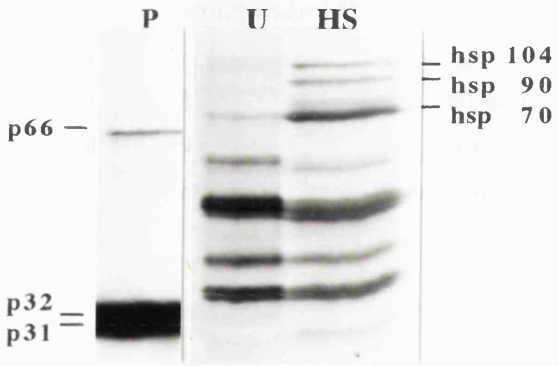
On Laemmli SDS gels O-, S- and N- phosphates are stable. Acyl phosphates are

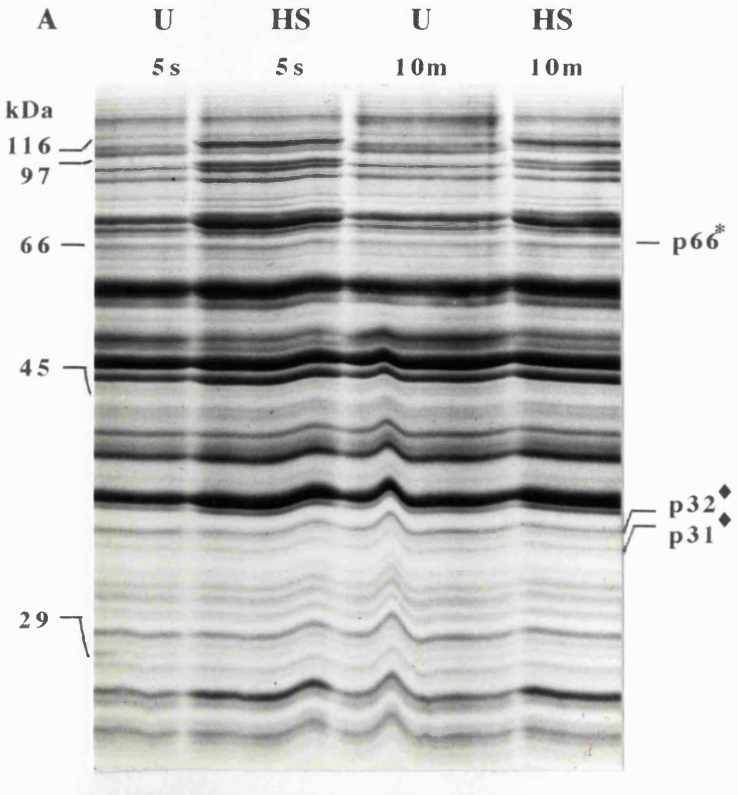
unstable, and would not be visualised by autoradiography after phosphorylation with [γ-^{32}P] ATP (see section 4.4.11). However, unlike O-P and S-P bonds, N-P linkages are extremely susceptible to acid hydrolysis (Duclos *et al.*, 1991). Accordingly, ^{32}P lost from proteins during staining/de-staining gels in acidic solutions described above must originate from N-phosphates. This loss of N-linked phosphate during fixation of polyacrylamide gels in acetic acid is well established (Huang *et al.*, 1991).

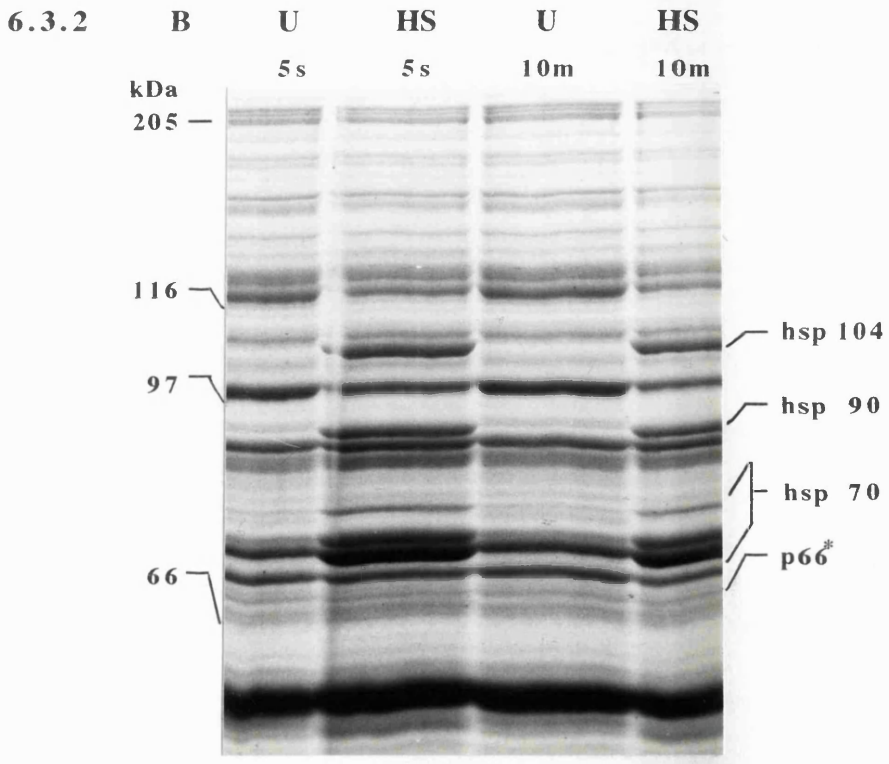
There are far fewer reports in the literature concerning protein kinases catalysing N-P linkages compared with those catalysing the formation of O-P linkages. Indeed, N linked phosphates have been largely overlooked; several reviews on protein phosphorylation do not even mention their existence (see Hunter, 1987; Kennelly and Krebs, 1991 and Roach, 1991). This is due to the technical difficulties associated with studying N-phosphates. It is the susceptibility of the N-P linkage to acid hydrolysis that is the most important factor here. Most kinase assays involve precipitation of proteins by trichloroacetic acid; fixation of proteins in polyacrylamide gels is usually carried out using acetic acid; HPLC is frequently carried out in the presence of trifluoroacetic acid, and, acid hydrolysis is an integral part of the Edman degradation. In all of these cases, N linked phosphates will be lost. This has led to the suggestion that N linked phosphates may be far more widespread than is generally believed (Duclos *et al.*, 1991). The few known instances of N linked phosphorylation are reviewed in section 6.4; possible ramifications of this type of phosphorylation in relation to the phosphoproteins described in this chapter are also discussed.

Figure 6.3.1 Phosphoprotein p66 does not co-migrate with any of the major hsps. S100 extracts from heat shocked cells were phosphorylated as described in Figure 6.2. Samples phosphorylated for 5 seconds and 10 minutes were mixed and their proteins resolved by 15% SDS-PAGE (**P**). Proteins of unstressed (**U**) and heat shocked (**HS**) cells, pulse labelled with [^{3}H] leucine (as described in section 2.6.1), were resolved on the same gel. hsps are indicated on the right (hs lane only), phosphoproteins are indicated on the left.

Figure 6.3.2 Visualising phosphoproteins by Coomassie staining. S100 extracts from unstressed (**U**) and heat shocked (**HS**) cells were phosphorylated as described in Figure 6.2. Following SDS-PAGE gels were stained with Coomassie blue, dried, and then exposed to film in order to determine which stained polypeptides co-migrate with the p31, p32 and p66 phosphoproteins. Proteins were resolved by 15% gels (**A**), and, to improve resolution of p66, by 9% gels **(B).** Molecular mass markers are indicated on the left, major hsps and the stained polypeptides migrating with the phosphoproteins are indicated on the right.







* On autoradiograms this protein was visualised in the **HS,10m** lanes only (see Fig. 6.2).

♦ On autoradiograms these proteins were only visualised in the **U,5s** & **HS,5s** lanes only (see Fig. 6.2).

6.3.3 The method by which the S100 is prepared affects the time needed to phosphorylate p66.

Once the S100 fraction had been isolated from yeast cells, the extract was prepared for *in vitro* phosphorylation. The most important aspect of this procedure was the removal of endogenous ATP. Removal of microsolutes can be achieved by dialysis or ultrafiltration. The advantage of the latter is that it can be carried out rapidly, unlike dialysis which is a comparatively lengthy procedure. Preliminary experiments involved preparing S100 in either of 3 ways:

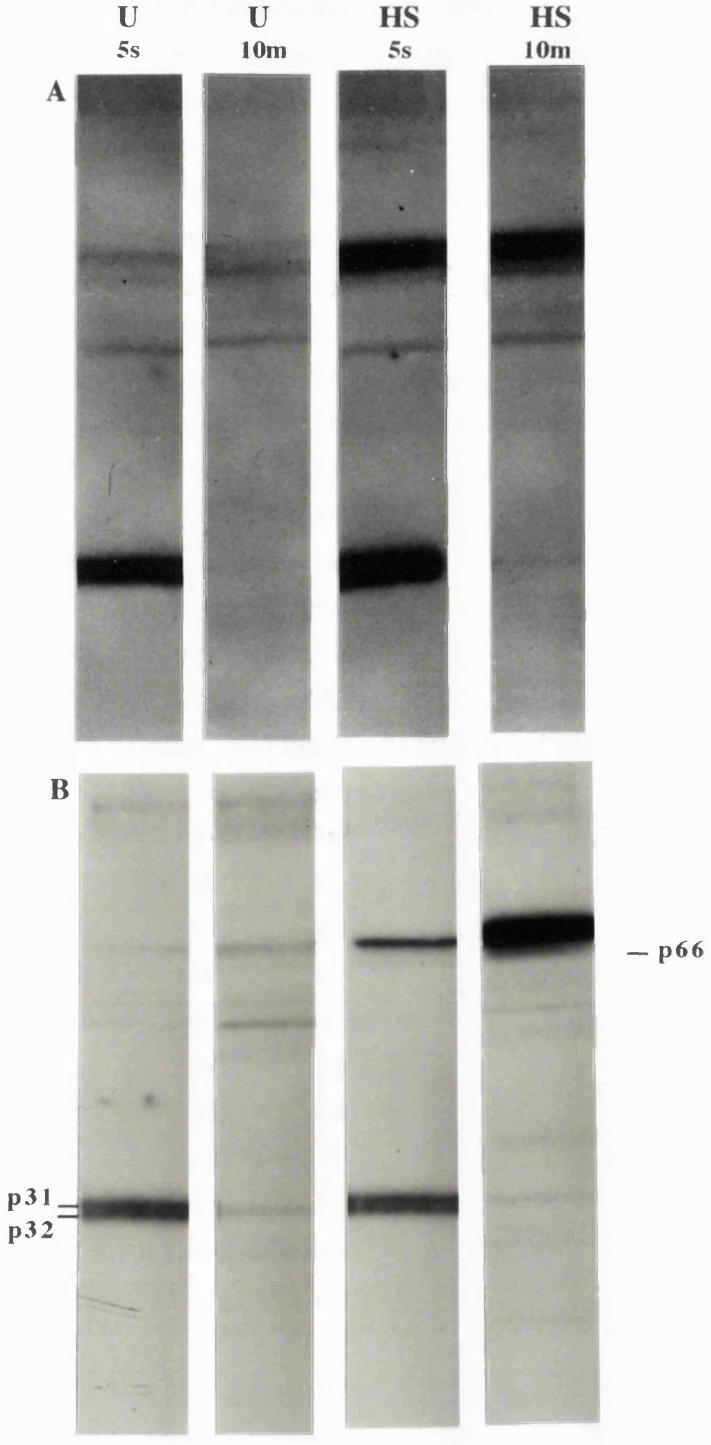
i) ultrafiltration, achieved by centrifugal concentration as described in section 2.16. This procedure took only 2 hours.
ii) dialysis against 5 changes of buffer over 6 hours, as described in section 2.16.
iii) dialysis against 5 changes of buffer over 12 hours, as described in section 2.16.

Identical *in vitro* phosphorylation reactions using 10nM [γ-^{32}P] ATP were carried out with all 3 preparations. The overall phosphoprotein profile was identical (see Fig. 6.4 A,B and Fig. 6.2). However, the time needed to phosphorylate p66 in extracts from heat shocked cells varied. Lengthy dialysis (12 hours) did not result in the phosphorylation of p66 in heat shock extracts after a 5 second incubation with ATP (Fig. 6.2). However, the polypeptide *was* phosphorylated after 10 minutes i.e between 5 seconds and 10 minutes. In contrast to this, intermediate dialysis (6 hours) did result in phosphorylation of p66 after 5 seconds, though the extent of phosphorylation was not as great as that seen after 10 minutes (Fig. 6.4 B). Finally, S100 extracts prepared by a method which takes even less time (centrifugal concentration) results in maximal phosphorylation of p66 after 5 seconds i.e. the extent of phosphorylation was the same as that seen after 10 minutes (Fig. 6.4 A).

The activity of a component of the phosphorylation system is affected by the length of time needed to prepare S100 extracts. This may be due to the absence of an accessory molecule involved in the reaction which could be i) a polypeptide of molecular weight >10kDa, since both membranes involved in dialysis and ultrafiltration procedures described in section 2.16 have molecular-weight cut-off points of ca. 10kDa, or, ii) an ion or a small molecule.

If samples were incubated with [γ-^{32}P] ATP for 10 minutes only, the phenomenon described above would not have been noticed. This effect was only apparent from the results of short term incubations with [γ-^{32}P] ATP. Not surprisingly, there are no reports in the literature describing a similar phenomenon, since most reports describe the results of long term reactions.

Figure 6.4 The way in which S100 extracts were prepared affects the time needed to phosphorylate p66*. S100 extracts from unstressed and heat shocked cells prepared by one of 3 ways were phosphorylated as described in Fig. 6.2. (**A**) 2 hour centrifugal concentration (as described in section 2.9.2), (**B**) 6 hour dialysis (as described in section 2.9.2) and by 12 hour dialysis (see Fig. 6.2).



* It is length of time taken to prepare S100 extracts that is likely to be the important factor here, rather than the procedure *per se* (see section 6.3.3)

6.3.4 Extracts from cells stressed by ethanol catalyse p66 phosphorylation.

Like heat shock, incubating cells in the presence of high but sub-lethal concentrations of ethanol induces hsp synthesis (Plesset *et al.*, 1982 and Fig. 6.5.2). To determine whether this type of stress can also lead to phosphorylation of p66, the S100 fraction from cells incubated in the presence of 8% ethanol for 50 minutes was incubated with 10nM [γ-^{32}P] ATP. The hsps produced by cells under these conditions are shown in Fig. 6.5.2. Phosphorylation of p66 did occur, but it was not as extensive as that catalysed by extracts from heat shocked cells (Fig. 6.5.1).

Induction of the ability for phosphorylation of p66 is protein synthesis dependent (see section 6.3.1). Extracts from cells heat shocked in the presence of cycloheximide will not phosphorylate p66. Accordingly, the best way by which one can determine if a specific hsp is implicated involves experiments like those described in section 6.3.1 using extracts from strains bearing disruptions for particular hsp genes. Strains of *S. cerevisiae* deleted for all the major hsp genes are available. Such an experiment was carried out with a strain deleted for two of the hsp70 genes (*SSA1* and *SSA2*), the result being that p66 was phosphorylated in extracts from *both* unstressed cells as well as heat shocked cells (not shown). This was not surprising given that deletion of these two genes results in the expression of hsps at high levels even at 23^{0}C (Craig and Jacobsen, 1984). Phosphorylation of p66 in extracts from this double deletion mutant indicates that hsps other than those encoded by *SSA1* and *SSA2* are involved. This necessitates performing *in vitro* phosphorylation of extracts from the strains disrupted for the other hsp genes. These experiments, would have been carried out if time had permitted. They are now being performed by L. Cheng and have yielded interesting results (see below).

Phosphorylation of p66 catalysed by extracts from cells incubated in the presence of 8% ethanol may provide a clue as to which hsp influences p66 phosphorylation. Under these circumstances phosphorylation of this polypeptide is nowhere near as extensive as that catalysed by extracts from heat shocked cells. This may be due to ethanol stress inducing relatively low amounts of the particular hsp involved. Comparison of the appropriate hsp profiles reveals that levels of hsp90 and hsp26 in ethanol stressed cells are far lower than those in heat shocked cells (Fig. 6.5.2). Of these, it seems likely that hsp90 is the more likely candidate, since it has been shown to modulate the activity of various protein kinases in cells of higher eukaryotes. Not surprisingly, recent experiments carried out by L. Cheng, using strains of *S. cerevisiae* in which levels of hsp90 are dramatically altered, result in striking effects on the phosphorylation of p66. For instance, p66 is extensively phosphorylated in extracts from *unstressed* cells derived from strains that overproduce hsp90 (L. Cheng, unpublished results, see section 6.4).

Figure 6.5.1 S100 extracts from ethanol stressed cells poorly phosphorylate p66. S100 extracts from **unstressed**, and **heat shocked** cells were phosphorylated as described in Fig. 6.2, except all assays were carried out using 1μg of protein instead of 10μg. In addition extracts from cells incubated for 50 minutes in the presence of 8% ethanol were also phosphorylated. Phosphoproteins were visualised as described in Fig. 6.1. Short (**A**) and long (**B**) term exposures are presented.

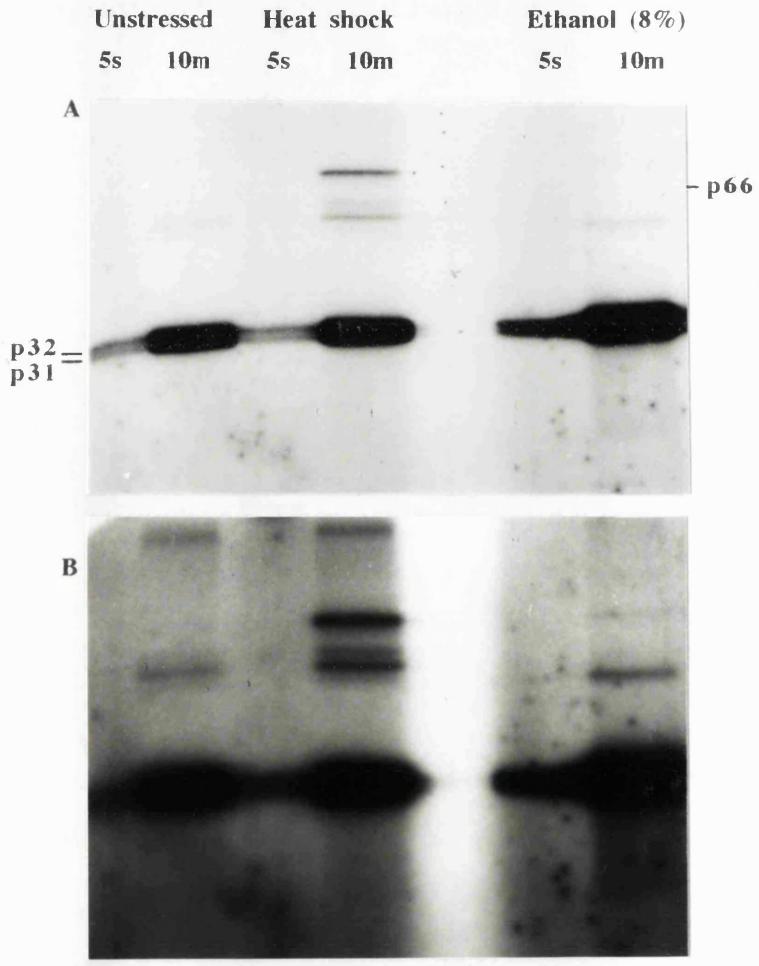
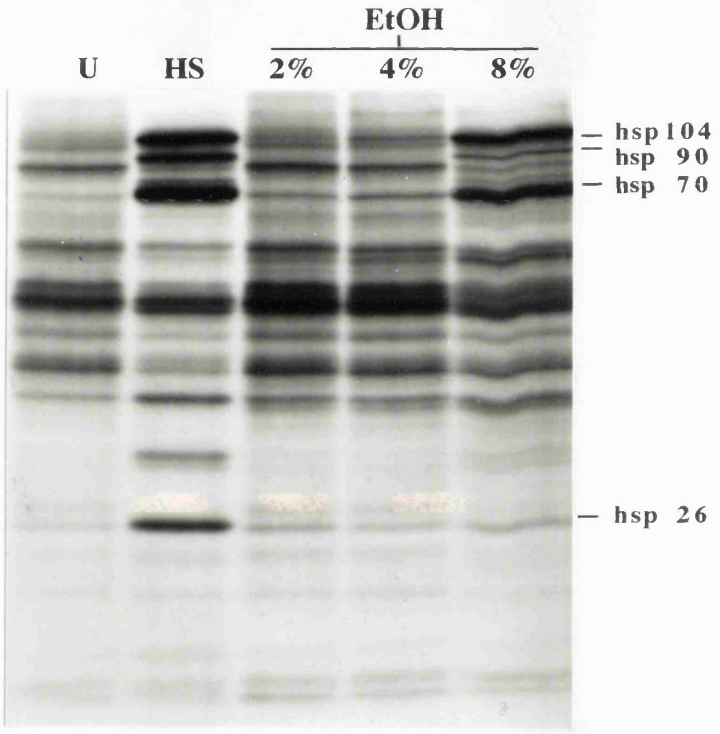


Figure 6.5.2 Comparing the stress proteins induced by heat shock and ethanol. Unstressed (**U**), heat shocked (**HS**) and ethanol stressed (**EtOH**) cells*, were pulse labelled with [^{3}H] leucine. Final concentrations of ethanol used were as indicated below. Proteins from total cell extracts were resolved by 15% SDS-PAGE and visualised by fluorography. hsps are indicated on the right.



* Unstressed cells pulse labelled for 40 minutes at 20^{0}C, heat shocked cells pulse labelled during 10-50 minutes after a 20-40^{0}C heat shock, EtOH stressed cells pulse labelled 10-50 minutes after the addition of absolute EtOH to give the final concentration indicated.

