A Study of Coenzyme A Metabolism and Function in Mammalian Cells

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Declaration

I, Pascale Monteil, declare that all the work presented in this thesis is the result of my own work. The work presented here does not constitute part of any other thesis. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. The work herein was carried out while I was a graduate research student at University College London Research Department of Structural and Molecular Biology under the supervision of Professor Ivan Gout. Mass Spectrometry work was carried out by N. Totty, Cancer Research UK.

Pascale Monteil

Abstract

CoA is well established as a metabolic cofactor in numerous oxidative and biosynthetic pathways. The levels of CoA usually remain within a tight range, however these have been shown to change in response to nutritional state, fibrate drugs and several pathological conditions. Although the mechanisms that alter CoA levels are not fully understood, the fluctuations in CoA can influence the cellular processes it regulates. The regulatory roles of CoA have mainly been studied in the context of feedback/ feed forward regulation of metabolic pathways or enzymes, yet very little is known about its role as a regulator of cellular function. In addition to its action as a cofactor, a few reports have indicated that CoA binds to and regulates proteins through allosteric and covalent modification, suggesting wider regulatory roles for CoA. The aim of this thesis was therefore to gain a greater understanding of the function of CoA as a regulator of cellular processes.

Initially, conditions for extracting and measuring CoA levels in cultured cells were developed and optimised. CoA levels were much lower in cultured HepG2 cells compared to liver and did not respond in the same way when treated with extracellular stimuli. A 2-fold increase in CoA levels, through over-expression of the enzyme PanK1β, did result in the novel finding that CoA has a negative effect on cell growth. This was accompanied by increased acetylation of a large number of proteins and also an increase in lactate production. In addition, affinity purification with CoA Sepharose, followed by mass spectrometry, pulled down several proteins, previously unknown to bind to CoA, which regulate a wide range of cellular processes. Together, the data from this thesis has provided a greater insight into the wider role of CoA as a regulator of cellular function.

Dedication

There are a number of people to whom I am greatly indebted for the completion of this thesis.

My parents, who gave me the educational platform that provided me with countless opportunities, enabling me to pursue my dreams.

My supervisor, Professor Ivan Gout, whose passion for science is truly inspiring, for offering me this PhD position, as well as his advice and expertise.