6.4 Discussion.

The cellular stress response has been extensively studied. In spite of this, little information is available regarding signal transduction and the resulting responses during physiological stress. Even less is known about the subsequent processes involved in recovery. On the whole, activity of many proteins is regulated by one (or more) of many different types of post translational modification. Among these are acetylation, methylation and phosphorylation. Of these, protein phosphorylation is the most widespread. It seems likely that various proteins implicated in stress inducible responses are covalently modified in this way. There are several reports concerning the effects of heat on protein phosphorylation; they are reviewed in section 6.1.

Incubation of S100 extracts with ultralow or near-physiological concentrations of ATP results in the phosphorylation of several proteins, notably p31, p32 and p66 (see section 6.3.1). Furthermore p31 and p32 are rapidly phosphorylated. Incubation time needed to phosphorylate p66 seems to depend upon how S100 extracts are prepared.

The question arises as to whether these events are catalysed by kinases or the molecules *per se* (i.e autophosphorylation). The physiological significance of autophosphorylation is questionable since there are examples of these reactions occurring *in vitro* but not *in vivo*. Also, it is noticeable that *in vitro* reaction conditions, though utilising near-physiological concentrations of ATP, usually involve molar excesses of the proteins in question. It should be noted that reactions described in this chapter were carried out using i) S100 extracts, so the concentration of any one particular protein is not likely to be very high and ii) 20μM ATP or less; physiological concentration of this metabolite in yeast is ca. 100μM. Until recently, reports describing the consequences of autophosphorylation were limited to effects on the catalytic parameters of the enzymes in question. However the advent of molecular genetic techniques has allowed more convincing proof of a physiological role of autophosphorylation. For instance, oligonucleotide directed mutagenesis of several autophosphorylation sites in the human insulin receptor lead to dramatic changes (ranging from reduction to abolition) of the signal transmitted by binding of insulin (Murakami and Rosen, 1991).

The definitive method for determining whether p31 and p32 are autophosphorylated would involve incubation of the purified proteins with [γ-^{32}P] ATP. It will be some time before these proteins are purified since so little is known about their biological function. However a requirement for purified protein can be bypassed. Proteins can be separated by SDS-PAGE, transferred to nitrocellulose or PVDF, renatured and then autophosphorylated (as described by Ferrel and Martin, 1989). It should be noted that this technique cannot be used to determine whether or not p66 is autophosphorylated since it has already been demonstrated that its phosphorylation *in vitro* is dependent on the presence of other proteins (see section 6.3.1). Proteins which undergo autophosphorylation reactions must

bind ATP (or ATP analogs). Accordingly, these proteins should be amenable to covalent labelling with a radioactive non-hydrolysable analog of ATP such as [α-^{32}P] Azido-ATP (Csermely and Kahn, 1991). Evidence of binding to ATP could be exploited for purification purposes.

Comparison of the phosphorylated proteins in control and heat shock extracts, separated by SDS-PAGE, revealed a striking difference in the profile of labelled proteins. A polypeptide of M_r 66kDa (p66) is extensively phosphorylated in heat shock extracts only. Though not an hsp itself, the phosphorylation of p66 is hsp dependent. Recent experiments suggest that one stress protein involved here is hsp90 (L. Cheng, unpublished results; also see section 6.3.4). This can be interpreted in a number of ways, some of which are listed below:

1) p66 is phosphorylated by a kinase which is activated by hsps.
2) hsps bind to and alter the conformation of p66 rendering it amenable to phosphorylation by kinases.
3) p66 may be phosphorylated by an hsp. This seems unlikely since none of the hsps so far discovered are protein kinases.

The involvement of hsp90 is not surprising (L. Cheng, unpublished results). In higher eukaryotes hsp90 forms complexes with a number of protein kinases including casein kinase II, double-stranded DNA-activated protein-kinase, heme regulated eIF-2α kinase, protein kinase C and various tyrosine kinases (see section 1.2.3; Csermely and Kahn, 1991 and references therein). Proteins associated with hsp90, which may include kinases involved in phosphorylating p66 or even p66 itself, might be isolated from S100 extracts by immunoprecipitation using anti-hsp90 antiserum. It should be noted that phosphorylation of p66 is unlikely to be involved in a system that detects stress as it is dependent on the presence of proteins which are themselves stress inducible.

Acid fixation of gels results in the loss of most of the ^{32}P attached to proteins phosphorylated *in vitro*. This is due to the susceptibility of N-P linkages to acid hydrolysis (the acceptor amino acid has most commonly been histidine, see section 6.3.2). Known cases of histidine phosphorylation are less numerous than for hydroxy amino acid residues (serine, threonine, tyrosine), mainly because of the widespread use of techniques that fail to preserve acid labile phosphoamino acids (see section 6.3.2). Among the reports of the existence of N-linked phosphates are components of the signal transduction systems involved in osmotic regulation, chemotaxis and control of nitrogen metabolism in prokaryotes (Stock *et al.*, 1990) and in various proteins from eukaryotes including eIF-2α kinase and certain nuclear proteins (Huang *et al.*, 1991 and references therein).

The existence of kinases catalysing N-P linkages has been controversial since the presence of N-linked phosphate does not prove the existence of N-kinases *in vivo*. However, a 32kDa protein histidine kinase has recently been purified from yeast (Huang *et al.*, 1991). This is the first member of this class of protein kinases to be isolated. The *in*

vivo substrates of this enzyme, and the consequences of phosphorylation of such substrates on histidine are unknown. This is in contrast to N-linked phosphorylation in prokaryotes, where a key part of certain signal transduction systems involves a protein that autophosphorylates at a histidine residue. This phosphoryl group is subsequently either hydrolysed or transferred to an aspartic acid residue of a second component. This has been demonstrated in several systems including regulation of outer-membrane porin synthesis in response to osmotic shock, and, chemotaxis in response to attractant and repellent gradients (Stock *et al.*, 1990). In the latter case, changing the appropriate histidine residue by site directed mutagenesis abolished the chemotactic response (Hess *et al.*, 1988).

The N-linked phosphorylation of p31, p32 and p66 may constitute part of a signal transduction system in yeast. As for bacteria, transfer of the phosphoryl group to aspartate may occur. The apparent disappearance of p31 and p32 seen after long term incubations with [γ^{32}P]-ATP (see Fig. 6.2 and section 6.3.1) may be due to this. Autoradiograms presented in this chapter would not have visualised the aspartyl-^{32}phosphates thereby generated. This is because phosphoproteins were resolved on Laemmli gels, the alkali pH of which causes hydrolysis of aspartyl phosphates (Amory *et al.*, 1980). Whether or not de-phosphorylation of p31 and p32 occurs simultaneously with phosphorylation of aspartate residues can be investigated by resolving phosphorylated proteins on acid gels (as described by Amory *et al.*, 1980) and by conventional SDS-PAGE .

The acidic staining/destaining solutions did not result in hydrolysis of all amino acid-phosphate linkages. Accordingly, p31, p32 and p66 are also phosphorylated to a much lower extent on hydroxy amino acids (serine, threonine, tyrosine). Determining which amino acids are involved can be achieved simply by direct hydrolysis of proteins transferred to PVDF, which eliminates the need for preparative extraction of proteins from gels (Kamps and Sefton, 1989). Released [^{32}P] phosphoamino acids can be analysed by well established procedures involving a combination of electrophoresis and thin layer chromatography (Hunter and Sefton, 1980). Since acid hydrolysis destroys N-P linkages this method cannot be used to determine which of the basic amino acids are involved in the N-P linkage. This can be achieved by the methods recently developed by Wei and Matthews (1992).

It cannot be over emphasised that the work described in this chapter is a preliminary study of *in vitro* phosphorylation reactions catalysed by S100 extracts from stressed cells. The reaction conditions used here will lead to detection of only a fraction of the protein kinases in the S100 fraction. Kinases have been described which are activated by a variety of agents including cyclic nucleotides, calcium ions, double stranded DNA and double stranded RNA. Addition of these to reaction mixtures may reveal more differences in the phosphoprotein profiles of heat shock extracts relative to control extracts. Also, the physiological donor of phosphoryl groups is usually ATP, though several protein kinases can also use GTP effectively *in vitro* (Krebs, 1986). Certain *in vitro* reactions may prefer

other nucleoside triphosphates as substrate, which could indicate that these may be the physiologically relevant phosphoryl donors.

The physiological relevance of *in vitro* studies is frequently questioned. Eventually, one may need to show that proteins phosphorylated *in vitro* are also phosphorylated *in vivo*. This can be demonstrated by pulse labelling cells during heat shock with $^{32}P_i$. Proteins of S100 extracts would then be analysed by gel electrophoresis.

It is already well established that induction of hsp synthesis involves the phosphorylation of a transcription factor. Protein phosphorylation could play a role in both detection of stress *per se* as well as in the subsequently induced responses. This makes sense bearing in mind that some of the consequences of heat shock could lead to activation of various kinases. In higher eukaryotes heat shock causes a rapid and significant increase in the intracellular free calcium. This could lead to stimulation of Ca^{2+}/calmodulin kinase (see section 1.8.5). Heat shock also increases cAMP, leading to increased protein kinase A activity (Thevelein, 1991), and, causes stimulation of phosphoinoside turn-over which will activate protein kinase C (see section 1.8.5).

Data presented in this chapter suggest that phosphorylation events in the cytosol are influenced by heat shock. The chemical nature and function of the proteins involved have yet to be determined. The most striking feature of this work is that a polypeptide of M_r 66kDa (p66) is phosphorylated in heat shock extracts only, in an hsp dependent manner. A limited N-terminal sequencing of this protein can be generated rapidly by sequencing p66 blotted onto PVDF, as demonstrated with hsp104 in section 3.4.3. The corresponding gene could be cloned by screening a genomic library with a degenerate oligonucleotide based on this N-terminal sequence. Subsequent studies could involve determining the cellular consequences of a gene disruption.

Chapter 7 Discussion.

7.1 Introduction.

In most organisms a mild sub-lethal stress increases the capacity for survival during a subsequent lethal stress. The mechanisms that implement this acquisition of tolerance have been the subject of numerous studies. The ultimate aim must be to control stress tolerance. In certain instances hastening cell death will be an advantage. Knowledge of the mechanisms which enable microorganisms to resist stress will assist those who devise strategies designed to limit the spread of food–borne pathogens. Also, the major impediment to the successful treatment of cancer is the intrinsic resistance to cytotoxic drugs. Cancer cells differ widely in their response to chemotherapy. Some tumours rarely respond and are innately resistant, others are moderately sensitive and a few are so sensitive they are killed easily (Walker *et al.*, 1987; Parris *et al.*, 1988). Accumulating evidence indicates that responses to chemotherapy and stress are linked. Induction of thermotolerance results in transient resistance to certain chemotherapeutic drugs such as adriamycin (Hahn and Li, 1990). Also, transfection of certain hsps into cells affords resistance to this drug (Huot *et al.*, 1991).

Alternatively, manipulating organisms in such a way as to enhance stress tolerance must also be a future goal. For instance, development of salt tolerant crops has enormous potential of making important contributions to food production. This is especially true now that changes in the global environment are leading to evermore cases of agricultural damage attributable to increasing salinity.

The most obvious approach to adopt in the study of stress tolerance is to catalogue the consequences of stress *per se*. Discovery of the physiological markers of tolerance, and the subsequent elucidation of the underlying mechanisms at the molecular level are important research goals in biology.

7.2 Activity of the H^+-ATPase joins the list of factors known to influence stress tolerance.

The cellular phenomena which are implicated in acquired thermotolerance are:

1. Expression of a highly conserved set of genes encoding heat shock proteins (hsps) (section 1.2). The degree to which hsps are involved in acquired thermotolerance has been vastly overestimated (see next page).
2. The physiological state of cells has a dramatic effect on both acquired and innate thermotolerance. The activity of cAMP-dependent protein kinase (protein kinase A) is likely to be the most important factor here. Evidence of the involvement of

protein kinase A has come from studies involving mutants of the yeast cAMP system. This is discussed more fully in section 1.7.1.

3. Intracellular acidification and the activity of the plasma-membrane-associated H^+-ATPase (work presented in Chapters 3 and 4; and Coote *et al.*, 1991). This is discussed in greater detail below. Apart from action of H^+-ATPase, ubiquitin and catalase T have also been implicated in thermotolerance. Strains lacking the gene encoding polyubiquitin are more sensitive to *prolonged* incubation at 38.5°C, a marginal growth temperature for *S. cerevisiae* (Finley *et al.*, 1987; also see section 1.2.7). Cells lacking catalase T (encoded by *CTT1*) are more sensitive to exposure to lethal temperatures than cells of an isogenic strain that contain this enzyme. It is likely that the role of catalase T is to protect against damage by oxidative free radicals and that any influence on thermotolerance is indicative of more severe oxidative damage at high temperatures (Wieser *et al.*, 1991).
4. The high amounts of the disaccharide trehalose that accumulate in the cytoplasm of yeast cells in response to heat shock or entry into stationary phase (Wiemken, 1990). This is protein synthesis independent. Though levels of trehalose correlate with levels of thermotolerance, it should be noted that definitive proof of the involvement of trehalose in stress tolerance is lacking. The use of cells deleted for the genes encoding one or more of the enzymes of trehalose biosynthesis should reveal to what extent levels of this disaccharide influence stress tolerance.

It has become obvious that acquired thermotolerance involves mechanisms that are both dependent and independent of protein synthesis. However, in the past too much emphasis has been placed on the contribution of the inducible heat-shock proteins (Watson, 1990). This was primarily due to the positive correlation between the amount of hsps present and the degree of tolerance. Also, the only organism known not to induce hsps (a *Hydra*), exhibits far less acquired thermotolerance when compared to a closely related member of the same species which is capable of inducing hsps (Bosch *et al.*, 1988). It is now well established that the role of hsps in thermotolerance in yeast is limited. Acquisition of thermotolerance is only slightly diminished in the presence of protein synthesis inhibitors. Also, a mutation in heat-shock transcription factor that results in a general block to the induction of hsps does not affect acquisition of thermotolerance (Smith and Yaffe, 1991). In agreement with this, disruption of certain *S. cerevisae* hsp genes does not reduce innate or acquired thermotolerance (hsp26, Petko and Lindquist, 1986; hsp12, Praekelt and Meacock, 1990). However, cells lacking hsp104 do acquire less thermotolerance with heat shock relative to the parental wild type (Sanchez and Lindquist, 1990). But, increasing the

adaptive mild heat-shock from 30 to 60 minutes largely eliminates this effect (DeVirgilio *et al.*, 1991). It is likely that in the natural habitat of yeast cells, build up of temperature to lethal levels will occur slowly. Accordingly, in such situations the influence of hsps (like hsp104) on stress tolerance will be negligible. The detectable effects of hsp deletions on thermotolerance may therefore be due purely to experimental conditions, the development of hsps during evolution having been for reasons other than the improvement of stress tolerance. A role in maintaining growth at high temperatures is more likely. Consistent with this, disruptions of the *HSP82* gene (encoding hsp90) (Borkovich *et al.*, 1989) or the *UBI4* gene (Finley *et al.*, 1987) or both *SSA1* and *SSA2* genes (encoding members of the hsp70 family) (Craig and Gross, 1991) reduce growth of yeast cells at higher temperatures but do not reduce acquired thermotolerance. Furthermore, hsps may be involved primarily in recovery from heat shock. Consistent with this is the finding that strains lacking certain members of the hsp70 family lack the rapid increase of trehalase activity that usually accompanies temperature shift down (De Virgilio, personal communication).

The overwhelming body of evidence illustrating that substantial induction of thermotolerance occurs in the absence of protein synthesis indicates that this induction must reflect physiological changes independent of *de novo* synthesis of hsps. Certain types of stress, heat shock among them, lead to increases in membrane permeability which in turn lead to influx of H^+ and subsequent intracellular acidification. Consequently it was not surprising that the activity of the plasma-membrane-associated H^+-ATPase, an enzyme which would be expected to counteract H^+ influx, was found be one of the determinants of stress tolerance (see Chapter 3 and Coote *et al.*, 1991). Consistent with this, activity of the enzyme *per se* increases during heat shock as assayed i) *in vivo* (Coote *et al.*, 1991) and ii) *in vitro*, using plasma membrane isolates from heat shocked cells (see Chapter 4). This reveals the existence of a system which activates the enzyme during heat shock. Possible mechanisms involved are discussed in section 7.2.1.

Levels of H^+-ATPase fall dramatically during heat shock. A 40 minute 20-40^{0}C heat-shock results in the loss of more than 50% of the polypeptide from the plasma membrane (Fig. 4.2). This is surprising given the importance of this enzyme in cellular physiology as a whole. The overall energy charge of the cell may be as important here as sustaining H^+-ATPase activity, since this enzyme consumes up to 50% of cellular ATP. Activating the enzyme during heat shock may lead to severe depletion in levels of ATP. The requirement for increased H^+ extrusion without compromising energy charge may be met by a combination of i) enzyme activation and ii) loss of a proportion of H^+-ATPase molecules. This type of dual regulation is unorthodox. Invariably, the demand for increased activity of any enzyme is met either by an activation of the enzyme *per se*, or by an increase in its levels.

The fall in levels of H^+-ATPase during heat shock and entry into stationary phase

implies the existence of a system that removes the enzyme from plasma membranes. On the whole very little is known about turn-over of plasma membrane proteins. In general these proteins, including H+-ATPase, have half lives ($t_{1/2}$) of ca. 11 hours (see section 4.4.8). During heat shock $t_{1/2}$ of H+-ATPase drops to <40 minutes (Chapter 4). A likely degradative mechanism involved here is the ubiquitin pathway, some components of which are heat shock inducible (Finley and Chau, 1991). One could examine its involvement in H+-ATPase turn-over by using mutants of this system. Alternatively, heat shock may increase the rate at which portions of the plasma membrane housing H+-ATPase are internalised. Vesicles bearing plasma membrane proteins could then be sent to the lysosome, as demonstrated for mammalian cells (Hare, 1990). *S. cerevisiae* endocytosis mutants are available and can be used to investigate this (Waltschewa, 1991). Whether the rapid loss of H+-ATPase is limited by protective action of heat inducible proteins is as yet unclear. An hsp specifically directed to the plasma membrane may act by protecting plasma membrane components during stress. Analysis of the protein composition of plasma membranes before and after heat shock revealed the existence of a heat inducible integral plasma membrane protein (hsp30, see Chapter 4).

7.2.1 Mechanisms that may activate H+-ATPase during heat shock.

The plasma membrane associated H+-ATPase is crucial to the existence of plant and fungal cells (see section 3.1). In plants this enzyme is regulated by almost all factors that control cell physiology such as organ development, hormones, phytotoxins, light, turgor and pH (see section 3.1). The mechanisms whereby these factors regulate H+-ATPase are as yet unclear. This is one of the most fundamental gaps in knowledge regarding the physiology of plants and fungi. A pertinent question concerns the nature of intracellular messengers involved in H+-ATPase activation. In yeast cells glucose (Serrano, 1983), acid media (Eraso and Gancedo, 1987) and heat shock (Chapter 4; Coote *et al.*, 1991) all activate this enzyme. All of these cause intracellular acidification. Perturbation of pH_i could be the first messenger in the pathway leading to H+-ATPase activation. It is known that yeast adenylate cyclase is activated by intracellular acidification (Thevelein, 1991), and, cAMP levels increase during heat shock (Camonis *et al.*, 1986; Boutelet *et al.*, 1986). Accordingly an increase in cAMP may be one activator of H+-ATPase. Consistent with this is the finding that in plants the hormone auxin and the phytotoxin fusicoccin both rapidly induce a decline in pH_i and both stimulate H+-ATPase (Serrano, 1989). An analogy can be made to the Na+/K+ ATPase of mammalian cells. This enzyme is regulated by intracellular concentrations of the extruded cation sodium (Rozengurt, 1980); H+-ATPase of fungi and plants may be regulated by intracellular concentration of H+ (pH_i).

In yeast, addition of weak organic acids causes a decline in pH_i which in turn induces thermotolerance (Coote *et al.*, 1991). Some of this thermotolerance is probably attributable to the observed increase in H+-ATPase activity (Coote *et al.*, 1991; Eraso and Gancedo, 1987). Acquisition of thermotolerance caused by incubation in the presence of weak acids is not accompanied by induction of hsps (L. Cheng, unpublished results).

Alterations in the level of a key messenger may regulate H+-ATPase by modulating the activity of protein kinases. Control by phosphorylation is the most frequent regulatory mechanism employed by eukaryotes. Apart from protein kinase A, the activity of which is dependent on cAMP (see above), there are other kinases in the cell that are sensitive to other messengers the concentrations of which change dramatically during heat shock. Elevated temperatures in mammalian cells induce turnover of phosphoinositols and result in an increase of intracellular Ca^{2+} levels. There are various kinases, in yeast as well as mammalian cells, that will respond to these changes (discussed more fully in section 4.5.2). Action of these kinases could stimulate H+-ATPase activity. Consistent with the putative role of kinases is the finding that phosphorylation of yeast H+-ATPase has been demonstrated *in vivo* and *in vitro* (see Chapter 4). Furthermore, *in vitro* studies reveal that the H+-ATPase is phosphorylated to a greater extent in plasma membranes from heat shocked cells relative to those from unstressed cells (Chapter 4). So far, one kinase that phosphorylates H+-ATPase has been purified. Its action has been shown to dramatically stimulate H+-ATPase *in vitro*. However, the second messengers regulating this kinase are as yet unknown (Kolarov *et al.*, 1988).