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Table of Contents

Declaration 2

Abstract 3

Dedication 4

List of Figures 13

Abbreviations 16

Chapter 1: 20

1.1 General Introduction 21

1.2 Coenzyme A 21

1.2.1 Coenzymes 21

1.2.2 Coenzyme A 21

1.2.3 CoA structure 23

1.2.4 CoA derivatives 23

1.3 Cellular Functions of CoA 25

1.3.1 Overview of CoA in metabolism 25

1.3.2 The role of CoA in energy generation 25

1.3.3 The role of CoA in Fatty Acid Oxidation 26

1.3.4 The role of CoA in Biosynthetic pathways 29

1.4 Regulation of Proteins and Cellular processes by CoA and CoA thioesters 33

1.4.1 The role of CoA in the regulation of cellular processes 33

1.4.2 Availability as a cofactor or substrate 33

1.4.3 Regulation through non-covalent interaction 34

1.4.3.1 Balance between CoASH/CoA thioesters 34

1.4.3.2 Allosteric and other non-covalent interactions 36

1.4.4 Regulation through covalent modifications 40

1.5 Control of CoA levels 43

1.5.1 CoA Biosynthetic Pathway 43

1.5.2 Regulatory enzymes in CoA Biosynthesis: Pantothenate Kinase 46

1.5.3 Regulatory enzymes in CoA Biosynthesis: CoA Synthase 48

1.5.4 Degradation of CoA 49

1.5.5 Conversion of CoASH to other esters 50

1.5.6 Compartmentalisation of CoA 50

1.6 CoA homeostasis 51

1.6.1 Basal levels of CoA 51

1.6.2 Physiological changes in CoA levels 52

1.6.3 Pathological conditions which change CoA levels 52

1.7 Aim of thesis 54

Chapter 2: 56

Materials and Methods 56

2.1 Materials 57

2.1.1 Common chemicals and Reagents 57

2.1.2 Antibodies and sera 57

2.1.3 Mammalian cells 57

2.1.4 Plasmids and Primers 58

2.2 Nucleic Acid Manipulation 58
2.2.1 Polymerise Chain Reaction 58
2.2.1.1 Primer Design 58
2.2.2 DNA Digestion using Specific Restriction Endonucleases 59
2.2.3 DNA Electropheresis and Purification of DNA Fragments 59
2.2.4 DNA Ligation 60
2.2.5 Transformation and Growth of Bacteria 60
2.2.5.1 Preparing Chemical Competent Cells 61
2.2.6 Purification of Plasmid DNA 61
2.3 Mammalian Cell Culture and Methodology 62
2.3.1 Maintenance of Cell lines 62
2.3.2 Freezing Cell lines 62
2.3.3 Defrosting Cell lines 63
2.3.4 Cell counting 63
2.3.5 Inducible gene expression in cell lines 63
2.3.6 Transient transfection 64
2.3.7 Generation of Stable cell lines 64
2.3.8 Serum deprivation and stimulation of cells 65
2.3.9 Treatment of Cells with Extracellular Stimuli 65
2.3.10 Growth Assay 66
2.4 CoA Isolation 66
2.4.1 Harvesting Mammalian cells 66