It is well established that the C-terminus of H+-ATPase plays a role in the activation of this enzyme by glucose metabolism (Portillo *et al.*, 1989). It is now thought that phosphorylation at this domain of the H+-ATPase by Ca^{2+}/calmodulin dependent kinase is involved (Portillo *et al.*, 1991). Consistent with this is the finding that glucose causes an increase in intracellular Ca^{2+} levels (Ca^{2+}_i) in yeast (Kaibuchi *et al.*, 1986). Interestingly, one of the consequences of heat shock is an increase in Ca^{2+}_i. This has been demonstrated in cells of higher eukaryotes (Calderwood *et al.*, 1988; Drummond *et al.*, 1986) but has not yet been reported to occur in yeast. The overall conservation of the responses to heat shock suggest that an increase in Ca^{2+}_i could occur in yeast, which in turn may activate Ca^{2+}/calmodulin kinase. Also, the *in vitro* kinase-mediated phosphorylation of oat root H+-ATPase is stimulated by addition of low concentrations of calcium and by a decrease in pH (Schaller and Sussman, 1988). This is indirect evidence that changes in Ca^{2+}_i and pH are potentially significant regulators of the activity of H+-ATPase, acting via kinase-mediated phosphorylation events.

Various groups are studying the H+-ATPase activation system. Given that the enzyme is regulated by so many different factors it seems logical that several mechanisms

are involved. Indirect proof of this comes from the finding that activation caused by acid media differs from that caused by glucose in terms of certain catalytic parameters (Eraso and Gancedo, 1987). The changes caused by heat shock to the K_m for MgATP, optimal pH and reaction velocity could be compared to those caused by glucose or acid media.

The H+-ATPase of plants and yeast commands the uptake of nutrients. At one point it was thought that altering gene dosage might increase growth rates of these organisms (Goffeau, 1988). The potential to increase innate stress tolerance by alteration of H+-ATPase levels or structure is also apparent bearing in mind the results presented in Chapter 3. However, both overexpression or reduced expression of the gene encoding H+-ATPase are detrimental to the cell (Eraso *et al.*, 1987; Vallejo and Serrano, 1989). Not surprisingly, it is now well established that modulation of H+-ATPase occurs exclusively at the level of specific activity. Levels of enzyme are tightly controlled (Eraso *et al.*, 1987), with the exception of heat shock where levels of polypeptide are reduced, and the remaining molecules are activated (Chapter 4). Overall, this implies that strategies designed to improve growth rate or stress tolerance by manipulation of H+-ATPase must be confined to modulating its regulatory mechanisms or stability during stress.

7.3 The response to osmotic shock.

Cells preconditioned by a non-lethal osmotic shock acquire resistance to a second more severe osmotic shock. Most organisms respond to osmostress by accumulating a pool of osmoprotectant compounds e.g glycerol in yeast (see section 4.5.3). Osmotically shocked yeast are also thermotolerant. However, the accumulation of glycerol is slow during osmotic shock (Singh and Norton, 1991), much slower than the increase in thermotolerance (Trollmo *et al.*, 1988). Consequently, the acquired thermotolerance must be due to other factors. hsps are not involved as sub-lethal osmotic shocks do not induce their synthesis (Chapter 4). However, both heat shock and osmostress activate H+-ATPase (Chapter 4). Proof for H+-ATPase activity as one of the determinants of osmotolerance was obtained by the use of strains isogenic but for a mutation at the *PMA1* locus (Chapter 3). It seems logical that salt tolerant yeasts such as *Debaromyces hansenii* and *Zygosaccharomyces rouxii* (Andre *et al.*, 1988) may possess a H+-ATPase with unique properties. Consistent with this are the results from recent work on *Zygosaccharomyces rouxii*. Unlike H+-ATPase in *S. cerevisiae*, the enzyme from *Z. rouxii* is not activated by glucose i.e *Z. rouxii* keeps activity constantly high (Watanabe *et al.*, 1991). Replacing the genomic copy of the *PMA1* gene in *S. cerevisiae* with that from *Z. rouxii* could render *S. cerevisiae* more tolerant to salt.

S. cerevisiae shares basic ion-transport mechanisms with plants. In plants, it has long been established that higher H+-ATPase activity correlates with resistance to salinity.

Such work has involved use of halophytes (plants adapted for growth in saline environments) and glycophytes (plants unable to grow in saline environments). For instance growing the halophyte *Atriplex nummularia* in the presence 400mM NaCl doubles the H^+ translocating activity of root vesicles (Braun *et al.*, 1986). This is in contrast to the glycophyte *Gossypium hirsutum*, where saline conditions have no effect on H^+ translocation (Hassidim *et al.*, 1986). Correlation between osmotolerance and H^+-ATPase activity is not sufficient proof of this enzyme playing a role in osmotolerance. However, the use of strains isogenic but for mutations at the *PMA1* locus (Chapter 3) prove that this correlation is not a coincidence.

To date, studies concerning changes induced by osmostress have almost entirely been limited to bacteria and plants. Regarding yeast, investigation has focussed on physiological processes (Blomberg *et al.*, 1988; Trollmo *et al.*, 1988; Blomberg and Adler, 1989). A preliminary analysis of the proteins induced by sub-lethal osmostress is presented in Chapter 4. One of the inducible proteins has the same M_r as the product of the recently-isolated salt-inducible *HAL1* gene of *S. cerevisiae* (Serrano, unpublished results; see section 4.5.3). This gene, which enables growth in the presence of high concentrations of salt, is conserved in plants. Yeast may therefore share stress inducible responses at the level of proteins *and* basic ion-transport mechanisms (see above). Molecular cloning and over expression of *HAL1* and of the other salt inducible proteins (Chapter 4) may eventually lead to improvement of salt tolerance in plants as well as yeast.

7.4 Discovery of a heat-inducible integral plasma-membrane protein.

Under heat shock conditions a single new plasma-membrane protein can be rapidly labelled with radioactive amino acids, other plasma membrane proteins becoming labelled to a far lesser extent (Fig. 4.3). This protein has an M_r of 30kDa and has been designated hsp30. hsp30 is an apparently-unglycosylated integral membrane-protein that can be phosphorylated *in vitro* (thus far the only yeast hsp that has been shown to become phosphorylated *in vitro*). A search for such a protein in species other than yeast might prove rewarding, given the extreme conservation of the heat shock response.

At this stage one can only speculate as to the function of hsp30. It seems likely that hsp30 is not involved in thermotolerance, given that inhibition of protein synthesis only slightly diminishes acquired thermotolerance (see section 7.2). The corresponding gene has been cloned recently (Boucherie, unpublished results). Its amino acid sequence reveals no similarities to any proteins in the current sequence databanks. The sequence contains one motif recognisable by protein kinase A and 4 motifs recognisable by protein kinase C. Interestingly, levels of the second messengers that control both of these kinases rise during heat shock. However it has not yet been demonstrated that hsp30 is phosphorylated at these

sites.

In recent years it has become apparent that hsps with protective roles are targeted to the golgi, the mitochondria and the endoplasmic reticulum; hsp30 may carry out a similar role at the cell membrane. As such it may facilitate the expression of heterologous membrane proteins in yeast. High level production of heterologous proteins in *E. coli* frequently results in their aggregation into inclusion bodies. Overexpressing heat shock chaperones in *E. coli*, has resulted in large increases in proper folding of certain heterologous proteins (Lee and Olins, 1992). Overexpressing hsp30 in yeast may yield similar results with respect to expressing heterologous membrane proteins in this expression host.

Strains in which the gene encoding hsp30 has been disrupted are viable (Boucherie, unpublished results) which is not surprising since hsp30 is only made during heat shock and other stresses (Chapter 4). Use of these strains may show to what extent hsp30 influences both the stability and activities of various membrane-associated proteins. Alternatively, hsp30 may in some way influence the distribution of ions across the cell membrane. Dissipation of the electrochemical H^+ gradient caused by heat shock could be limited by this hsp. In mammalian cells heat shock induces two waves of Ca^{2+} influx into the cytosol. The first happens almost instantaneously and, as such, is unlikely to be influenced by membrane associated hsps. In this case the increase in Ca^{2+}_i is due to release of Ca^{2+} from internal stores. The second occurs ca. 30 minutes after heat shock and is caused by influx of Ca^{2+} from the extracellular environment. Activation of an ion channel (which may be hsp30) seems a logical mechanism here. Overall the possible role of changes in Ca^{2+}_i have been neglected. In yeast, the effects of Ca^{2+} ionophores on thermotolerance or on H^+-ATPase activity could be investigated.

7.5 Proteins can be divided into two classes regarding their response to stress.

In vivo, many enzymes have been described as being inactivated during heat shock. Among them are DNA polymerase (Spiro *et al.*, 1982), Na^+/K^+ ATPase (Burdon and Cutmore, 1982), Ca^{2+} ATPase (Cheng *et al.*, 1987) and interferon-induced double-stranded RNA-dependent protein-kinase (Dubois *et al.*, 1989). In the past this has been investigated by following loss of activity, caused by stress, of reporter enzymes such as β-galactosidase and luciferase expressed in mouse or *Drosophila* cells. Invariably, the polypeptides are not degraded but become insoluble even in the presence of non-ionic detergents (Nguyen and Bensaude, 1989). Remarkably, this effect is more dramatic *in vivo* than *in vitro*.

On the other hand, there are very few reports describing activation of enzyme activity caused by heat shock e.g the kinase that phosphorylates eIF-2α (see section 6.1)

and enzymes involved in turn-over of phosphoinositides (see section 1.8.5). To the list of activities stimulated by heat shock can be added i) the H^+-ATPase (Chapter 4 and Coote *et al.*, 1991) and ii) *in vitro* phosphorylation of a cytosolic protein (p66) that is present both prior to and during stress (see Chapter 6). The latter is dependent on stress-induced protein synthesis. Searching for enzymes that are stimulated during stress could reveal mechanisms that make important contributions to i) thermotolerance or ii) growth at high temperatures. Alternatively, it may be easier to look for changes in post-translational regulatory mechanisms that are influencing these enzymes. This would reveal proteins whose activities are modulated by stress, as well as proteins which regulate these effects. For instance, changes in phosphorylation (the most common regulatory mechanism) can be investigated by labelling *in vivo* with [^{32}P] phosphoric acid or *in vitro* with radiolabelled nucleoside triphosphates. *In vitro* experiments (Chapter 6) revealed the phosphorylation of a protein in heat shock lysates only. Experiments designed to determine the significance of this are in hand.

7.6 Proteins phosphorylated *in vitro* were multiply phosphorylated.

Cytosolic (S100) extracts from unstressed and heat shocked cells were incubated with [γ^{32}P]-ATP. Both O-phosphates, formed by phosphorylation of hydroxy amino acids and N-phosphates, formed by phosphorylation of basic amino acids were detected (section 6.3.2). This was not surprising since it has become evident that most phosphoproteins contain multiple sites (Roach, 1991). It is thought that multiple protein modifications expand the types of structural changes that can be induced in proteins (Roach, 1991). Alternatively, phosphorylation at different sites may reflect action of different kinases and therefore regulation by different factors.

The chemistry of phosphorylation at histidine (yielding N-phosphates) is quite different from the more frequently observed phosphorylations at serine, tyrosine and threonine (yielding O-phosphates). The free energy of phosphorylation for seryl-phosphate is higher than that for histidinyl-phosphate (Stock *et al.*, 1990). Greater intrinsic stability of phosphoserine is well suited for the induction of unfavourable conformation changes that may control enzyme activities. According to Stock *et al.* (1990), the large negative free-energy of de-phosphorylation for phosphohistidine is suited to its being a phosphate donor, as is the case in the bacterial chemotactic response (Hess *et al.*, 1988). Whether phosphohistidine detected in yeast (Chapter 4) acts in the same way remains to be seen (experiments designed to investigate this are outlined in section 6.4).

References.

Aaronson, L.R., Hager, K.M., Davenport, J.W., Mandala, S.M., Chang, A., Speicher, D.W. and Slayman, C.W. (1988) *J. Biol. Chem. 263*, 14552-14558.

Achstetter, T. and Wolf, D.H. (1985) *Yeast 1*, 139-157.

Adams, C.C. and Gross, D.S. (1991) *J. Bacteriol. 173*, 7429-7435.

Adamska, I. and Kloppstech, K. (1991) *Eur. J. Biochem. 198*, 375-381.

Amory, A. and Goffeau, A. (1982) *J. Biol. Chem. 257*, 4723-4730.

Amory, A., Foury, F. and Goffeau, A. (1980) *J. Biol. Chem. 255*, 9353-9357.

Ananthan, J., Goldberg, A.L. and Voellmy, R. (1986) *Science 232*, 522-524.

Andre, L., Hemming, A. and Adler, L (1991) *FEBS Lett. 286*, 13-17.

Ang, D., Liberek, K., Skowrya, D., Zylicz, M. and Georgopoulos, C. (1991) *J. Biol. Chem. 266*, 24233-24236.

Angelidis, C.E., Lazaridis, I. and Pagoulatos, G.N. (1991) *Eur. J. Biochem. 199*, 35-39.

Anraku, Y., Unemoto, N., Hirata., R. and Wada, Y. (1989) *J. Bioenerg. Biomembr. 21*, 589-603.

Arrigo, A.-P. (1980) *Mol. Gen. Genet. 178*, 517-524.

Ashburner, M. and Bonner, J.J. (1979) *Cell 17*, 241-254.

Attfield, P. (1987) *FEBS Lett. 225*, 259-263.

Bagi, G. and Hidvegi, E..J. (1990) *Int. J. Rad. Biol. 58*, 633-650.

Bailyes, E.M., Richardson, P.J. and Luzio, J.P. (1990) in *Biological Membranes a practical approach* (Findlay, J.B.C. and Evans, W.H., eds.) pp. 73-101 IRL Press, Oxford.

Balke, N.E. and Hodges, T.K. (1977) *Plant Sci. Lett. 10*, 319-325.

Bansal, G.S., Norton, P.M. and Latchman, D.S. (1991) *Expt. Cell Res. 195*, 303-306.

Bardwell, J.C.A. and Craig, E.A. (1988) *J. Bacteriol 170*, 2977-2983.

Barnett, T., Altschuler, M., McDaniel, C.N. and Mascarendas, J.P. (1980) *Dev. Genet. 1*, 331-340.

Baulieu, E-E. and Catelli, M-G. (1989) in *Stress-Induced Proteins* (Pardue, M.L., Feramisco, J.R. and Lindquist, S., eds.) pp. 203-219 Alan R. Liss, New York.

Behrens, M.M. and Maia, J.C.C. (1986) *Biochem. Int. 12*, 503-512.

Behrens, M.M. and Mazon, M.J. (1988) *Biochem. Biophys. Res. Commun. 151*, 561-567.

Benito, B., Moreno, E. and Lagunas, R. (1991) *Biochim. Biophys. Acta 1063*, 265-268.

Bienz, M. and Gurdon, J.B. (1982) *Cell 29*, 811-819.

Blomberg, A., Larsson, C., and Gustafsson, L. (1988) *J. Bacteriol. 170*, 4562-4568.

Blomberg, A. and Adler, L. (1989) *J. Bacteriol. 171*, 1087-1092.

Blumberg, H. and Silver, P.A. (1991) *Nature 349*, 627-630.

Bochner, B.R., Zylicz. M. and Georgopoulos, C. (1986) *J. Bacteriol. 168*, 931-935.

Bond, J.S. (1989) in *Proteolytic Enzymes - A Practical Approach* (Beynon, R.J. and Bond, J.S., eds.) pp. 232-240 IRL Press, Oxford.

Boorstein, W.R. and Craig, E.A. (1990) *EMBO J. 9*, 2543-2553.

Bordier, C. (1981) *J. Biol. Chem. 256*, 1604-1607.

Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J. and Lindquist, S. (1989) *Mol. Cell. Biol. 9*, 3919-3930.

Bosch, T.C.G., Krylow, S.M., Bode, H.R. and Steele, R.E. (1988) *Proc. Natl. Acad. Sci. 85*, 7927-7931.

Bossier, P., Fitch, I.T., Boucherie, H. and Tuite, M.F. (1989) *Gene 78*, 323-330.

Bouche, G., Amalric, F., Caizergues-Ferrer, M. and Zaita, J.P. (1979) *Nucleic Acids Res. 7*, 1739-1747.

Boucherie, H. (1985) *J. Bacteriol. 161*, 385-392.

Boutelet, F., Petitjean, A. and Hilger, F. (1985) *EMBO J. 4*, 2635-2641.

Boutry, M., Michelet, B. and Goffeau, A. (1989) *Biochem. Biophys. Res. Commun. 162*, 567-574.

Bowman, E.J. and Bowman, B.J. (1982) *J. Bacteriol. 151*, 1326-1337.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248-254.

Braun, Y., Hassidim, M., Lerner, H.R. and Reinhold, L. (1986) *Plant Physiol. 81*, 1050-1056.

Bukau, B. and Walker, G.C. (1990) *EMBO J. 9*, 4027-4036.

Burdon, R.H. (1986) *Biochem. J. 240*, 313-324.

Burdon, R.H. and Cutmore, M.M. (1982) *FEBS Lett. 140*, 45-48.

Busa, W.B. and Nuccitelli, R. (1984) *Am. J. Phys. 246*, R409-R438.

Busturia, A. and Lagunas, R. (1986) *J. Gen. Microbiol. 132*, 379-385.

Caizergues-Ferrer, M., Bouche, G., Amalric, F. and Zalta, J.-P. (1980) *Eur. J. Biochem. 108*, 399-404.

Calderwood, S.K. and Hahn, G.M. (1983) *Biochim. Biophys. Acta. 756*, 1-8.

Calderwood, S.K., Stevenson, M.A. and Hahn, G.M. (1987) *J. Cell Physiol. 130*, 369-376.

Calderwood, S.K., Stevenson, M.A. and Hahn, G.M. (1988) *Radiat. Res. 113*, 414-425.

Calvert, R. and Gratzer, W.B. (1978) *FEBS Lett.. 86*, 247-249

Camonis, J.H., Kalekine, M., Gondre, B., Garreau, M., Boy-marcotte, E. and Jacquet, M. (1986) *EMBO J. 5*, 375-380.

Caplan, A.J. and Douglas. M.G. (1991) *J. Cell Biol. 114*, 609-621.

Carson, S.D. and Konigsberg, W.H. (1981) *Anal. Biochem. 116*, 398-401.

Cartwright, C.P., Veazey, F.J. and Rose, A.H. (1987) *J. Gen. Microbiol. 133*, 857-865.

Castagna, M., Takai, Y., Kaibuchi, K., Sato, K., Kikkawa., U., and Nishizuka, Y. (1982) *J. Biol. Chem. 257*, 7847-7851.

Castellanos, R.M.P. and Mazon, M.J. (1985) *J. Biol. Chem. 260*, 8240-8242.

Chamberlain, J.P. (1979) *Anal. Biochem. 98*, 132-135.

Chandrasekhar, G.N., Tilly, K., Woolford, C., Hendrix, R. and Georgopoulos, C. (1986) *J. Biol. Chem. 261*, 12414-12419.

Chang, A. and Slayman, C.W. (1990) *J. Cell. Biol. 111*, 340a.

Chappell, T.G., Welch, W.J., Schlossman, D.M., Palter, K.B., Schlesinger, M.J. and Rothman, J.E. (1986) *Cell 45*, 3-13.

Cheng, K.K., Hui, S.W. and Lepock, J.R. (1987) *Cancer Res. 47*, 1255-1262.

Cheng, L., Hirst, K. and Piper, P.W. (1992) *Biochim. Biophys. Acta 1132*, 26-34.

Cheng, M.Y., Hartl, F-U., Martin, J., Pollock, R.A., Kalousek, F., Neupert, W., Hallberg, E.M., Hallberg, R.L. and Horwich, A.L. (1989) *Nature 337*, 620-625.

Chiang, H-L., Terelecky, S.R., Plant, C.P. and Dice, F.J. (1989) *Science 246*, 382-384.

Chirico, W.J., Waters, M.G. and Blobel G. (1988) *Nature 332*, 805-810.

Chothia, C. (1992) *Nature 357*, 543-544.

Chung, C.H. and Goldberg, A.L. (1981) *Proc. Natl. Acad. Sci. USA 78*, 4728-4732.

Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) *J. Biol. Chem. 252*, 1102-1106.

Clos, J., Westwood, J.T., Becker, P.B., Wilson,S., Lambert, K. and Wu, C. (1990) *Cell 63*, 1085-1097.

Cole, M.B. and Keenan., M.H.J. (1987) *Yeast 3*, 23-32.

Collinson, L.P. and Dawes, I.N. (1992) *J. Gen. Microbiol. 138*, 329-335.

Coote, P.J., Cole, M.B. and Jones, M.V. (1991) *J. Gen. Micro. 137*, 1701-1708.

Cossins, A.R. and Bowler, K. (1987) in *Temperature biology of animals* pp. 155-201 Chapman and Hall, London.

Coull, J.M., Pappin, D.J.C., Aebersold, M.J. and Koster, H. (1991) *Anal. Biochem. 194*, 110-120.

Courtneidge, S.A. and Bishop, J.M. (1982) *Proc. Natl. Acad. Sci. USA 79*, 7117-7121.

Craig, E.A. (1985) *CRC Cririca1 Reviews in Biochemistry 18*, 239-280.

Craig, E.A. (1990) in *Stress Proteins in Biology and Medicine* (Morimoto, R., Tissieres, A. and Georgopoulos, C., eds.) pp. 301-321 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Craig, E.A. and Gross, C.A. (1991) *Trends Biochem. Sci. 16*, 135-140.

Craig, E.A. and Jacobsen, K. (1984) *Cell 38*, 841-849.

Craig, E.A., Kramer, J., Shilling, J., Werner-Washburne, M., Holmes, S., Kosic-Smithers, J. and Nicolet C.M. (1989) *Mol. Cell. Biol 9*, 3000-3008.

Craig, E.A., McCarthy, B.J. and Wadsworth S.C. (1979) *Cell 16*, 575-583.

Csermely, P. and Kahn, C.R. (1991) *J. Biol. Chem. 266*, 4943-4950.