2.4.2 CoA extraction 67

2.4.3 Alkaline Hydrolysis 67

2.4.4 *N*-ethylmaleimide treatment 68

2.5 CoA analysis 68

2.5.1 Recycling Assay 68

2.5.2 HPLC 70

2.5.2.1 Sample preparation 70

2.5.2.2 Standard preparation 71

2.6 CoA binding Partners 71

2.6.1 CoA Sepharose preparation 71

2.6.2 Determination of coupling efficiency by Ellman's test 72

2.6.3 Preparation of Rat Liver homogenates 72

2.6.4 Preparation of mammalian cell lysates 73

2.6.5 Affinity binding with CoA Sepharose 73

2.7 Isolation and Analysis of Cellular Protein 74

2.7.1 Preparation of mammalian cell lysates 74

2.7.2 Determination of protein concentration in solution 75

2.7.3 SDS-PAGE 75

2.7.3.1 Protein sample preparation 75

2.7.3.2 Gel Electropheresis 76

2.7.4 Visualization of proteins 76

2.7.4.1 Coomassie Stain 76

2.7.4.2 Silver staining 76

2.7.5 Immunoblot analysis (Western Blot) 77

2.7.5.1 Transfer to Nitrocellulose membrane 77

2.7.5.2 Immunoblotting of transferred membranes 77

2.7.5.3 Developing Immunoblots 78

2.7.6 Stripping and re-probing western blots 78

2.7.7 LDH assay 78

2.7.8 G-6-PDH assay 79

2.8 Metabolic Studies 79

2.9 Statistical Analysis 80

Chapter 3: Results 81

3.1 Introduction 82

3.2 Detection Methods 84

3.2.1 Recycling Assay 85

3.2.1.1 Reaction time 85

3.2.1.2 Measuring CoASH and acetyl CoA separately 88

3.2.2 HPLC 88

3.2.3 Mass Spectrometry 97

3.3 Sample preparation 97

3.3.1 Deproteinisation 99

3.3.2 Preserving CoA esters 99

3.3.2.1 The effect of pH on the recycling assay 99

3.3.2.2 Compatibility with the Recycling Assay 102

3.3.3 Concentration 102

3.3.4 Reducing Agent 105

3.4 Standardisation 105

3.5 Percentage recovery 107

Chapter 4: Results 110

4.1 Introduction 111

4.2 Results 113

4.2.1 Levels of CoA in Tissues and Cultured cells 113

4.2.2 Characterisation of CoA levels under different conditions in cultured cells 121

4.2.2.1 The effect of FBS 121

4.2.2.2 The effect of Glucose and Insulin 122

4.2.2.3 The effect of Fatty Acids 124

4.2.3 The effect of signalling pathway inhibitors 127

4.2.3.1 Regulation of CoA levels via the mTOR pathway 127

4.2.3.2 Regulation of CoA levels via the PI3K pathway 130

4.2.3.3 Regulation of CoA levels via the MAPK pathway 130

4.2.4 Manipulation of cellular CoA levels to identify cellular functions affected by changes in CoA 130

4.2.4.1 Regulation of CoA levels by Bezafibrate 130

4.2.4.2 Overexpression of CoASy 132

4.2.4.3 The effect of Hopantenate on CoA levels 135

4.2.4.4 The effect of Pantethine on CoA levels 137

4.2.4.5 Delivery of CoA using liposomes and cell penetrating peptides 137

4.2.5 Generation of a stable cell lines over-expressing PanK1β and its characterisation 138

4.2.5.1 Generation of Hek293 stably expressing PanK1β 138

4.2.5.2 Analysis of CoA levels in Hek293 over-expressing PanK1β 140

4.2.5.3 The effect of PanK1β over-expression on cell growth 140

4.2.5.4 The effect of PanK1β over-expression on signalling pathways involved in cell growth 145

4.2.5.5 The effect of PanK1β over-expression on metabolic pathways 148

4.2.5.6 The effect of PanK1β over-expression on protein acetylation 151

4.3 Discussion 153

Chapter 5: Results 158

5.1 Introduction 159

5.2 Results 162

5.2.1 Coupling of CoA to Cyanogen Bromide Activated Sepharose 162

5.2.1.1 Chemistry of coupling CoA to Sepharose 162

5.2.1.2 Binding and orientation to CoA Sepharose beads 162

5.2.1.3 Do proteins that bind to CoA Sepharose bind specifically to CoA? 165

5.2.2 Affinity Purification and SDS PAGE Analysis 167

5.2.3 Identification of CoA binding proteins 171

5.2.4 Functional Studies 178

5.3 Discussion 182

Chapter 6: Discussion.....185

References 192

List of Figures

Table 1.1: Common coenzymes 22

Figure 1.1: The structure of CoA 24

Figure 1.2: The structure of the different forms of CoA. 24

Figure 1.3: Acetyl CoA is a central metabolite in metabolism. 27

Figure 1.4: Acetyl CoA is essential for progress in the citric acid cycle. 28

Figure 1.5: The role of CoA in Fatty acid oxiation 30

Figure 1.6: Fatty Acid synthesis 32

Figure 1.7: Summary of molecular mechanisms regulating PDK2 activity.. 35

Figure 1.8: CoA Biosynthetic Pathway 45

Figure 2.1: PCR primers for PanK1β. 59

Table 2.1: Components of the recycling assay 69

Table 2.2: Standard preparation for HPLC 71

Figure 3.1 Overview of CoA measurement in cultured cells. 86

Figure 3.2: Overview of the recycling assay 87

Figure 3.3: Determining the optimal assay time. 89

Table 3.1: Percentage recovery after NEM treatment of CoA standards. 90

Figure 3.4: CoA standard curve measured by the recycling assay. 90

Figure 3.5: Chromatogram of CoASH and CoA esters. 92

Figure 3.6: Standard curves of CoA and acetyl CoA measured by HPLC. 93

Figure 3.7: HPLC chromatograms of tissue and cultured cell PCA extracts. 94

Figure 3.8: Chromatogram of cultured cell extracts with internal standards 95

Figure 3.9: KOH treatment hydrolyses CoA thioesters to CoA in HepG2 cells.. 96

Figure 3.10: Mass spectrum of 100 pmol free CoASH standard 98

Figure 3.11: Hek293 mass spectrum.. 98

Figure 3.12: HPLC profiles of CoA/ acetyl CoA standards. 100

Figure 3.13: Stability of CoA/ acetyl CoA in neutralised PCA. 101

Figure 3.14: The effect of pH on the recycling assay 101

Figure 3.15: The effect of PCA cell extracts on the recycling assay 103

Figure 3.16: CoA levels in cultured cells collected by different methods.. 104

Figure 3.17: Lyophilisation affects % recovery of CoA and acetyl CoA.. 106

Figure 3.18: The effect of TCEP on the recycling assay 106

Figure 3.19: Methods of standardisation. 108

Figure 3.20: Percentage recovery of CoA and acetyl CoA in cell extracts is good..... 109