Curran, B., Davies, M.W., Hirst, K., Seward, K. and Piper, P.W. (1988) *Yeast 4*, S324.

De Virgilio, C., Piper, P., Boller, T. and Wiemken, A. (1991) *FEBS Lett. 288*, 86-90.

De Virgilio, C., Simmen, U., Hottiger, T., Boller, T. and Wiemken, A. (1990) *FEBS Lett. 273*, 107-110.

DeBenedetti, A. and Baglioni, C. (1986) *J. Biol. Chem. 261*, 338-342.

Deshaies, R.J., Koch, B.D., Werner-Wasburne, M., Craig, E.A. and Schekman, R. (1988) *Nature 332*, 800-805.

Deutch, C.E. and Perry, J.M. (1974) *J. Gen. Microbiol. 80*, 259-266.

Downton, W.J.S. (1984) *CRC Crit. Rev. Plant Sci.1*, 183-201.

Drapeau, G.R., Boily, Y. and Houmard, J. (1972) *J. Biol. Chem. 247*, 6720-6726.

Drummond, I.A., McClure, S.A., Poenie, M., Tsien, R.Y. and Steinhardt, R.A. (1986) *Mol. Cell. Biol. 6*, 1767-1775.

Dubois, M-F., Galabru, J., Lebon, P., Safer, B. and Hovanessian, A.G. (1989) *J. Biol. Chem. 264*, 12165-12171.

Duclos, B., Marcandier, S. and Cozzone, A.J. (1991) *Methods Enzymol. 201*, 10-21.

Duncan, R. and Hershey, J.W.B. (1984) *J. Biol. Chem. 259*, 11882-11889.

Eilers, M. and Schatz, G. (1988) *Cell 52*, 481-483.

Eraso, P. and Gancedo, C. (1987) *FEBS Lett. 224*, 187-192.

Eraso, P., Cid, A. and Serrano, R. (1987) *FEBS Lett. 224*, 193-197.

Erickson, J.W. and Gross, C.A. (1989) *Genes Dev. 3*, 1462-1471.

Feinberg, B. and McLaughlin, C.S. (1988) in *Yeast: a practical approach* (Campbell, I. and Duffus, J.H., eds.) pp.147-161 IRL Press, Oxford.

Ferrell, J.E., Jr. and Martin, G.S. (1989) *J. Biol. Chem. 264*, 20723-20729.

Findlay. J.B.C. (1990) in *Biological Membranes a practical approach* (Findlay, J.B.C. and Evans, W.H., eds.) pp. 179-217 IRL Press, Oxford.

Findly, R.C., Gillies, R.J. and Shulman, R.G. (1983) *Science 219*, 12223-1225.

Finley, D. and Chau, V. (1991) *Annu. Rev. Cell Biol. 7*, 25-69.

Finley, D., Ciechanover, A. and Varshavsky, A. (1984) *Cell 37*, 43-55.

Finley, D., Ozkaynak, E. and Varshavsky, A. (1987) *Cell 48*, 1035-1046.

Flannery, A.V., Beynon, R.J. and Bond, J.S. (1989) in *Proteolytic Enzymes - A Practical Approach* (Beynon, R.J. and Bond, J.S., eds.) pp. 145-162 IRL Press, Oxford.

Friedman, D.E., Olson, E.R., Georgopoulos, C., Tilly, K., Herskowitz, I. and Banuett, F. (1984) *Microbiol. Rev. 48*, 299-325.

Gadd, G.M., Chalmers, K. and Reed, R.H. (1987) *FEMS Microbiol Lett. 48*, 249-254.

Gaitanaris, G.A., Papavassiliou, A.G., Rubock, P., Silverstein, S.J. and Gottesman, M.E. (1990) *Cell 61*, 1013-1020.

Gallagher, S.R. and Leonard, R.T. (1982) *Plant Physiol. 70*, 1335-1340.

Gallo, G.J., Schuetz, T.J. and Kingston, R.E. (1991) *Mol. Cell Biol. 11*, 281-288.

Gancedo, J.M., Lopez, S. and Ballesteros, F. (1982) *Mol. Cell. Biochem. 43*, 89-95.

Georgopoulos, C., Ang, D., Liberek, K. and Zylicz, M. (1990) in *Stress Proteins in Biology and Medicine* (Morimoto, R., Tissieres, A. and Georgopoulos, C., eds.) pp. 191-221 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Georgopoulos, C.P. (1977) *Mol. Gen. Genet. 151*, 35-39.

Georgopoulos, C.P. and Hohn, B. (1978) *Proc. Natl. Acad. Sci. USA 75*, 131-135.

Gething, M.J. and Sambrook, J. (1991) *Nature 355*, 33-45.

Ghislain, M., Schlesser, A. and Goffeau, A. (1987) *J. Biol. Chem. 262*, 17549-17555.

Gifford, L.A., Miller, J.N. and Stretton, R.J. (1971) *J. Chromatog. 55*, 430-433.

Gillies, R.J., Ugurbil, K, Hollander, D. and Shulman, R.G. (1981) *Proc. Natl. Acad. Sci. USA 78*, 2125-2129.

Glover, C.V.C. (1982) *Proc. Natl. Acad. Sci. USA 79*, 1781-1785.

Goffeau, A. (1988) *Brazilian J. Med. Biol. Res. 21*, 1233-1240.

Goloubinoff, P., Christeller, J.T., Gatenby, A.A. and Lorimer, G.H . (1989) *Nature 342*, 844-889.

Gottesman, S., Squires, C., Pichersky, E., Carrington, M., Hobbs, M., Mattick, J.S., Dalrymple, B., Kuramitsu, H., Shiroza, T., Foster, T., Clark, W.P., Ross, B., Squires, C.L. and Maurizi, M.R. (1990) *Proc. Natl. Acad. Sci. USA 87*, 3513-3517.

Green, P.J., Pines, O. and Inoye, M. (1986) *Annu. Rev.Biochem. 55*, 569-597.

Gross,C.A., Straus, D.B. and Erickson, J.W. (1990) in *Stress Proteins In Biology And Medicine* (Morimoto, R.I., Tissieres, A. and Georgopoulos, C., eds.) pp. 167-189 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Grossman, A.D., Erickson, J.W. and Gross, C.A. (1984) *Cell 38*, 383-390.

Guijarro, J.M and Lagunas, R. (1984) *J. Bacteriol. 160*, 874-878.

Hahn, G.M and Li, G.C. (1990) in *Stress proteins in biology and medicine* (Morimoto, R.I., Tissieres, A. and Georgopoulos, C., eds.) pp. 79-100 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Hahn, G.M., Shiu, E.C., West, B., Goldstein, L. and Li, G.C. (1985) *Cancer Res. 45*, 4138-4143.

Hall, B. (1983) *J. Bacteriol. 156*, 1363-1365.

Hames, B.D. (1990) in *Gel Electrophoresis of Proteins A Practical Approach* (Hames, B.D. and Rickwood, D., eds.) pp. 1-147 IRL Press, Oxford.

Hancock, J.F., Magee, A.I., Childs, J.E. and Marshall, C.J. (1989) *Cell 57*, 1167-1177.

Hare, J.F. (1990) *Biochim. Biophys. Acta 1031*, 71-90.

Harlow, E. and Lane, D. (1988) [a] in *Antibodies A Laboratory Manual* pp. 635-657 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Harlow, E. and Lane, D. (1988) [b] in *Antibodies A Laboratory Manual* pp. 471-510 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Harlow, E. and Lane, D. (1988) [c] in *Antibodies A Laboratory Manual* pp. 53-137 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Harper, J.F., Surowy, T.K. and Sussman, M.R. (1989) *Proc. Natl. Acad. Sci. USA 86*, 1234-1238.

Hassidim, M., Braun, Y., Lerner, H.R. and Reinhold, L. (1986) *Plant Physiol. 81*, 1057-1061.

Hawkes, R. (1982) *Anal. Biochem. 123*, 143-146.

Hemmingsen, S., Woolford. C., Van der Vier. S., Tilly, K., Dennis, D., Georgopoulos, C., Hendrix, R. and Ellis, R.J. (1988) *Nature 333*, 330-334 .

Hensold, J.O., Hunt, C.R., Calderwood, S.K., Housman, D.E and Kingston, R.E. (1990) *Mol. Cell Biol.10*, 1600-1608.

Hess, J.F., Bourret, R.B. and Simomn, M.I. (1988) *Nature 336*, 139-143.

Hightower, L.E. (1991) *Cell 66*, 191-197.

Hirst, K.A. (1990) in *Studies on the yeast heat shock response*. Ph.D. thesis, University of London.

Holcomb, C.L., Hansen, W.J., Etcheverry, T. and Shekman, R. (1988) *J. Cell Biol. 106*, 641-648.

Hottiger, T., Schumtz, P. and Wiemken, A. (1987) *J. Bacteriol. 169*, 5518-5522.

Houmard, J. and Drapeau, G.R. (1972) *Proc. Natl. Acad. Sci. USA 69*, 3506-3509.

Houslay, M.D. and Stanley, K.K (1982) in *Dynamics of Biological Membranes* p94-138 John Wiley and Sons Ltd.

Howard, K.J., Holley. S.J., Yamamoto, K.R. and Distelhorst, C.W. (1990) *J. Biol. Chem. 265*, 11928-11935.

Huang, J.,Wei, Y., Kim, Y., Osterberg, L. and Matthews , H.R. (1991) *J. Biol. Chem. 266*, 9023-9031.

Hunter, T. (1987) *Cell 50*, 823-829.

Hunter, T. (1991) *Methods Enzymol. 200*, 3-37.

Hunter, T. and Sefton, B.M. (1980) *Proc. Natl. Acad. Sci. USA 77*, 1311-1315.

Huot, J., Roy, G., Lambert, H., Chretien, P. and Landry, J. (1991) *Cancer Res. 51*, 5245-5252.

Hutchison, K.A., Czar, M.J. and Pratt, W.B. (1992) [a] *J. Biol. Chem. 267*, 3190-3195.

Hutchison, K.A., Brott, B.K., De Leon, J.H., Perdew, G.H, Jove, R. and Pratt, W.B. (1992) [b] *J. Biol. Chem. 267*, 2902-2908.

Iida, H. (1988) *Mol. Cell. Biol 8*, 5555-5560.

Iwasaki, M., Saito, H., Yamamoto, M., Kovach, K.S., Hirogome and Sugano, H. (1989) *Biochim. Biophys. Acta 992*, 1-8.

Jacobsen, B.K. and Pelham, H.R.B. (1991) *EMBO J. 10*, 369-375.

Johnston, G.C. and Singer, R.A. (1980) *Mol. Gen. Genet. 178*, 357-360.

Johnston, G.C., Pringle, J.R. and Hartwell, L.H. (1977) *Expt. Cell Res. 105*, 79-98.

Jurivich, D.A., Chung, J. and Blenis, J. (1991) *J. Cell. Physiol. 148*, 252-259.

Kaibuchi, K., Miyajima, A., Arai, K-I. and Matsumoto, K. (1986) *Proc. Natl. Acad. Sci. USA 83*, 8172-8176.

Kamps, M.P. and Sefton, B.M. (1989) *Anal. Biochem. 176*, 22-27.

Kelley, P.M. and Schlesinger M.J. (1978) *Cell 15*, 1277-1286.

Kennedy, I.M., Burdon, R.H. and Leader, D.P. (1984) *FEBS Lett. 169*, 267-273.

Kennedy, T.E., Wager-Smith, K., Barzilai, A., Kandel, E.R. and Sweatt, J.D. (1988) *Nature 336*, 499-500.

Kennelly, P.J. and Krebs, E.G. (1991) *J. Biol. Chem. 266*, 15555-15558.

Kingston, R.E., Schuetz, T.J. and Larin, Z. (1987) *Mol. Cell Biol. 7*, 1530-1534.

Kirk, N. and Piper, P.W. (1991) *Yeast 7*, 539-546.

Kobayashi, H., Suzuki, T. and Unemot, T. (1986) *J. Biol. Chem. 261*, 627-630.

Kobota, S., Yoshida, Y., Tumaoka, H. and Furumichi,H (1977) *J. Biol. Chem 81*, 197-206.

Kolarov, J., Kulpa, J., Baijot, M. and Goffeau, A. (1988) *J. Biol. Chem. 263*, 10613-10619.

Krebs, E.G. (1986) in *The enzymes* (3rd edition) Volume XVII (Boyer, P.D. and Krebs, E,G., eds.) pp. 3-20.

Laemmli, U.K. (1970) *Nature 227*, 680-685.

Lambert, N. (1989) in *Protein purification methods: a practical approach* (Harris, E.L.V. and Angal, S., eds.) pp. 101-108 IRL Press, Oxford.

Landry, J., Crete, P., Lamarche, S. and Chretien, P. (1988) R*adiation Research 113*, 426-436.

Larson. J.S., Schuetz, T.J. and Kingston R.E. (1988) *Nature 335*, 372-375.

Leao, C. and VanUden, N. (1985) *Appl. Microbiol. Biotechnol. 22*, 359-363.

Lee, S.C. and Olins, P.O. (1992) *J. Biol. Chem. 267*, 2849-2852.

Leenders, H.J., Kemp, A., Konintex, J.F. and Rosing, J. (1974) *Expt. Cell Res. 86*, 25-30.

Lees-Miller, S.P. and Anderson, C.W. (1989) [a] *J. Biol. Chem. 264*, 2431-2437.

Lees-Miller, S.P. and Anderson, C.W. (1989) [b] *J. Biol. Chem. 264*, 17275-17280.

Legagneux, V., Morange, M. and Bensaude, O. (1990) *Eur. J. Biochem. 193*, 121-126.

Lemaux, P.G., Herendeen, S.L., Bloch, P.L. and Neidhardt, F.C. (1978) *Cell 13*, 427-434.

Lepock, J.R., Frey, H.E. and Inniss, K.E. (1990) *Biochim. Biophys. Acta 1055*, 19-26.

Lepock, J.R., Frey, H.E., Rodahl, A.M. and Kruov, J. (1988) *J. Cell Physiol. 137*, 14-24.

Leustek, T., Dalie, B., Dekel. A-S., Brot, N. and Weissbach, H. (1989) *Proc. Natl. Acad. Sci. USA 86*, 7805-7808.

Lew, R.R (1989) *Plant Physiol. 91*, 213-216.

Li, G.C. (1983) *J. Cell Physiol 115*, 116-122.

Li, G.C. and Werb, Z. (1982) *Proc. Natl. Acad. Sci. USA 79*, 3218-3222.

Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. and Zylicz, M. (1991) *Proc. Natl. Acad. Sci. USA 88*, 2874-2878.

Liberek, K., Skowyra, D., Zylicz, M., Johnson, C. and Georgopoulos, C. (1991) *J. Biol. Chem. 266*, 14491-14496.

Lillie, S.H. and Pringle, J.R. (1980) *J. Bacteriol. 143*, 1384-1394.

Lindquist, S. (1980) *J. Mol. Biol. 137*, 151-158.

Lindquist, S. (1981) *Nature 293*, 311-314.

Lindquist, S. (1986) *Ann. Rev. Biochem. 55*, 1151-1191.

Lindquist, S. and Craig, E.A. (1988) *Annu. Rev. Genet. 22*, 631-677.

Livak, K.J., Freund, R., Schweber, M., Wensink P.C. and Meselson, M. (1978) *Proc. Natl. Acad. Sci. USA 75*, 5613-5617.

Londesborough, J. and Nuutinen, M. (1987) *FEBS Lett. 219*, 249-253.

Lopez, S. and Gancedo, J.M. (1979) *Biochem. J. 178*, 769-776.

Lubben, T.H., Gatenby, A.A, Donaldson, G.K, Lorimer, G.H. and Vitanen, P.V. (1990) *Proc. Natl. Acad. Sci. USA 87*, 7683-7687.

Luke, M.M., Sutton, A. and Arndt, K.T. (1991) *J. Cell Biol. 114*, 623-638.

Mackenzie, K.F., Singh, K.K. and Brown, A.D. (1988) *J. Gen. Microbiol. 134*, 1661-1666.

Maiorella, B.L., Blanch, H.W. and Wilke, C.R. (1984) *Biotech. Bioeng. 26*, 1155-1166.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035-10038.

Matsudaira, P. (1990) *Methods Enzymol. 182*, 602-613.

Matsumoto, K., Uno, I. and Ishikawa, T. (1985) *Yeast 1*, 15-24.

Matsumoto, K., Uno, I., Oshima, Y. and Ishikawa, T. (1982) *Proc. Natl. Acad. Sci. USA 79*, 2355-2359.

Matts, R.L., Xu, Z., Pal, J.K. and Chen, J.-J. (1992) *J. Biol. Chem. 267*, 18160-18167.

Mazon, M.J., Behrens, M.M., Portillo, F. and Pinon, R. (1989) *J. Gen. Microbiol. 135*, 1453-1460.

McAlister, L. and Finkelstein, D.B. (1980) *Biochem. Biophys. Res. Commun. 93*, 819-824.

McCarty, J.S. and Walker, G.C. (1991) *Proc. Natl. Acad. Sci. USA 88*, 9513-9517.

McDonough, J.P. and Mahler, H.P. (1982) *J. Biol. Chem. 257*, 14579-14581.

Minami, Y, Kawasaki, H., Miyata, Y., Suzuki, K., and Yahara, I. (1991) *J. Biol. Chem. 266*, 10,099-10,103.

Mitts, M.R., Grant, D.B. and Heideman, W. (1990) *Mol. Cell. Biol. 10*, 3873-3883.

Mizzen, L.A., Chang, C., Garrels, J.G. and Welch, W.J. (1989) *J. Biol. Chem. 264*, 19,872-19,878.

Morimoto, R.I., Abravaya, K., Mosser, D. and Williams, G.T. (1990) in *Stress proteins* (Schlesinger, M.J., Santoro, M.G. and Garaci, E., eds.) pp. 1-17 Springer-Verlag, Berlin.

Morimoto, R.I. and Milarski, K.L. (1990) in *Stress Proteins In Biology And Medicine* (Morimoto, R.I., Tissieres, A. and Georgopoulos, C., eds.) pp. 323-359 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Mosser, D.D., Kotzbauer, P.T., Sarge, K., Morimoto, R.I. (1990) *Proc. Natl. Acad. Sci. USA 87*, 3748-3752.

Murakami, M.S. and Rosen, O.M. (1991) *J. Biol. Chem. 266*, 22653-22660.

Neidhardt, F.C., Van Bogelen, R.A. and Vaughn, V. (1984) *Ann. Rev. Genet. 18*, 295-329.

Nelson, N. and Taiz, L. (1989) *TIBS 14*, 113-116.

Neves, M.J., Jorge, J.A., Francois, J.M. and Terenzi, H.F. (1991) *FEBS Lett. 283*, 19-22.

Nguyen, V.T., Morange, M. and Bensaude, O. (1989) *J. Biol. Chem. 264*, 10487-10492.

Nishizuka, Y. (1986) *Science 233*, 305-312.

Nishizuka, Y. (1988) *Nature 334*, 661-665.

North, M.J. (1989) in *Proteolytic enzymes: a practical Approach* (Beynon, R.J. and Bond, J.S., eds.) pp. 105-123 IRL press, Oxford.

Nuccitelly, R. and Heiple, J.M. (1982) in *Intracellular pH: Its Measurement Regulation and Utilisation in Cellular Functions* (Nuccitelly, R. and Deamer, D.W., eds.) 567-586 Alan R. Liss, New York.

O' Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) *Cell 12*, 1133-1142.

Olsen, A.S., Triemer, D.F. and Sanders, M.M. (1983) *Mol. Cell Biol. 3*, 2017-2027.

Ostermann, J., Voos, W., Kang, P.J., Craig, E.A., Neupert, W. and Pfanner, N. (1990) *FEBS Lett. 277*, 281-284.

Padan, E., Zilberstein, D. and Shuldiner, S. (1981) *Biochim. Biophys. Acta 650*, 151-156.

Panaretou, B and Piper, P.W. (1990) *J. Gen. Microbiol. 136*, 1763-1770.

Panaretou, B and Piper, P.W. (1992) *Eur. J. Biochem. 206*, 635-640.

Parag, H.A, Raboy, B. and Kulka, R.G. (1987) *EMBO J. 6*, 55-61.

Pardo, J.M. and Serrano, R. (1989) *J. Biol. Chem. 264*, 8557-8562.

Park, H. and Craig, E.A. (1989) *Mol. Cell Biol. 9*, 2025-2033.

Parris, C.N., Arlett, C.F., Lehmann, A.R., Green, M.H.L. and Masters, J.R.W. (1988) *Int. J. Radiat. Biol. 53*, 599-608.

Parsell, D. and Sauer, R. (1989) *Genes Dev. 3*, 1226-1232.

Parsell, D.A., Sanchez, Y. Stitzel, J.D. and Lindquist, S. (1991) *Nature 353*, 270-273.

Patton, J.L. and Lester, R.L. (1992) *Arch. Biochem. Biophys. 292*, 70-76.

Peake, P., Basten, A., and Britton, W.J. (1991) *J. Biol. Chem. 31*, 20828-20832.

Pekkala, D. and Silver, J. C. (1987) *Expt. Cell Res. 168*, 325-337.

Pelham, H.R.B. (1984) *EMBO J. 3*, 3095-3100.

Pelham, H.B.R. (1986) *Cell 46*, 959-961.

Pelham, H.R.B. (1990) in *Stress Proteins in Biology and Medicine* (Morimoto, R., Tissieres, A. and Georgopoulos, C., eds.) pp. 287-299 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Pelham, H., Lewis, M. and Munro, S. (1985) in *Sequence specificity in transcription and translation* (Calendar, R. and Gold, L., eds.) pp. 681-688 Alan R. Liss, New York.

Pena, P.D.L., Barros, F., Gascon, S., Ramos, S. and Lazo, P.S. (1982) *Eur. J. Biochem. 123*, 447-453.

Perdew, G.H. and Whitelaw, M.L. (1991) *J. Biol Chem. 266*, 6708-6713.

Perisic, O., Xiao, H. and Lis, J.T. (1989) *Cell 59*, 797-806.

Perlin, D.S., Harris, S.L., Seto-Young, D., and Haber, J.E. (1989) *J. Biol. Chem. 264*, 21857-21864.

Perona, R. and Serrano, R. (1988) *Nature 334*, 438-440.

Petko, L. and Lindquist, S. (1986) *Cell 45*, 885-894.

Picard, D., Khursheed, B., Garabedian, M.J., Fortin, M.G., Lindquist, S. and Yamamoto, K.R. (1990) *Nature 348*, 166-168.

Pilecki, M., Grankowski, N., Jacobs, J. and Gasior, E. (1992) *Eur. J. Biochem. 206*, 259-267.

Piper, P.W. (1987) *Sci. Prog.Oxf. 71*, 531-544.

Piper, P.W., Curran, B., Davies, M.W., HIrst, K. Lockheart, A and Seward, K. (1988) *Mol. Microbiol. 2*, 353-361.

Piper, P.W., Curran, B., Davies, M.W., Lockheart, A. and Reid, G. (1986) *Eur. J. Biochem 161*, 525-531.

Piper, P.W., Davies, M.W., Curran, B., Lockheart, A., Spalding, A. and Tuite, M.F. (1987) *Curr. Genet. 11*, 595-598.

Plesofsky-Vig, N. and Brambl, R. (1990) *J. Biol. Chem. 265*, 15432-15440.

Plesset, J., Palm, C. and McLaughlin, C.S. (1982) *Biochem. Biophys. Res. Commun. 108*, 1340-1345.

Portillo, F., Eraso, P. and Serrano, R. (1991) *FEBS Lett. 287*, 71-74.

Portillo, F., Larrinoa, I.F. and Serrano, R. (1989) *FEBS Lett. 247*, 381-385.

Portillo, F. and Mazon, M. J. (1985) *FEBS Lett. 192*, 95-98.

Portillo, F. and Serrano, R. (1988) *EMBO J. 7*, 1793-1798.

Portillo, F. and Serrano, R (1989) *Eur. J. Biochem 186*, 501-507.

Praekelt, U.M. and Meacock, P.A. (1990) *Mol. Gen. Genet. 223*, 97-106.

Price, B.D. and Calderwood, S.K. (1991) *Mol. Cell. Biol. 11*, 3365-3368.

Pryde, J.G. (1986) *Trends Biochem. Sci. 11*, 160-163.

Quinn, P.J. and Williams, W.P. (1985) in *Photosynthetic mechanisms and the environment* (Barber, J. and Barber, N.R., eds.) pp. 1-47, Elsevier, Amsterdam.

Rabindran, S.K., Giorgi, G, Clos, J. and Wu, C. (1991) *Proc. Natl. Acad. Sci. USA 88*, 6906-6910.

Ramos. S., Balbin, M., Raposo, M., Valle, E. and Pardo, L.A. (1989) *J. Gen. Microbiol. 135*, 2413-2422.

Reading, D.S., Hallberg, R.L. and Myers, A.M. (1989) *Nature 337*, 655-659.

Reed, R.H., Chudek, J.A., Foster, R. and Gadd, G.M. (1987) *Appl. Environ. Microbiol. 53*, 2119-2123.

Richter, W.W., Zang, K.D. and Issinger, O.-G. (1983) *FEBS Lett. 153*, 262-266.

Ritossa, F.M. (1962) *Experientia 18*, 571-573.

Roach, P.J. (1991) *J. Biol. Chem. 266*, 14139-14142.

Roe, S. (1989) in *Protein purification methods a practical appropach* (Harris, E.L.V. and Angal, S., eds.) pp. 175-244 IRL Press, Oxford.

Roitt, I., Brostoff, J. and Male, D. (1986) in *Immunology* pp. 8.1-8.10 Gower Medical Publishing Ltd.

Roldan, M., Donaire, J.P., Pardo, J.M. and Serrano, R. (1991) *Plant Science 79*, 163-172.

Rosa, M.F and Sa-Correia, I. (1991) *Appl. Environ. Microbiol. 57*, 830-835.

Rosa, M.F and Sa-Correia, I. (1992) *Enzyme Microb. Technol. 14*, 23-27.

Rose, M., Misra, L.M. and Vogel, J.P. (1989) [a] *Cell 57*, 1211-1221.

Rose, A.H. and Veazey, F.J. (1988) in *Yeast - A Practical Approach* (Campbell, I and Duffus, J.H., eds.) pp 255-275 IRL Press, Oxford.

Rose, I.A. and Warms, J.V.B (1987) *Proc. Natl. Acad. Sci. USA 84*, 1477-1481.

Rose, D.W., Welch, W.J., Kramer, G. and Hardesty, B. (1989) [b] *J. Biol. Chem. 264*, 6239-6244.

Rothman, J.E. (1989) *Cell 59*, 591-601.

Rozengurt, E. (1980) *Curr. Top. Cell. Regul. 17*, 59-88.

Sambrook, J., Fritsch, E.F. and Maniatis, S.T. (1989) in Book 3 of *Molecular Cloning - A Laboratory Manual* second edition pp. 18.3-18.88 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sanchez, E.R., Meshinchi, S., Tienrungroj, W., Schlesinger, M.J., Toft, D.O. and Pratt, W.B. (1987) *J. Biol. Chem. 262*, 6986-6991.

Sanchez, Y. and Lindquist, S. (1990) *Science 248*, 1112-1115.

Sanderman, H. (1978) *Biochim. Biophys. Acta 515*, 209-237.

Schafer, W.R., Trueblood, C.E., Yang, C-C., Mayer, M.P., Rosenberg, S., Poulter, C.D., Kim, S-H. and Rine, J. (1990) *Science 249*, 1133-1139.

Schagger, H. and Von Jagow, G. (1987) *Anal. Biochem. 166*, 368-379.

Schaller, G.E. and Sussman, M.R. (1988) *Planta 173*, 509-518

Scharf, K.-D. and Nover, L. (1982) Cell 30, 427-437.

Schenberg-Frascino, A. and Moustacchi, E. (1972) *Mol. Gen. Genet. 115*, 243-257.

Scherer, P.E., Krieg, U.C., Hwang, S.T., Vestweber, D. and Schatz, G. (1990) *EMBO J. 9*, 4315-4322.

Schlesser, A., Ulaszewski, S., Ghislain, M. and Goffeau, A. (1988) *J. Biol. Chem. 263*, 19480-19487.

Schmidt, R., Ackerman, R., Kratky, Z., Wasserman and Jacobsen, B. (1983) *Biochim. Biophys. Acta. 732*, 421-427

Schoffl, F. and Key, J.L. (1982) *J. Mol. Appl. Genet. 1*, 307-314.

Schuetz, T.J., Gallo, G.J, Sheldon, L, Tempst, P. and Kingston, R.E. (1991) *Proc. Natl. Acad. Sci. USA 88*, 6911-6915.

Serrano, R. (1978) *Mol. Cell. Biochem. 22*, 51-63.

Serrano, R. (1983) *FEBS Lett. 156*, 11-14.

Serrano, R. (1984) *Curr. Top. Cell. Regul. 23*, 87-126.

Serrano, R. (1988) *Biochim. Biophys. Acta 947*, 1-28.

Serrano, R. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 40*, 61-94.

Serrano, R., Kielland-Brandt, M.C. and Fink, G.R. (1986) *Nature 319*, 689-693.

Serrano, R., Montesinos, C., Roldan, M., Garrido, G., Ferguson, C., Leonard, K., Monk, B.C., Perlin, D.S. and Weilar, E.W (1991) *Biochim. Biophys. Acta 1062*, 157-164.

Seufert, W. and Jentsch, S. (1990) *EMBO J. 9*, 4535-4541.

Shin, D-Y., Matsomoto, K., Iida, H., Uno, I. and Ishikawa, T. (1987) *Mol. Cell. Biol. 7*, 244-250.

Simon, M.C., Kitchener, K., Kao, H-T, Hickey, E., Weber, L., Voellmy, R., Heintz, N. and Nevins, J.R. (1987) *Mol. Cell. Biol 7*, 2884-2894.

Singh, K.S. and Norton, R.S. (1991) *Arch. Microbiol. 156*, 38-42.

Singh, N.K., Nelson, D.E, Kuhn, D., Hasegawa, P.M. and Bressan, R.A. (1989) *Plant Physiol 90*, 1096-1101.

Sinibaldi, R.M. and Storti, R.V. (1982) *Biochem. Genet. 20*, 791-807.

Skou, J.C. (1988) *Methods Enzymol. 156*, 1-25.

Skowyra, D., Georgopoulos, C. and Zylicz, H. (1990) *Cell 62*, 939-944.

Skriver, K. and Mundy, J. (1990) *Plant Cell 2*, 503-512.

Smith, B.J. and Yaffe, M.P. (1991) *Proc. Natl. Acad. Sci. USA 88*, 11091-11094.

Smith, D.F., Faber, L.E. and Toft, D.O. (1990) *J. Biol. Chem. 265*, 3996-4003.

Sorger, P.K. (1990) *Cell 62*, 793-805.

Sorger, P.K. (1991) *Cell 65*, 363-366.

Sorger, P.K. and Pelham, H.R.B. (1988) *Cell 54*, 855-864.

Sorger, P.K., and Pelham, H.R.B. (1987) *EMBO J. 6*, 3035-3041.

Spiro, I.J., Denman, D.L. and Dewey, W.C. (1982) *Rad. Res. 89*, 134-149.

Squires, C.L., Pedersen, S., Ross, B.M. and Squires , C. (1991) *J. Bacteriol. 173*, 4254-4262.

Stock, J.B., Stock, A.M. and Mottonen, J.M. (1990) *Nature 344*, 395-400.

Strauch, K.L., Johnson, K. and Beckwith, J. (1989) *J. Bactreriol. 171*, 2689-2696.

Straus, D.B., Walter, W.A. and Gross, C.A. (1987) *Nature 329*, 348-351.

Struhl, K. (1983) *Nature 305*, 391-397.

Subjeck, J.R., Shyy, T, Shen, J., Johnson, R.J. (1983) *J. Cell. Biol. 97*, 1389-1395.

Sutton, C. (1989) in *Protein purification methods a practical appropach* (Harris, E.L.V. and Angal, S., eds.) pp. 268-282 IRL Press, Oxford.

Swida, U., Kreutzfelfdt, C., Ramezani-Rad, M. and Kaufer, N. (1982) *FEMS Microbiol. Lett. 15*, 313-318.

Tanaka, K., Matsumoto, K. and Toh-E, A. (1989) *Mol. Cell. Biol. 9*, 757-768.

Tanaka, K., Matsumoto, K. and Toh-e, A. (1988) *EMBO J. 7*, 495-502.

Tanner, W. and Lehle, L. (1987) *Biochim. Biophys. Acta 906*, 81-89.

Thevelein, J. (1991) *Mol. Microbiol. 5*, 1301-1307.

Thomas, T.C. and McNamee, M.G. (1990) *Methods Enzymol. 182*, 499-520.

Tilly, K. and Georgopoulos, C. (1982) *J. Bacteriol. 149*, 1082-1088.

Tilly, K., McKittrick, N., Zylicz, M. and Georgopoulos, C. (1983) *Cell 34*, 641-646.

Towbin, H., Staehelin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA 76*, 4350-4354.

Treger, J., Hiechman, K.A. and McEntee, K. (1988) *Mol. Cell Biol. 8*, 1132-1136.

Trollmo, C., Andre, L., Blomberg, A. and Adler, L. (1988) *FEMS Microbiol. Lett. 56*, 321-326.

Uchida, E., Ohsumi, Y. and Anraku, Y. (1985) *J. Biol. Chem. 260*, 1090-1095.

Ulaszewski, S., Balzi, E. and Goffeau, A. (1987) [b] *Mol. Gen. Genet. 207*, 38-46.

Ulaszewski, S., Coddington, A. and Goffeau, A. (1986) *Curr. Genet. 10*, 359-364.

Ulaszewski, S., Grenson, M. and Goffeau, A. (1983) *Eur. J. Biochem. 130*, 235- 239.

Ulaszewski, S., Hilger, F. and Goffeau, A. (1989) *FEBS Lett. 245*, 131-136.

Ulaszewski, S., Van Herck, J-C., Dufour, J-P., Kulpa, J., Nieuwenhuis, B. and Goffeau, A. (1987) [a] *J. Biol. Chem. 262*, 223-228.

Vai, M., Popolo, L. and Alberghina, L. (1986) *FEBS Lett. 206*, 135-141.

Vallejo, C.G. and Serrano, R. (1989) *Yeast 5*, 307-319.

Van Dyck, L., Petretski, J. H., Wolosker, H., Rodrigues, G., Schlesser, A., Ghislain, M. and Goffeau, A. (1990) *Eur. J. Biochem 194*, 785-790.

Vernon, D.M and Bohnert, H.J. (1992) *EMBO J. 11*, 2077-2085.

Viegas, C.A. and Sa-Correia, I. (1991) *J. Gen. MIcrobiol. 137*, 645-651.

Voellmy, R., Goldschmidt-Clermont, M., Southgate, R., Tissieres A., Levis, R. and Gething, W. (1981) *Cell 23*, 261-270.

Waegemann, K., Paulsen, H. and Soll H. (1990) *FEBS Lett. 261*, 89-92.

Walker, M.C., Parris, C.N. and Masters, J.R.W.(1987) *JNCI 79*, 213-216.

Walker-Caprioglio, H.M. and Parks, L.W. (1987) *Appl. Environ. Microbiol. 53*, 33-35.

Waltschewa, L, Kotyk, A. and Venkov, P. (1991) *Yeast 7*, 211-217.

Watanabe, Y., Yamaguchi, M., Sanemitsu, Y, and Tamai, Y. (1991) *Yeast 7*, 599-606.

Watson, K. (1984) in *Membrane fluidity* (Kates, M and Manson, L.A., eds.) pp 517-541 Plenum Publishing Corporation.

Watson, K. (1987) in *The Yeasts 2*, (2nd edition) pp 41-66 Academic Press Inc, London.

Watson, K., Dunlop, G. and Cavicchioli, R. (1984) *FEBS Lett. 172*, 299-302.

Wei, Y. and Matthews, H.R. (1991) *Methods Enzymol. 200*, 388-414.

Weitzel, G., Pilatus, U. and Rensing, L. (1987) *Expt. Cell Res. 170*, 64-79.

Welch, W.J. (1990) in *Stress proteins in biology and medicine* (Morimoto, R.I., Tissieres, A. and Georgopoulos, C., eds.) pp. 223-278 Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Welch, W.J. and Mizzen, L.A. (1988) *J. Cell. Biol. 106*, 1117-1130.

Welch, W.J. and Suhan, J.P. (1985) *J. Cell Biol. 101*, 1198-1211.

Welch, W.J., Mizzen, L.A. and Arrigo, A-P. (1989) in *Stress-Induced Proteins* (Pardue, M.L., Feramisco, J.R. and Lindquist, S., eds.) pp. 187-202 Alan R. Liss, New York.

Weretilnyk, E.A and Hanson, A.D. (1990) *Proc. Natl. Acad. Sci. USA 87*, 2745-2749.

Werner-Washburne, M., Becker, J., Kosic-Smithers, J. and Craig, E.A. (1989) *J. Bacteriol. 171*, 2680-2688.

Werner-Washburne, M., Stone, D.E. and Craig, E.A. (1987) *Mol. Cel. Biol. 7*, 2568-2577.

Wetzstein, M. and Schumann, W. (1990) *Nucleic Acids Res. 18*, 128-?.

Whelan, S.A. and Hightower, L.E. (1985) *J. Cell Physiol. 125*, 251-258.

Widelitz, R.B., Magun, B.E. and Gerner, E.W. (1986) *Mol. Cell. Biol. 6*, 1088-1094.

Wiemken, A. (1990) *Antonie van Leeuwenhoek 58*, 209-217.

Wieser, R., Gerhard, A., Wagner, A., Schuller, C., Marchler, G., Ruis, H., Krawiec, Z. and Bilinski, T. (1991) *J. Biol. Chem. 266*, 12406-12411.

Yahara, I., Lida, H. and Koyasu, S. (1986) Cell Structure And Function 11, 65-73.

Yamamori, T. and Yura, T. (1980) *J. Bacteriol. 142*, 843-851.

Yanagita, Y., Abdel-Ghany, M., Raden, D., Nelson, N. and Racker, E. (1987) *Proc. Natl. Aad. Sci. USA 84*, 925-929.

Yost, H.J. and Lindquist, S. (1986) *Cell 45*, 185-193.

Zimmerman, J.L., Petri, W. and Meselson, M. (1983) *Cell 32*, 1161-1170.

Zylicz, M., LeBowitz, J.H., McMacken, R., and Georgopoulos, C. (1983) *Proc. Natl. Acad. Sci. 80*, 6431-6435.

Eur. J. Biochem. 206, 635–640 (1992)

The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and the entry to stationary phase

Barry PANARETOU and Peter W. PIPER

Department of Biochemistry and Molecular Biology, University College London, England

(Received February 25, 1992) – EJB 92 0261

Recent studies have revealed that the action of the proton-translocating ATPase of the plasma membrane of yeast is an important determinant of several stress tolerances and affects the capacity of cells to synthesise heat shock proteins in response to heat shock [Panaretou, B. & Piper, P. W. (1990) *J. Gen. Microbiol. 136*, 1763-1770; Coote, P. J., Cole, M. B. & Jones, M. V. (1991) *J. Gen. Microbiol. 137*, 1701-1708]. This study investigated the changes to the protein composition of the *Saccharomyces cerevisiae* plasma membrane that result from a heat shock to dividing cultures and the entry to stationary growth caused by carbon source limitation. Plasma membranes were prepared from exponential, heat-shocked and stationary yeast cultures. The proteins of these membrane preparations were then analysed by polyacrylamide gel electrophoresis and immunoblot measurement of ATPase levels.

The protein composition of plasma membranes displayed two prominent changes in response to both heat shock and the entry to stationary phase: (a) a reduction in the level of the plasma membrane ATPase; and (b) the acquisition of a previously uncharacterised 30 kDa heat-shock protein (hsp30). The ATPase decline with heat shock probably exerts an important influence over the ability of the cell to maintain ATPase activity, and therefore intracellular pH, during extended periods of stress. Through *in vivo* pulse-labelling of plasma membrane proteins synthesised before and during heat shock, followed by subcellular fractionation, it was shown that hsp30 is the only protein induced by the yeast heat-shock response that substantially copurifies with plasma membranes. It might therefore exert a stress-protective function specifically at this membrane.

The heat-shock response is an inducible stress response of practically all living cells. It is triggered by a number of stress agents in addition to heat shock and it apparently increases the capacity of the cell to counteract heat damage [1–7]. A major feature of the response is its strong induction of a small number of heat-shock proteins [1–7]. Some of these proteins probably serve to sequester damaged, denatured or otherwise improperly folded protein (as complexes with hsp70 family of proteins [1–4]), while other heat-shock proteins (proteases in prokaryotes, components of the ubiquitination pathway for intracellular protein turnover in eukaryotes) almost certainly assist in the degradation of damaged protein [2–5]. Increasing the levels of heat-shock proteins may serve therefore to increase the ability of the cell to survive intracellular accumulations of aberrant or improperly folded protein. The heat shock response may itself be triggered by shortages of free, uncomplexed hsp70 [1], a shortage that might result when all the available hsp70 has associated with the increased amounts of damaged protein in heat-stressed cells. Evidence that heat-shock proteins probably fulfil an important function is also provided by their high evolutionary conservation, a conservation that must reflect the perpetuation of functions essential for cell survival [1–4].

The potentially cytotoxic effects of heat stress are thought to result from damage to protein assemblies and to membranes. Most heat-shock studies have focussed on the induced heat-shock proteins. It has been found that some of these proteins are highly homologous with, and probably function in a similar manner to, the constitutively synthesised chaperonin proteins present in unstressed cells [1–4]. In ATP-dependent processes many of these chaperonins assist in the unfolding and refolding of polypeptide chains prior to, and after, polypeptide transport across intracellular membranes [2–4, 6, 7]. Similarly certain heat-shock proteins may sequester partially unfolded protein and assist in its renaturation [1]. To date only a small number of heat-shock studies have investigated the effects of stress on membrane structure *per se*. These have demonstrated temperature effects on membrane fluidity [8] and have revealed that long-term acclimation to high temperatures is correlated with an increased saturation of membrane lipids [9]. Certain inducers of stress responses (e.g. heat shock and ethanol) will permeabilise membranes, thereby causing pronounced disturbance to transmembrane ion gradients. An electrochemical gradient crucial for many systems is that gradient maintained across the cytoplasmic

Correspondence to P. W. Piper, Department of Biochemistry and Molecular Biology, University College London, London WC1E 6BT, England

Fax. +44 71 380 7193.

Abbreviation. hsp70, heat-shock protein of molecular mass 70 kDa.

membrane (bacteria) or plasma membrane (fungal and plant cells). This is needed for several vital cellular functions (e.g. nutrient uptake, maintenance of potassium balance, and the regulation of intracellular pH). In fermentative bacteria, fungi and plants it is established and maintained largely through the action of a proton-translocating ATPase [10–14]. However this gradient, despite its importance for diverse cell functions, remains especially vulnerable to dissipation by several stress agents.

We recently reported that the activity of the plasma membrane ATPase of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* influences the tolerances of these organisms to a variety of stresses, as well as the extent to which heat-shocked *S. cerevisiae* can synthesise heat-shock proteins [15]. The importance of this enzyme in heat tolerance (thermotolerance) has since been confirmed in an independent investigation, a study which also identified hypersensitisation to thermal death as a major effect of the plasma membrane ATPase inhibitor diethylstilbesterol [16]. By making the plasma membrane more permeable, heat-shock and other inducers of thermotolerance in yeast lower intracellular pH [16, 17]. This intracellular pH drop in turn stimulates the plasma membrane ATPase [16], causing an increased proton efflux from the cell that serves partly to counteract the internal acidification resulting from the stress-induced increase in membrane permeability. In the fermentative bacterium *Streptococcus faecalis* cytoplasmic acidification causes a similar increase in proton extrusion through an enhanced activity of a proton-translocating ATPase [10].

While evidence for the importance of plasma membrane integrity in stress survival is increasing, there have been no reports to date of eukaryotic stress proteins associating with this membrane. It is nevertheless conceivable that cells target proteins with a damage-limitation function to the plasma membrane as part of their inducible protective responses. We have therefore investigated the proteins of purified plasma membranes from exponentially growing and stationary, unstressed and heat-shocked *S. cerevisiae* as an initial step towards establishing to what extent the plasma membrane, or the enzymes and carrier systems associated with this membrane, are altered in response to stress.

EXPERIMENTAL PROCEDURES

Strains

The multiply protease-deficient *S. cerevisiae* strain BJ2168 (a *leu2-3,112,trp1,ura3-52,prb1-1122,pep4-3,prc407,gal2:* gift of P. Sorger) was used for the studies reported here.

Cell culture, heat shock and pulse-labelling

For each experiment two 1-l standard defined minimal medium [18] cultures of BJ2168 with 2% glucose as carbon source were grown to mid-exponential phase (5×10^6 cells/ml) at 20 °C with leucine, uracil and tryptophan (each at 20 mg/l) supplements. One culture (unstressed cells) was then harvested, the other (heat-shocked cells) being shifted to 40 °C for 40 min prior to harvesting. Plasma membranes were then prepared from both cultures as decribed below.

For studying membrane proteins labelled *in vivo* both before and during heat shock, two 50-ml portions of the above cultures growing at 20 °C were labelled either for 40 min at 20 °C or at 10–40 min after the heat shock from 25 °C to 40 °C. Either 0.05 mCi/ml L-[^{35}S]methionine (1000 Ci/mmol; New England Nuclear), or 0.05 mCi/ml [4,5-^{3}H]leucine (53 Ci/mmol; New England Nuclear) was added to the culture medium.

Plasma membrane isolation

Cells were disrupted by vortexing with glass beads and, following subcellular fractionation, plasma membranes were isolated by the banding of the cell membranes of the 22 000 × g pellet fraction on discontinuous sucrose gradients according to the well established method of Serrano [12, 19–21]. This procedure was modified slightly by the inclusion of protease inhibitors (purchased from Sigma) in all buffers; the inhibitors were: 1 mM phenylmethylsulphonyl fluoride, 1 mM *N* tosyl-L-phenylalanine chloromethane (both added just before use from 100 mM stocks in ethanol) and 2 μg/ml pepstatin A (from a 2.5 mg/ml stock in methanol). On the preparative sucrose gradients less than a third of the vanadate-sensitive (plasma membrane) ATPase activity bands together with mitochondria at a density of 1.173 g/cm^3, the major fraction banding at the higher density of 1.223 g/cm^3 [21]. Only the fraction banding at higher density corresponds solely to plasma membranes according to the criteria given below, this 1.223-g/cm^3 fraction being further purified by a second identical discontinuous sucrose gradient banding for experiments reported here.

Criteria used to confirm purity of 1.223 g/cm^3 plasma membrane fraction

A number of tests were applied to show that membranes banding at 1.223 g/cm^3 on the preparative gradients were plasma membranes of high purity. The ATPases of the mitochondrial and vacuolar membranes are not inhibited by orthovanadate, a specific inhibitor of plasma membrane ATPase [22, 23]. It is therefore acknowledged that one of the best methods of assessing the purity of yeast plasma membrane preparations is to determine the extent of inhibition of the associated ATPase by orthovanadate [22]. Assaying ATPase activity as in [22], the ATPase activity of the 1.223-g/cm^3 membrane fraction consistently showed greater than 90% inhibition by orthovanadate and less than 6% inhibition by oligomycin. In contrast, the ATPase of the 1.173-g/cm^3 membrane fraction on the preparative gradients, a fraction which consists predominantly of mitochondria [19, 21], was inhibited about 80% by oligomycin. The absence of cytochrome peaks in the absorption spectra of the 1.223-g/cm^3 fraction (even in concentrated membrane suspensions) and the extremely low levels of the microsomal marker enzyme NADPH–cytochrome *c* reductase [24] in this fraction were further indications of the lack of any major contamination by mitochondrial and microsomal membranes.

Gel analysis and immunoblot analysis

Protein determinations were performed using the Bio-Rad protein assay kit and bovine serum albumin as standard. Protein samples were prepared by incubation at 37 °C in protein gel sample buffer containing sodium dodecyl sulphate, then analysed on one-dimensional 12.5% or 15% SDS/polyacrylamide gels [25]. Gel staining with Coomassie brilliant blue, detection of labelled proteins by fluorography, semidry electroblotting of proteins onto nitrocellulose and antibody probing of blots were according to standard procedures [26].

A polyclonal antiserum against whole plasma membrane ATPase [12] was the gift of R. Serrano. Polyclonal rabbit antisera to yeast hsp26 and hsp90 were raised in this laboratory, using as immunogen the corresponding heat-shock protein as a fragmented gel band.

RESULTS

Heat shock causes prominent changes to the plasma membrane-associated proteins of yeast

Cultures of *S. cerevisiae* BJ2168 in exponential growth at 20 °C were harvested both in the unstressed state and following a heat shock to 40 °C. The heat shock employed (40 min at 40 °C, 1–2 °C above the maximum growth temperature of this *S. cerevisiae* strain on fermentative media) is relatively severe and promotes strong induction of heat-shock proteins [27]. Membrane fractions were isolated from these cultures by banding on discontinuous sucrose gradients (see Experimental Procedures). The heavier-banding membrane fraction of density 1.223 g/cm^3 has been shown in a number of studies to consist almost entirely of plasma membranes [19–21]. In this investigation this fraction was also assessed to be of high purity by application of a number of criteria (see Experimental Procedures) and its proteins gave highly reproducible banding patterns on Coomassie-blue-stained one-dimensional gels. These gels consistently revealed two prominent changes to plasma membrane protein composition with heat shock: (a) a reduction in the levels of the most abundant plasma membrane protein, the 100-kDa plasma membrane ATPase; and (b) the appearance of a new protein band with an approximate molecular mass of 30 kDa. These changes are visible in Fig. 1. Western blots of these membrane protein samples (Fig. 1) confirmed the decline in the ATPase, with no appearance of lower-molecular-mass bands with epitopes recognised by the anti-ATPase antiserum that might have corresponded to ATPase degradation products.

Extensive precautions were taken to ensure that these changes in plasma membrane proteins do not reflect proteolytic artefacts. Most of the proteolysis in yeast cell extracts is due to the vacuolar proteases [28]. This study was therefore conducted on a strain essentially lacking such activities. Also protease inhibitors were included in all the solutions used in plasma membrane isolation, although their omission during the isolations was found not to result in any major alteration to the gel profiles of plasma membrane proteins (not shown).

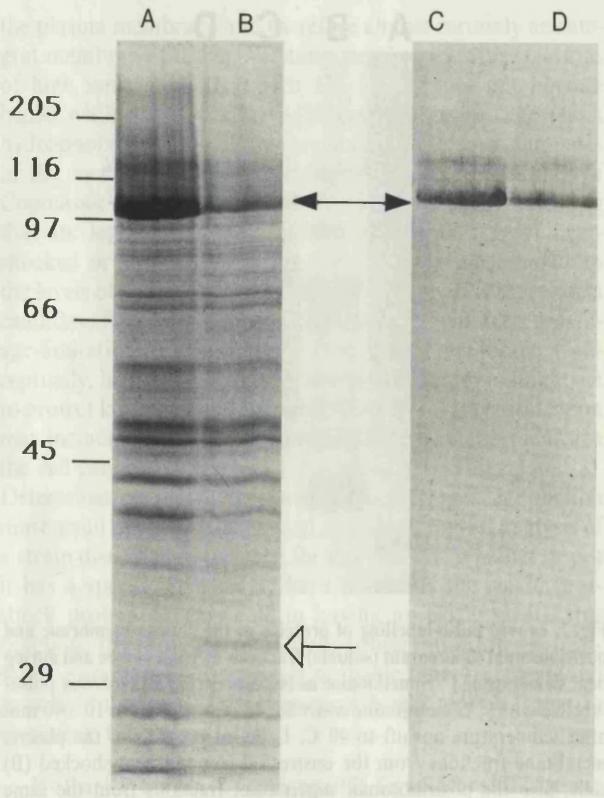


Fig. 1. Proteins of isolated plasma membranes of unstressed and heat-shocked *S. cerevisiae*. (A, B) 12.5% gel, stained with Coomassie blue, of plasma membrane proteins of (A) unstressed cells and (B) cells given a 40-min 20–40 °C heat shock. Molecular masses (kDa) of markers are indicated on the left of the gel. On the right are indicated the 100-kD plasma membrane ATPase (*solid arrow*) and the hsp30 of heat-shocked cell plasma membranes (*open arrow*). Each sample comprised 50 µg total membrane protein. (C, D) Western blot analysis of the same plasma membrane protein samples (also 50 µg protein/track) from (C) unstressed cells and (D) cells given a 40-min 20–40 °C heat shock. A polyclonal antiserum to whole plasma membrane ATPase [12] was used in the detection of this protein.

The decline in plasma membrane ATPase levels is due partly to a lack of appreciable synthesis of this enzyme in heat-shocked cells

To determine whether any of the stained bands from the membranes of heat-shocked cells corresponds to proteins synthesised *de novo* after the heat shock, cells were pulse-labelled in the absence and presence of a heat shock and their membranes isolated (see Experimental Procedures). [^{35}S]Methionine was used initially as isotope in these studies of plasma membrane proteins labelled *in vivo*. The results revealed very little, if any, incorporation of *de novo* synthesised ATPase into the plasma membranes during the heat shock (Fig. 2; sample B). Immunogold labelling of yeast sections has previously shown that there is no significant pool of plasma membrane ATPase in internal membranes [21]. This, when considered together with the lack of any appreciable ATPase labelling in heat-shocked *S. cerevisiae*, leads to the conclusion that the decline in ATPase levels with heat shock (Fig. 1) is due to turnover in the absence of synthesis. Probably cells are unable to synthesise plasma membrane ATPase when in a state of severe stress (see Discussion below).

The 30-kDa protein is the only heat-shock protein that substantially copurifies with plasma membranes

The 30-kDa heat-inducible protein was the major nascent protein associated with the plasma membrane in heat-stressed cells (Fig. 2, sample B). This polypeptide was not labelled prior to the heat shock either in the plasma membrane fraction (Fig. 2, sample A), the soluble fraction (Fig. 2, sample C) or other cell fractions (data not shown). It is therefore a heat-shock protein (designated hsp30 in Figs 1–3). Methionine labelling (Fig. 2) would not have detected any methionine-free plasma-membrane-associating heat-shock protein and at least one heat-shock protein of yeast (hsp26 in Fig. 3) has previously been found to lack methionine [29, 30]. The pulse-labellings of plasma membrane proteins in Fig. 2 were therefore repeated using [^{3}H]leucine as isotope in place of [^{35}S]methionine. Apart from hsp30, no previously unidentified heat-shock proteins were detected in the plasma membranes of heat-shocked cells with the use of this more abundant amino acid for *in vivo* labelling (Fig. 3, sample B). Small

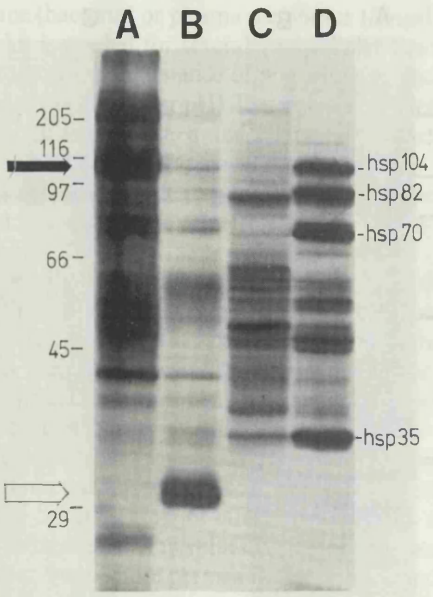


Fig. 2. *In vivo* pulse-labelling of proteins of the plasma membrane and postribosomal supernatant (soluble) fractions of yeast before and during heat shock, using [^{35}S]methionine as isotope. Strain BJ2168 was pulse-labelled with [^{35}S]methionine either for 40 min at 20 °C or 10–40 min after temperature upshift to 40 °C. Labelled proteins of the plasma membrane fractions from the unstressed (A) and heat-shocked (B) cells; also the postribosomal supernatant fractions from the same labellings (C and D respectively) were separated on a 12.5% gel and detected by fluorography. Molecular masses (kDa) of markers are indicated on the left, as also are the positions of the 100-kDa plasma membrane ATPase (*solid arrow*; samples A and B) and hsp30 of heat-shocked cell plasma membranes (*open arrow*; sample B only). Major labelled heat-shock proteins present in the soluble fraction of heat-stressed cells (D) are indicated on the right.

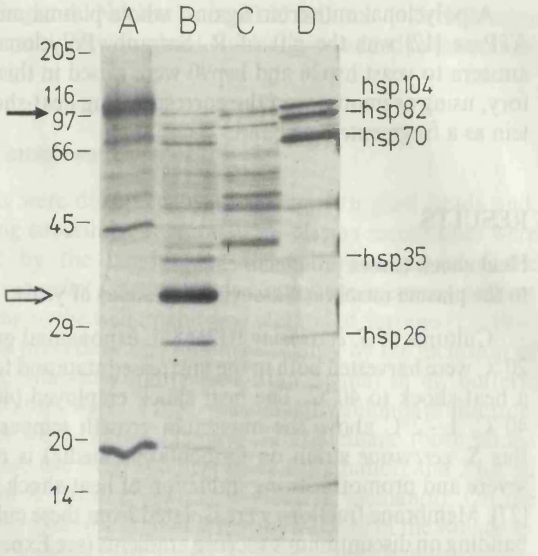


Fig. 3. *In vivo* pulse-labelling of proteins of the plasma membrane and postribosomal supernatant fractions of yeast before and during heat shock, using [^{3}H]leucine as isotope. This experiment was identical to that in Fig. 2, except that [^{3}H]leucine was used to label the proteins of unstressed and heat-shocked cells and a higher percentage (15%) gel was used for protein separation. The autoradiograph shows labelled proteins of the plasma membrane fractions from unstressed (A) and heat-shocked (B) cells; also of postribosomal supernatant fractions from the same labellings (C and D respectively). Molecular masses (kDa) of markers are indicated on the left as also are the positions of the 100-kDa plasma membrane ATPase (*solid arrow*; samples A and B) and hsp30 of heat-shocked cell plasma membranes (*open arrow*; sample B only). Major heat-shock proteins present in the soluble fraction of heat-stressed cells (D) are indicated on the right.

amounts of major heat-shock proteins substantially isolated in the postribosomal supernatant fraction from heat-stressed cells (hsp104, hsp82, hsp70, hsp35 and hsp26 [1–4, 29–32]; see sample D of Figs 2 and 3) were found to be associated with plasma membrane preparations from heat-shocked cells. However label incorporation into these bands on gels of plasma membrane proteins was consistently only a small percentage of the labelling of hsp30 (see sample B of Figs 2 and 3).

hsp30 does not correspond to any previously reported yeast heat-shock protein. Staining of protein gels and *in vivo* pulse-labelling studies conducted in conjunction with subcellular fractionation of heat-shocked yeast (not shown) revealed that the cellular levels of most well characterised heat-shock proteins are considerably in excess of total cell levels of hsp30. Many of these other heat-shock proteins are isolated predominantly in the high-speed supernatant fraction during subcellular fractionation of heat-shocked yeast (see sample D of Figs 2 and 3). There are however exceptions to this rule (e.g. the mitochondrial hsp60 [7]). hsp30 is, however, the most abundant heat-shock protein in the plasma membrane fraction of density 1.223 g/cm^3 even though its total levels in heat-stressed cells are considerably less than total cellular levels of hsp104, hsp82, hsp70, hsp35 and hsp26. Much smaller amounts of hsp30 were also identified in the lighter membrane fraction banding at density 1.173 g/cm^3 on the preparative gradients (not shown), a fraction which primarily consists of mitochondrial membranes, together with a small proportion of the plasma membranes [21]. Our data therefore indicate hsp30 as being unique in that it is the only protein induced by the heat shock response which substantially copurifies with the plasma membrane.

hsp30 is induced during entry to the stationary phase of growth, a property it shares with several heat-shock proteins of yeast

A number of heat-shock proteins are induced as yeast cells undergo the transition from exponential growth to stationary phase, these becoming major cell proteins in stationary cultures [2, 29, 33–36]. To determine if hsp30 is similarly induced at stationary phase, plasma membranes were prepared from cells which had 12–18 h previously entered stationary phase through carbon source (glucose) limitation. Analysis of the proteins of this membrane preparation clearly showed the acquisition of hsp30 and a decline in levels of the ATPase (Fig. 4), changes identical to the two major changes to plasma membrane protein composition seen with heat shock (Fig. 1). hsp30 therefore resembles other yeast heat-shock proteins in that it is induced both by heat stress and during entry to the stationary phase.

DISCUSSION

The integrity of the plasma membrane is probably an important factor determining survival of a number of different stresses, yet the changes at this membrane in stressed cells are still poorly understood. The stimulation of plasma membrane ATPase activity in yeast by several inducers of the heat-shock response (see the introduction) almost certainly helps to restore intracellular pH in response to its decline consequent upon increases in membrane permeability. This study has

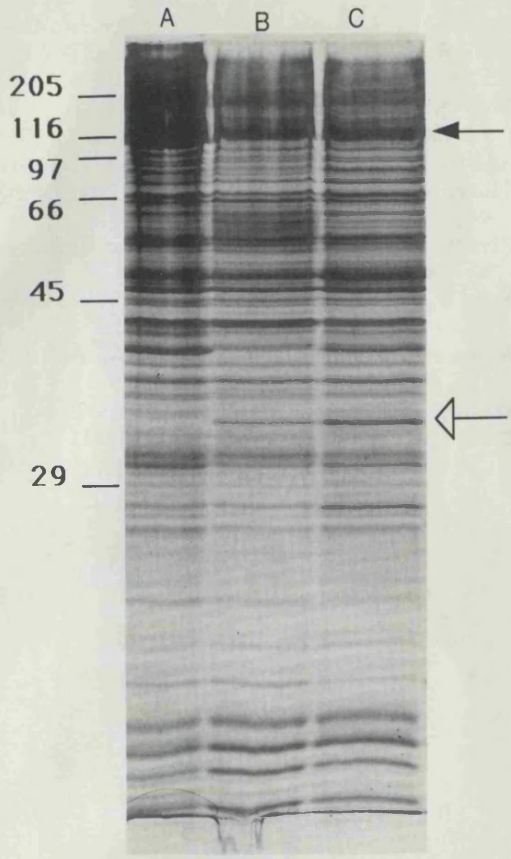


Fig. 4. hsp30 is present in plasma membranes of stationary phase cultures. 15% gel, stained with Coomassie blue, of plasma membrane proteins from (A) unstressed, (B) stationary and (C) heat-shocked cells. 100 μg total membrane protein was fractionated in each lane. Molecular masses (kDa) of markers are indicated on the left of the gel, the 100-kDa plasma membrane ATPase (*solid arrow*) and hsp30 (*open arrow*) being indicated on the right.

identified a reduction in levels of the most abundant plasma membrane protein, the proton-translocating ATPase, during both heat shock (Fig. 1) and entry to stationary phase (Fig. 4). The ATPase decline with heat shock probably exerts an important influence over the ability of the cell to maintain its intracellular pH during extended periods of stress. This turnover of the ATPase in heat-stressed cells, at a time when it is not being replenished by *de novo* synthesis (Figs 2 and 3), probably progressively reduces the capacity for sustained plasma membrane ATPase activity *in vivo* at 40 °C. This in turn will steadily reduce the ability of the cell to maintain heat-shock-induced acid efflux at high temperature and to counteract the intracellular acidification caused by heat-induced increases in membrane permeability [16, 17]. Remarkably, the major gene encoding the ATPase is still actively transcribed in *S. cerevisiae* at heat-shock temperatures [15], even though very little of its product is incorporated into the plasma membrane at these temperatures (Figs 2 and 3). Possibly the oligomeric ATPase, with its multiple transmembrane domains [12], is too complex a structure to assemble correctly in cells that are in a state of severe stress.

With the induction of the heat-shock response a single heat-shock protein (hsp30) becomes associated with the plasma membrane. It is not found in other cell fractions, either before or after heat shock, and is therefore a heat-shock protein that is targetted to the plasma membrane. We also find that hsp30 is highly hydrophobic, tightly associated with the plasma membrane and therefore almost certainly an integral membrane protein. Washing membranes with solutions of high ionic strength (up to 1M NaCl) does not liberate hsp30, while phase separation [37] experiments indicate a high hydrophobicity (unpublished results). hsp30 also accumulates in the membrane at comparatively high level, scanning of Coomassie-stained gels such as those in Figs 1 and 4 indicating that its levels in isolated plasma membranes from heat-shocked or stationary cells are approximately equimolar to the levels of the much larger 100-kD ATPase. It is therefore a candidate for a heat-shock protein which might exert a damage-limitation role specifically at the plasma membrane. Conceptually, hsp30 might influence membrane order or might act to protect key membrane proteins. Targets for such protection may include the plasma membrane ATPase, an enzyme which the cell cannot replenish in the stressed state (Figs 2 and 3). Determination of whether hsp30 has any specific function must await detailed biochemical and physiological analysis of a strain disrupted in the gene for this protein. Whether or not it has a specific role, or perhaps resembles the hsp26 heat-shock protein of yeast [38] in having no easily identifiable function, the strong evolutionary conservation of the heat-shock response [1–7] makes it probable that structural (and possibly functional) homologues of hsp30 will be found in organisms other than yeast.

We thank P. Sorger, A. Goffeau and R. Serrano for gifts of strains and antisera. B. P. was a recipient of a Science and Engineering Research Council studentship.

REFERENCES

1. Craig, E. A. & Gross, C. A. (1991) *Trends Biochem. Sci. 16*, 135–140.
2. Lindquist, S. & Craig, E. A. (1988) *Annu. Rev. Genet. 22*, 631–677.
3. Watson, K. (1990) *Adv. Microb. Physiol. 31*, 183–223.
4. Schlesinger, M. J. (1990) *J. Biol. Chem. 265*, 12111–12114.
5. Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C. & Wolf, D. H. (1991) *EMBO J. 10*, 555–562.
6. Rothman, J. E. (1989) *Cell 59*, 591–601.
7. Reading, D. S., Hallberg, R. L. & Myers, A. M. (1989) *Nature 337*, 655–659.
8. Watson, K. (1987) *Temperature relations in the yeasts*, 2nd edn (Rose, A. H., ed.) vol. 2, pp. 41–71, Academic Press, London.
9. Quinn, P. J. & Williams, W. P. (1985) in *Photosynthetic mechanisms and the environment* (Barber, J. & Baker, N. R., eds) pp. 1–47, Elsevier, Amsterdam.
10. Kobayashi. H., Suzuki, T. & Unemot, T. (1986) *J. Biol. Chem. 261*, 627–630.
11. Goffeau, A. & Slayman, C. W. (1981) *Biochim. Biophys. Acta 639*, 197–223.
12. Serrano, R., Kielland-Brandt, M. C. & Fink, G. R. (1986) *Nature 319*, 689–693.
13. Serrano, R. (1988) *Biochim. Biophys. Acta 947*, 1–28.
14. Ulazewski, S., Grenson, M. & Goffeau, A. (1983) *Eur. J. Biochem. 130*, 235–239.
15. Panaretou, B. & Piper, P. W. (1990) *J. Gen. Microbiol. 136*, 1763–1770.
16. Coote, P. J., Cole, M. B. & Jones, M. V. (1991) *J. Gen. Microbiol. 137*, 1701–1708.
17. Weitzel, G., Pilatus, U. & Rensing, L. (1987) *Expt. Cell Res. 170*, 64–79.
18. Sherman, F., Fink, G. R. & Hicks, J. B. (1983) *Methods in yeast genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
19. Serrano, R. (1978) *Mol. Cell Biochem. 22*, 51–63.
20. Serrano, R. (1988) *Methods Enzymol. 157*, 533–544.

21. Serrano, R., Montesinos, C., Roldan, M., Garrido, G., Ferguson, C., Leonard, K., Monk, B. C., Perlin, D. S. & Weilar, E. W. (1991) *Biochim. Biophys. Acta 1062*, 157–164.
22. Rose, A. H. & Veazey, F. J. (1988) in *Yeast, a practical approach* (Campbell, I. and Duffus, J. H., eds) pp. 255–275, IRL Press, Oxford.
23. Uchida, E., Ohsumi, Y. & Anraku, Y. (1985) *J. Biol. Chem. 260*, 1090–1095.
24. Kobota, S., Yoshida, Y., Tumaoka, H. & Furumichi, H. (1977) *J. Biol. Chem. 81*, 197–206.
25. Laemmli, U. K. (1970) *Nature 227*, 680–685.
26. Harlow, E. & Lane, D. (1988) *Antibodies, a laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
27. Piper, P. W., Curran, B., Davies, M. W., Hirst, K., Lockheart, A. & Seward, K. (1988) *Mol. Microbiol. 2*, 353–361.
28. Achstetter, T. & Wolf, D. H. (1985) *Yeast 1*, 139–157.
29. Rossi, J. M. & Lindquist, S. (1989) *J. Cell. Biol. 108*, 425–439.
30. Susek, R. E. & Lindquist, S. L. (1989) *Mol. Cell. Biol. 9*, 5265–5271.
31. Borkovich, K. A., Farrelly, F. W., Finkelstein, D. B., Taulein, J. & Linquist, S. (1989) *Mol. Cell. Biol. 9*, 3919–3930.
32. Sanchez, Y. & Lindquist, S. (1990) *Science 248*, 1112–1115.
33. Tanaka, K., Matsumoto, K. & Toh-e, A, (1988) *EMBO J. 7*, 495–502.
34. Praekelt, U. M. & Meacock, P. A. (1990) *Mol. Gen. Genet. 223*, 97–106.
35. Kurtz, S., Rossi, J., Petko, L. & Lindquist, S. (1986) *Science 231*, 1154–1157.
36. Boorstein, W. R. & Craig, E. A. (1990) *Mol. Cell. Biol. 10*, 3262–3267.
37. Bordier, C. (1981) *J. Biol. Chem. 256*, 1604–1607.
38. Petko, L. & Linquist, S. (1986) *Cell 45*, 885–894.

Journal of General Microbiology (1990), **136**, 1763–1770. *Printed in Great Britain*

Plasma-membrane ATPase action affects several stress tolerances of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as well as the extent and duration of the heat shock response

BARRY PANARETOU and PETER W. PIPER*

Department of Biochemistry, University College London, London WC1E 6BT, UK

(*Received 2 January 1990; revised 17 April 1990; accepted 4 May 1990*)

The *pma1.1* mutations of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* decrease plasma-membrane ATPase activity. This study investigated how they affect different stress tolerances, and the extent and duration of the heat-shock response. *pma1.1* mutants exhibited higher resistance to ethanol and osmotic stress, but lower tolerance to ultraviolet damage, as compared to wild-type cells. *pma1.1* mutations also increased tolerance of the lethal temperature of 48 °C in cells in which no heat-shock response had been induced. However, after induction of a heat-shock response and elevated thermotolerance by a 25–38 °C upshift, then maintaining cells at 38 °C for 40 min, *pma1.1* lowered subsequent tolerances of much higher lethal temperatures. Analysis of pulse-labelled *S. cerevisiae* proteins revealed reduced heat-shock protein synthesis in the *pma1.1* mutant after a 25–38 °C heat shock. This may explain the greater increases in thermotolerance in wild-type as compared to *pma1.1* cells after both were given identical 25–38 °C shocks. With more severe treatment (25–42 °C), heat-shock protein synthesis in wild-type cells, although initially high, was switched off more rapidly than in the *pma1.1* mutant. These results indicate that plasma-membrane ATPase action exerts a major influence over several stress tolerances, as well as the extent and duration of heat-shock protein synthesis following induction of the heat-shock response.

Introduction

The ability of organisms to withstand cytotoxic agents or stressful situations is intimately affected both by their physiological state and by their capacity to induce protective responses. One of the best-characterized stress-induced responses is that elicited by heat shock. This 'heat shock response' (reviewed by Craig, 1986; Lindquist & Craig, 1988) entails the transient induction of a small number of heat-shock proteins (HSPs), and a simultaneous blocking of the synthesis of most of the proteins made prior to the shock. One of its most marked consequences is an increase in thermotolerance, measured as the ability to withstand normally lethal temperatures. A heat-shock response is displayed by most organisms and many of the induced genes show a remarkable degree of evolutionary conservation. Although experimentally the response is usually induced by temperature upshift, several potentially cytotoxic chemicals are also potent inducers. All these inducers may act through a common trigger, possibly the intracellular accumulation of aberrant or misfolded protein (Ananthan *et al.*, 1986; Pelham, 1987).

Stress tolerances can be affected by many factors in addition to the actions of those proteins induced through the triggering of a stress response. Thus thermotolerance in *Saccharomyces cerevisiae* cannot be attributed solely to the actions of HSPs. It is also affected by growth state, being lowest in cells in rapid exponential growth and high in stationary-phase (G_0) cells (Iida & Yahara, 1984; Iida, 1988). The high thermotolerance of G_0 cells may be due in part to the induction of HSP genes with the decline in cyclic AMP levels as cells enter stationary phase (Boucherie, 1985; Iida & Yahara, 1984; Shin *et al.*, 1987). A few of the proteins made in heat-shocked *S. cerevisiae* correspond to glycolytic enzymes that are also made by unstressed cells (Piper *et al.*, 1986, 1988). Furthermore, we recently identified the gene for plasma membrane ATPase (*PMA1*) as one of the few in *S. cerevisiae* still efficiently transcribed after heat shock to 42 °C, approximately 3 °C above the maximum growth temperature. Polyadenylated RNA pulse-labelled *in vivo* at 42 °C hybridizes to relatively few yeast sequences, one of them being the *PMA1* transcribed region (Curran *et*

Abbreviation: HSPs, heat-shock proteins.

al., 1988). This led us to investigate mutants altered in plasma-membrane ATPase activity to obtain evidence of whether the activity of this important enzyme is a major determinant of the tolerances of budding (*S. cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeast to heat shock and other stresses.

Plasma-membrane ATPase is an electrogenic proton pump crucial to all fungal and plant cells. It generates the electrochemical H^+ gradient essential for such important cellular functions as the secretion of acids, the uptake of nutrients, the maintenance of K^+ levels and the regulation of intracellular pH (Ulaszewski *et al.*, 1983, 1987; Serrano *et al.*, 1986; Eraso *et al.*, 1987; Eraso & Gancedo, 1987). One of the effects of heat-shock stress, at least in *S. cerevisiae* (Weitzel *et al.*, 1987), is a dissipation of this H^+ gradient. The resultant cytoplasmic acidification should be counteracted by plasma-membrane ATPase action, even though this enzyme has not previously been considered a possible influence on the tolerances or responses of fungi and plants to heat shock.

The plasma-membrane ATPases of *Neurospora crassa*, *S. cerevisiae* and *Sch. pombe* all display extensive sequence homology, as well as similar hydrophobicity profiles, thought to indicate 8–10 transmembrane domains (Aaronson *et al.*, 1988). Plasma-membrane ATPases from flowering plants also have up to 36% amino acid sequence homology with these fungal enzymes (Harper *et al.*, 1989; Pardo & Serrano, 1989; Boutry *et al.*, 1989). This ATPase is so conserved in structure and function that should it be shown to influence stress tolerances in one organism, there would be grounds to suspect that its action might also determine tolerance levels in diverse fungi and plants.

Methods

Strains. The *S. cerevisiae pma1.1*, *pma1.2*, *pma1.3* and *pma1.4* mutants (strains MG2129, MG2130, MG2131 and MG2132 respectively), their PMA1$^+$ prototrophic parent ε1278b (Ulaszewski *et al.*, 1983), the *Sch. pombe* strain Jv66 (h$^-$ *pma1.1*, *ade-413*) and its parent strain 972 (h$^-$ *ade-413*) (Ulaszewski *et al.*, 1986) were all kindly provided by A. Goffeau (University of Louvain, Belgium).

Media. All tolerance experiments were done on cells that had been maintained in exponential growth at 25 °C on YEPD medium (2% bactopeptone, 1% yeast extract, 2% glucose, all w/v) for more than 50 generations. Just prior to each experiment, *S. cerevisiae* cultures were briefly sonicated, just sufficiently for no cell aggregates to be seen by light microscopy. With the *Sch. pombe* cultures this sonication step was unnecessary.

Determinaton of killing kinetics. Thermotolerance experiments were done on exponential (0·5–1 × 10^7 cells ml^{-1}) YEPD cultures. The non-acute heat-shock treatment used for induction of thermotolerance was a rapid shift of part of each culture (the 'induced' cells) from 25 °C to 38 °C, maintaining cultures at 38 °C for 40 min prior to an immediate shift to 52 °C for a variable period. An identical portion of each culture (the cells 'uninduced' for thermotolerance) was immediately transferred from 25 °C to 48 °C for variable times, killing of these cells at temperatures higher than 48 °C being too rapid for accurate measurement. At intervals from 0 to 15 min, while cultures were at 48 °C or 52 °C, aliquots were rapidly diluted into 5 ml YEPD at room temperature (21–23 °C) and cells plated on YEPD plates within 20 min of the exposure to high temperature.

For measurements of ultraviolet killing, cells were diluted appropriately in YEPD and 0·15 ml aliquots spread on YEPD plates to give 300 cells per plate. Immediately after plating, the cells were exposed to an ultraviolet light source for various times.

To measure tolerance of high ethanol or salt concentrations, cells in exponential growth at 25 °C on YEPD (0·5–1 × 10^7 cells ml^{-1}) were diluted appropriately in YEPD at 25 °C and additions made of either absolute ethanol or 4 M-NaCl (to final concentrations of 12·5%, v/v, and 2·5 M respectively). Aliquots of 0·1 ml were removed immediately (for the zero time point) and at subsequent intervals, these being immediately diluted 100- to 1000-fold in YEPD prior to plating on YEPD plates.

In all of these experiments, killing was measured from the colonies (including petites) on YEPD plates incubated at 28 °C. Each experiment was repeated at least five times, with similar results, each survival shown in Figs. 1, 2 and 4 being the results from a representative experiment. Since each time point involved the counting of at least 300 colonies the major error in these viability measurements was in the preparation of serial dilutions. This error was estimated as no more than 15%.

Pulse labelling of S. cerevisiae proteins during heat shock. From 1 × 10^8 to 2 × 10^8 cells from exponential YEPD cultures of *S. cerevisiae* ε1278b and MG2129 were collected by centrifugation (3000 *g* for 5 min), resuspended in 20 ml CMD medium at 25 °C (2% glucose, 0·67% yeast nitrogen base without amino acids), recentrifuged, resuspended in 20 ml CMD medium and incubated for 20 min at 25 °C prior to heat shock. During this 20 min period the cultures were subdivided into 1 ml aliquots in glass tubes. To heat shock the cells, aliquots were shifted to either 38 °C (Fig. 3*a*) or 42 °C (Fig. 3*b*) and, at variable times after this temperature upshift, were labelled with [^{35}S]methionine (10 μCi ml^{-1}; 370 kBq ml^{-1}). Each pulse-labelling was for 15 min. A control 1 ml aliquot was pulse-labelled for 1 h at 25 °C without any heat shock. The cells from each labelling were rapidly chilled, then used in the preparation of samples of total cell protein which were analysed on one dimensional 12·5% polyacrylamide gels as described by Piper *et al.* (1986).

Results

Influence of the pma1.1 mutations of S. cerevisiae and Sch. pombe on thermotolerance

When *S. cerevisiae* or *Sch. pombe* cells in exponential growth at 25 °C are placed at 48 °C they rapidly lose viability. Neither organism can synthesize HSPs above 42–43 °C (see Discussion). In both species, *pma1.1* caused higher thermotolerance levels at 48 °C, as manifested by the slightly greater survival of mutant as compared to wild-type cells in Figs 1(*a*) and 2(*a*). Similar results were obtained with *S. cerevisiae pma1.2*, *pma1.3* and *pma1.4* (data not shown). If, prior to exposure to lethal temperatures, the *S. cerevisiae* or *Sch. pombe*

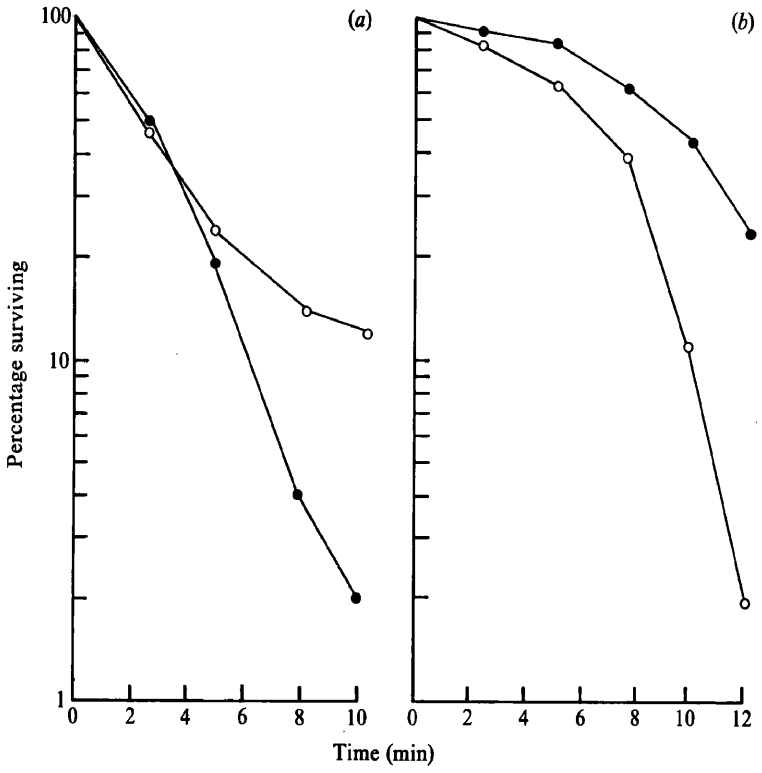


Fig. 1. High temperature tolerance of the *S. cerevisiae pma1.1* mutant (strain MG2129) (○) and its wild-type parent (ε1278b) (●). Cultures were either (*a*) uninduced for thermotolerance, being shifted to 48 °C from 25 °C; or (*b*) had their thermotolerance elevated by prior heat shock from 25 °C to 38 °C for 40 min before being shifted to 52 °C.

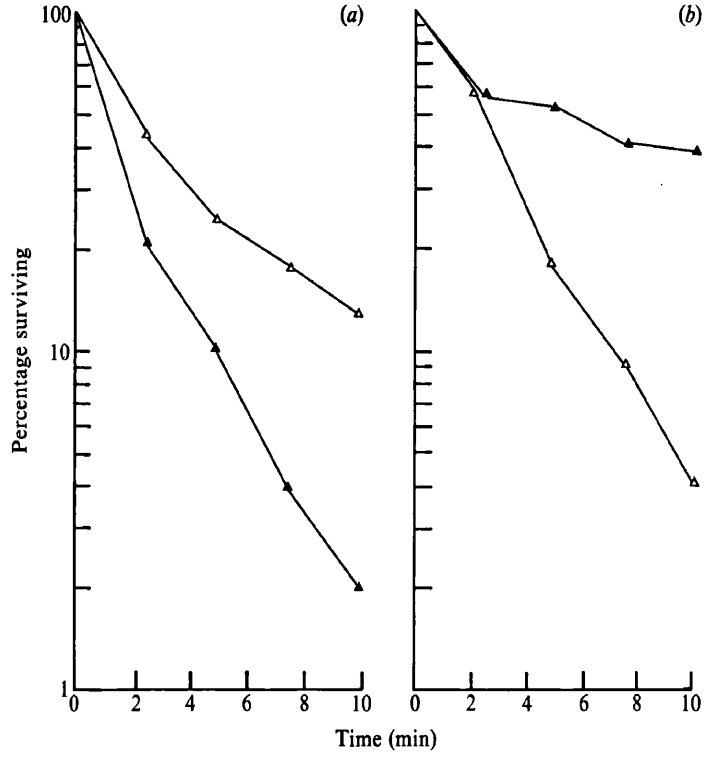


Fig. 2. High temperature tolerance of the *Sch. pombe pma1.1* mutant (strain JV66) (△) and its wild-type parent (972) (▲). Cultures were either (*a*) uninduced for thermotolerance, being shifted to 48 °C from 25 °C; or (*b*) had their thermotolerance elevated by prior heat shock from 25 °C to 38 °C for 40 min before being shifted to 52 °C.

cultures were given a non-acute heat shock (25–38 °C temperature upshift then a 40 min incubation at 38 °C) this effect of *pma1.1* on thermotolerance was reversed (Figs 1*b* and 2*b*). This effect was also demonstrated with *S. cerevisiae pma1.2*, *pma1.3* and *pma1.4* (not shown). The non-acute heat shock to 38 °C enables the induction of HSPs, associated with a marked elevation of thermotolerance (see Introduction). In both *S. cerevisiae* and *Sch. pombe* cells induced for this heat-shock response, *pma1.1* reduced viability at high temperature. Comparison of Fig. 1(*a*) with Fig. 1(*b*), and of Fig. 2(*a*) with Fig. 2(*b*), reveals that *pma1.1* reduces the increase in thermotolerance as the heat-shock response is induced by a 25–38 °C upshift.

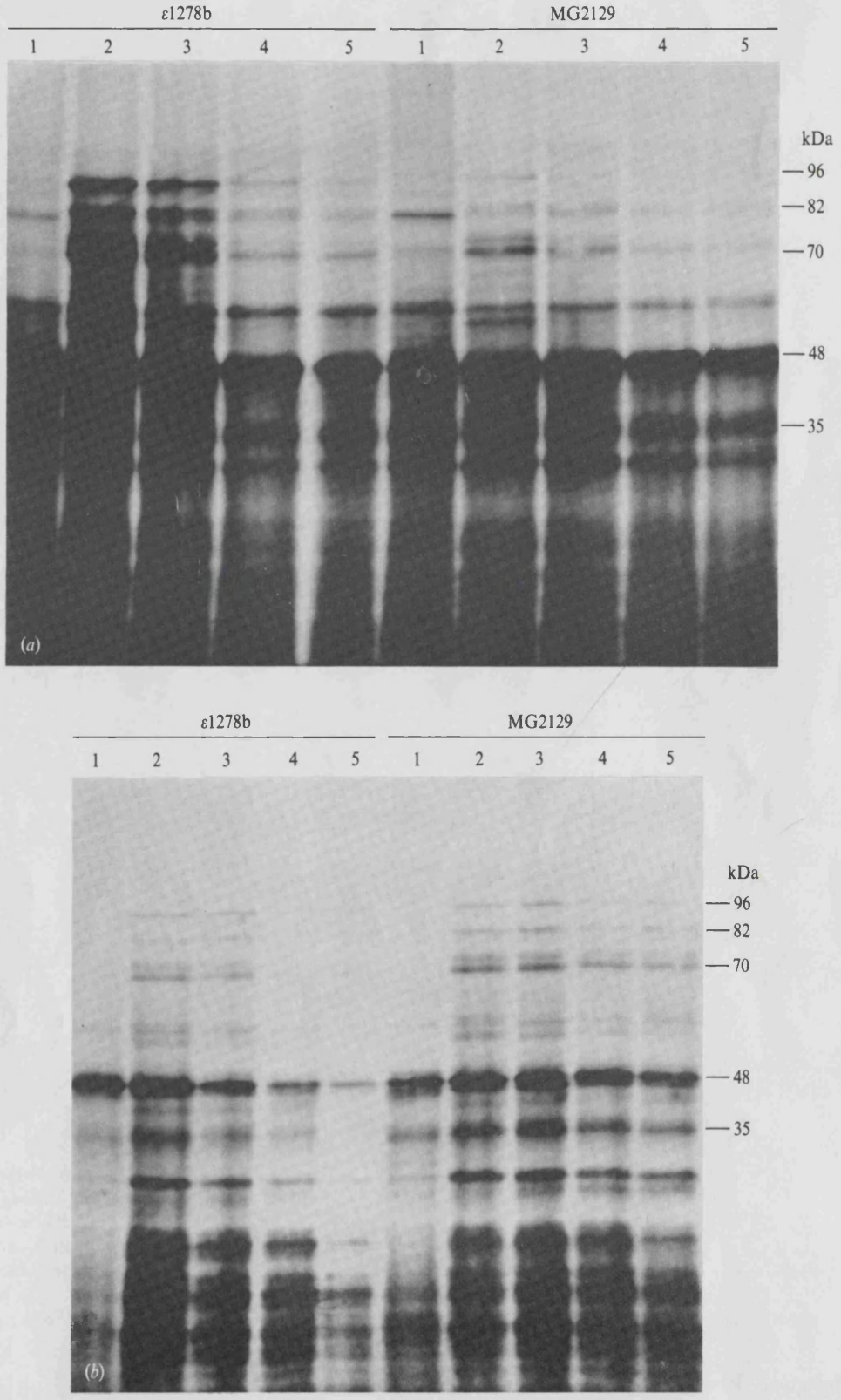


Fig. 3. Proteins pulse-labelled in heat-shocked cells of *S. cerevisiae* ε1278b and *pma1.1* mutant MG2129 after heat shock either to 38 °C (*a*); or to 42 °C (*b*). Cells were pulse-labelled with [^{35}S]methionine 10–25 min (2), 25–40 min (3), 40–55 min (4), or 55–70 min (5) after temperature upshift. Track 1 shows proteins labelled in unstressed cells at 25 °C. All gel samples contained protein from the same number of cells so the relative intensity of bands on the autoradiographs indicates relative protein labelling under these conditions. The indicated bands of 70, 82 and 96 kDa are the large heat-shock proteins HSP70, HSP82 and HSP96.

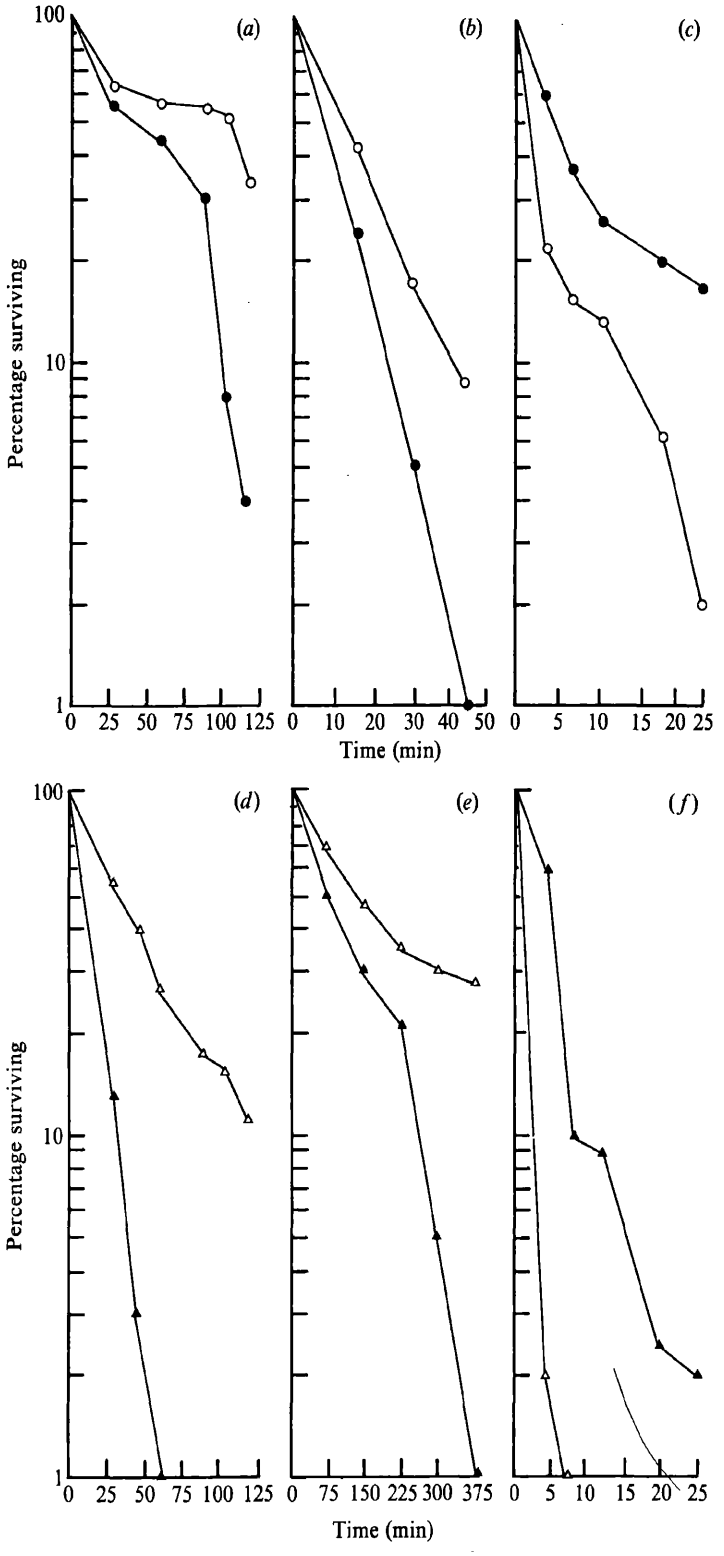


Fig. 4. Survival of yeast exposed to (*a*, *d*) 12·5% (v/v) ethanol; (*b*, *e*) 2·5 M-NaCl; and (*c*, *f*) ultraviolet irradiation. (*a–c*) Survival data for *S. cerevisiae* ε1278b (●) and *pma1.1* mutant MG2129 (○); (*d-f*) data for *Sch. pombe* 972 (▲) and *pma1.1* mutant JV66 (△).

Influence of pma1.1 on HSP synthesis in S. cerevisiae

S. cerevisiae ε1278b and MG2129 were labelled with [^{35}S]methionine both before, and at intervals after, a 25–38 °C heat shock. Their labelled proteins were then separated on a one-dimensional gel (Fig. 3*a*). After this relatively mild heat shock (38 °C is below the maximum growth temperature on glucose media), cells resumed an almost normal pattern of protein synthesis within about 1 h (Fig. 3*a*). However, synthesis of the major HSPs was dramatically reduced in the *pma1.1* mutant strain. Therefore a partial suppression of the heat-shock

response in mutant, compared to wild-type, cells is the probable reason for the lowered induction of thermotolerance by 25–38 °C heat shock in *pma1.1 S. cerevisiae* (Fig. 1).

When this labelling was repeated under conditions of considerably more severe (25–42 °C) heat shock a rather different result was obtained (Fig. 3*b*). The temperature of 42 °C is about 3 °C above the maximum for growth, and close to the maximum temperature at which *S. cerevisiae* will still display synthesis of HSPs (Piper *et al.*, 1986, 1988). Normally, after cells are shifted to 42 °C, an initial 'burst' of HSP synthesis is followed by a progressive cessation of all protein synthesis over about 1 h. This is apparent from the labelling of *S. cerevisiae* ε1278b in Fig. 3(*b*). HSP synthesis in the *pma1.1* mutant at 42 °C, although initially similar to that of wild-type cells, was not subject to such rapid inhibition and was sustained for at least 70 min at 42 °C (Fig. 3*b*).

These experiments indicate that, depending on the severity of the heat shock, *pma1.1* can affect either the extent or the duration of HSP synthesis in *S. cerevisiae*. They were not repeated on *Sch. pombe* strains JV66 and 972 because growth of these strains in minimal medium is severely restricted by their adenine auxotrophy even in the presence of exogenous adenine (Ulaszewski *et al.*, 1987).

Influence of pma1.1 on ethanol tolerance, osmotolerance and tolerance of ultraviolet irradiation

The *pma1.1* mutations of *S. cerevisiae* and *Sch. pombe* increased tolerance of brief exposures to high ethanol and high salt concentrations, and decreased tolerance of ultraviolet irradiation (Fig. 4). Similar results were obtained with *S. cerevisiae pma1.2*, *pma1.3* and *pma1.4* (data not shown).

Discussion

S. cerevisiae pma1.1, *pma1.2*, *pma1.3* and *pma1.4*, and *Sch. pombe pma1.1*, were isolated as spontaneous mutants showing resistance to Dio-9, a non-specific inhibitor of plasma-membrane ATPase (Ulaszewski *et al.*, 1983, 1986). They exhibit several similar changes in the *in vitro* properties of plasma-membrane ATPase, which is why they were investigated in parallel in this study. These changes include: (i) a lower specific activity; (ii) a modified K_m for MgATP; and (iii) strong resistance to vanadate (Ulaszewski *et al.*, 1983, 1987). The *Sch. pombe pma1.1* mutation corresponds to a single amino acid substitution in the ATPase, namely Gly to Asp at residue 268 (Ghislain *et al.*, 1987).

These mutants were used to obtain evidence as to whether plasma-membrane ATPase action exerts a major influence on tolerances to elevated temperatures and other forms of stress. The fact that mutation to Dio-9 resistance causes identical changes to several tolerances in two very distantly related species shows that the profound metabolic changes that ensue from alteration to plasma-membrane ATPase activity are an important determinant of these tolerance levels. Also, protein pulse-labelling in *S. cerevisiae* indicated that *pma1.1* can affect the extent and duration of HSP synthesis induced by the heat-shock response.

When *S. cerevisiae* or *Sch. pombe* cells actively growing at 25 °C are shifted to 48 °C there is rapid protein synthesis arrest; no HSPs are synthesized and these cells, uninduced for thermotolerance, quickly die. Pulse-labelling studies have shown that neither yeast can synthesize HPSs above 42–43 °C (Piper *et al.*, 1986; also unpublished observations). At 48 °C *pma1.1* conferred higher thermotolerance (Figs 1*a* and 2*a*). This result was not entirely unexpected for *S. cerevisiae*, since *pma1.1* causes slightly slower growth of prototrophic strains on YEPD, and thermotolerance levels are generally thought to be inversely related to the growth rate of this organism (Iida & Yahara, 1984; Iida, 1988). Figs 1(*b*) and 2(*b*) show survival of the same *S. cerevisiae* and *Sch. pombe* cultures after they had been shifted from 25 °C to 38 °C for 40 min and then exposed to a considerably higher temperature (52 °C). This non-acute 38 °C heat shock induces the synthesis of heat shock proteins, and causes a marked elevation of thermotolerance (for evidence of this in *S. cerevisiae* see Piper *et al.*, 1986, 1987, 1988). However, the increase in this thermotolerance was less for *pma1.1* than for wild-type cells given the same heat shock, in both *S. cerevisiae* and *Sch. pombe* (Figs 1 and 2). As a result, *pma1.1* cells were killed more rapidly in such cultures induced for thermotolerance, the converse of the situation found in the uninduced cultures. Both this observation and the reduction in HSP synthesis (Fig. 3*a*) are consistent with *pma1.1* causing a partial suppression of the heat-shock response normally caused by a 25–38 °C temperature shift. Important considerations here may be the extent of cytoplasmic acidification that accompanies heat shock (Weitzel *et al.*, 1987) and the rate at which it is counteracted by plasma-membrane ATPase action. Since the *pma1.1* mutant of *Sch. pombe* maintains a smaller transmembrane H^+ gradient than wild-type *Sch. pombe*, with a cytoplasmic pH 0·4 units lower (Ulaszewski *et al.*, 1987), there are grounds for expecting smaller decreases in cytoplasmic pH with heat-shock stress in this mutant. This may in turn lead to a reduced heat-shock response. The thermal stability of the ATPase must also be an important factor. Although the wild-type enzyme is the more active under most assay

conditions, *in vitro* studies have shown the *S. cerevisiae pma1.1* enzyme to be less prone to inactivation at 50 °C (Ulaszewski *et al.*, 1983).

The *pma1.1* mutants of *S. cerevisiae* and *Sch. pombe* exhibit greater tolerances of ethanol and high salt than the wild-type (Fig. 4). Low cyclic AMP levels in *S. cerevisiae* also cause higher ethanol tolerance (Iida, 1988). Ethanol diffuses rapidly into *S. cerevisiae* cells (Guijarro & Lagunas, 1984) and affects membrane order, as shown by Walker-Caprioglio & Parks (1987), using the lipophilic probe 1,6-diphenyl-1,3,5 hexatriene. This change in membrane order causes enhanced proton influx (Leao & VanUden, 1985), and ethanol therefore has marked effects on the maintenance of cytoplasmic pH and H^+-coupled transport of solutes across the plasma membrane. In contrast to ethanol tolerance, osmotolerance may primarily reflect intracellular levels of osmoregulatory solutes. Fungi that grow in media of high osmotic strength synthesize polyols (e.g. glycerol, arabitol) at high levels; these increase osmotolerance by functioning as compatible solutes. Their levels change with growth state and are major determinants of osmoregulation. Glycerol, probably the most important osmotic effector in exponentially-growing salt-stressed yeast (Reed *et al.*, 1987), is made at higher levels in yeast exposed to hypertonic media (Maiorella *et al.*, 1984).

The reduction in plasma-membrane ATPase activity due to *pma1.1* causes a slight reduction in growth rate on rich medium and a considerable reduction in growth on minimal medium containing ammonia as nitrogen source. Amino-acid and purine auxotrophies reduce the growth rate still further (Ulaszewski *et al.*, 1987). Physiological changes due to adaptation of *pma1.1* cells to slower growth presumably cause some of the tolerance effects observed in this study, but may not be the only cause of the observed differences. Thus *pma1.1* might even allow higher plasma-membrane ATPase activity under high-ethanol or dehydrating conditions, just as it renders this enzyme less susceptible to vanadate inhibition (Ulaszewski *et al.*, 1983). The available evidence highlights the need to relate tolerance measurements to other physiological studies (e.g. those measuring intracellular pH shifts under stress conditions) and *in vitro* studies of plasma-membrane ATPase action. However, the results with *pma1.1* mutant strains described in this paper show that plasma-membrane ATPase action cannot be ignored as a factor affecting tolerances of a variety of potentially cytotoxic agents (Figs 1, 2 and 4) and as a determinant of the extent and duration of at least one inducible stress response (Fig. 3).

We thank A. Goffeau for providing strains. This work was supported by the Central Research Fund of London University, and the award of a SERC Studentship to B.P.

References

AARONSON, L. R., HAGER, K. M., DAVENPORT, J. W., MANDALA, S. M., CHANG, A., SPEICHER, D. W. & SLAYMAN, C. W. (1988). Biosynthesis of the plasma membrane H^+-ATPase of *Neurospora crassa*. *Journal of Biological Chemistry* **263**, 14552–14558.

ANANTHAN, J., GOLDBERG, A. L. & VOELLMY, R. (1986). Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. *Science* **232**, 522–524.

BOUCHERIE, H. (1985). Protein synthesis during transition and stationary phase under glucose limitation in *Saccharomyces cerevisiae*. *Journal of Bacteriology* **161**, 385–392.

BOUTRY, M., MICHELET, B. & GOFFEAU, A. (1989). Molecular cloning of a family of plant genes encoding a protein homologous to plasma membrane H^+-translocating ATPase. *Biochemical and Biophysical Research Communications* **162**, 567–574.

CRAIG, E. A. (1986). The heat shock response. CRC *Critical Reviews in Biochemistry* **18**, 239–280.

CURRAN, B., DAVIES, M. W., HIRST, K., SEWARD, K. & PIPER, P. W. (1988). Genes transcribed efficiently both before and after heat shock: their promoter operation and roles in stress protection. *Yeast* **4**, S324.

ERASO, P. & GANCEDO, C. (1987). Activation of yeast plasma membrane ATPase by acid pH during growth. *FEBS Letters* **224**, 187–192.

ERASO P., MAZÓN, M. J. & GANCEDO, J. M. (1987). Internal acidification and cAMP increase are not correlated in *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **165**, 671–674.

GHISLAIN, M., SCHLESSER, A. & GOFFEAU, A. (1987). Mutation of a conserved glycine residue modifies the vanadate sensitivity of the plasma membrane H^+-ATPase from *Schizosaccharomyces pombe*. *Journal of Biological Chemistry* **262**, 17549–17555.

GUIJARRO, J. M. & LAGUNAS, R. (1984). *Saccharomyces* does not accumulate ethanol against a concentration gradient. *Journal of Bacteriology* **160**, 874–878.

HARPER, J. F., SUROWY, T. K. & SUSSMAN, M. R. (1989). Molecular cloning and sequence of cDNA encoding the plasma membrane proton pump (H^+-ATPase) of *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 1234–1238.

IIDA, H. (1988). Multistress resistance of *Saccharomyces cerevisiae* is generated by insertion of retrotransposon Ty into the 5′ noncoding region of the adenylate cyclase gene. *Molecular and Cellular Biology* **8**, 5555–5560.

IIDA, H. & YAHARA, Y. (1984). Durable synthesis of high molecular weight heat shock proteins in G_0 cells of yeast and other eukaryotes. *Journal of Cell Biology* **99**, 199–207.

LEAO, C. & VANUDEN, N. (1985). Effects of ethanol and other alkanols on the temperature relations of glucose transport and fermentation in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology* **22**, 359–363.

LINDQUIST, S. & CRAIG, E. A. (1988). The heat shock proteins. *Annual Review of Genetics* **55**, 1151–1191.

MAIORELLA, B. L., BLANCH, H. W. & WILKE, C. R. (1984). Feed component inhibition in ethanolic fermentation by *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **26**, 1155–1166.

PARDO, J. M. & SERRANO, R. (1989). Structure of a plasma membrane H^+-ATPase gene from the plant *Arabidopsis thaliana*. *Journal of Biological Chemistry* **264**, 8557–8562.

PELHAM, H. R. B. (1987). Heat shock proteins – coming in from the cold. *Nature, London* **332**, 776–777.

PIPER, P. W., CURRAN, B., DAVIES, M. W., LOCKHEART, A. & REID, G. (1986). Transcription of the phosphoglycerate kinase gene of *Saccharomyces cerevisiae* increases when fermentative cultures are stressed by heat shock. *European Journal of Biochemistry* **161**, 525–531.

PIPER, P. W., DAVIES, M. W., CURRAN, B., LOCKHEART, A., SPALDING, A. & TUITE, M. F. (1987). The influence of cell ploidy on the thermotolerance of *Saccharomyces cerevisiae*. *Current Genetics* **11**, 595–598.

PIPER, P. W., CURRAN, B., DAVIES, M. W., HIRST, K., LOCKHEART A. & SEWARD, K. (1988). Catabolite control of the elevation of PGK

mRNA levels by heat shock in *Saccharomyces cerevisiae*. *Molecular Microbiology* **2**, 353–361.

REED, R. H., CHUDEK, J. A., FOSTER, R. & GADD, G. M. (1987). Osmotic significance of glycerol accumulation in exponentially growing yeasts. *Applied and Environmental Microbiology* **53**, 2119–2123.

SERRANO, R., KIELLAND-BRANDT, M. C. & FINK, G. R. (1986). Yeast plasma membrane ATPase is essential for growth and has homology with ($Na^+ + K^+$), K^+- and Ca^{2+}-ATPases. *Nature, London* **319**, 689–693.

SHIN, D.-Y., MATSUMOTO, K., IIDA, H., UNO, I. & ISHIKAWA T. (1987). Heat shock response of *Saccharomyces cerevisiae* mutants altered in cyclic AMP-dependent protein phosphorylation. *Molecular and Cellular Biology* **7**, 244–250.

ULASZEWSKI, S., GRENSON M. & GOFFEAU, A. (1983). Modified plasma membrane ATPase in mutants of *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **130**, 235–239.

ULASZEWSKI, S., CODDINGTON, A. & GOFFEAU, A. (1986). A new mutation for multiple drug resistance and modified plasma membrane ATPase activity in *Schizosaccharomyces pombe*. *Current Genetics* **10**, 359–364.

ULASZEWSKI, S., VAN HERCK, J. C., DUFOUR, J. P., KULPA, J., NIEUWENHUIS, B. & GOFFEAU, A. (1987). A single mutation confers vanadate resistance to the plasma membrane H^+-ATPase from the yeast *Schizosaccharomyces pombe*. *Journal of Biological Chemistry* **262**, 223–228.

WALKER-CAPRIOGLIO, H. M. & PARKS, L. W. (1987). Autoconditioning factor relieves ethanol-induced growth inhibition of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* **53**, 33–35.

WEITZEL, G., PILATUS, U. & RENSING, L. (1987). The cytoplasmic pH, ATP content and total protein synthesis rate during heat-shock protein inducing treatments in yeasts. *Experimental Cell Research* **170**, 64–79.