Table 4.1: Summary of CoA and acetyl CoA levels in tissues and cultured cells.. 114

Figure 4.1: Summary of Total CoA levels measured in tissue and cell extracts.. 118

Figure 4.2: The effect of FBS starvation and stimulation on CoA levels in cultured cells. 123

Figure 4.3: The effect of Glucose on CoA levels in HepG2 cells. 125

Figure 4.4: The effect of insulin on CoA levels.. 126

Figure 4.5: The effect of Fatty Acids on CoA levels in HepG2 cells.. 128

Figure 4.6: The effect of mTOR signalling on CoA levels.. 129

Figure 4.7: The effect of a PI3K signalling on CoA levels. 131

Figure 4.8: The effect of MAPK signalling on CoA levels.. 131

Figure 4.9: Bezafibrate increases CoA levels in HepG2 cells. 133

Figure 4.10: CoA levels in cells over-expressing CoA Synthase 134

Figure 4.11: The effect of Pantothenate and Hopan on CoA levels in HepG2 cells.. 136

Figure 4.12: Delivering CoA into HepG2 cells using cell penetrating peptides. 139

Figure 4.13: Molecular cloning of PanK1β. 141

Figure 4.14: Generating stable cell lines overexpressing PanK1β. 142

Figure 4.15: The level of CoA increases in cells over-expressing PanK1β. 143

Figure 4.16: HPLC profile of malonyl CoA peak in cells over-expressing PanK1β. 144

Figure 4.17: The effect of PanK1β over-expression on cell growth.. 146

Figure 4.18: The effect of PanK1β over-expression on various signalling proteins.. 147

Figure 4.19: Fatty acid oxidation in cells over-expressing PanK1β..... 149

Figure 4.20: Glucose oxidation in cells over-expressing PanK1β. 149

Figure 4.21: Lactate production in cells over-expressing PanK1β. 150

Figure 4.22: The effect of PanK1β over-expression on protein acetylation. 152

Figure 5.1: Strategy for the Analysis of CoA binding proteins. 161

Figure 5.2: Schematic of CoA coupling to cyanogen coated beads 163

Figure 5.3: The binding and orientation of CoA binding to CNBr activated Sepharose beads. 164

Figure 5.4: Proteins do not bind to CoA through its thiol group. 166

Figure 5.5: Proteins are specifically eluted from CoA beads by CoA. 168

Figure 5.6: Schematic showing the potential interactions between proteins and CoA. 169

Figure 5.7: Affinity purification of CoA binding partners 170

Figure 5.8: Affinity purification of CoA binding partners in rat liver. 172

Figure 5.9: Summary table of CoA-binding proteins. 173

Table 5.1: Full list of CoA binding proteins 174

Figure 5.10: Example MS-MS spectra of selcted CoA binding proteins. 177

Figure 5.11: CoA binding assays for specific enzymes. 180

Figure 5.12: The effect of CoA on G6PDH and LDH. 181

Abbreviations

4'PP	4' Phosphopantetheine
ACBP	Acyl CoA binding protein
ACC	Acetyl CoA carboxylase
ACL	ATP citrate lyase
ACP	Acyl Carrier Protein
ACS	Acyl CoA Synthase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocator
ATP	Adenosine triphosphate
BF	Bezafibrate
BSA	Bovine serum albumin
C	Carbon
CNBr	Cyanogen Bromide
CO_2	Carbon dioxide
CoA	Coenzyme A
CoASH	Unesterified Coenzyme A
CoASy	CoA Synthase
COS-7	CV1 (simian) Origin cells carrying SV40 genetic material
CPT1	Carnitine palmitoyl transferase 1
CPT2	Carnitine palmitoyl transferase 2
DAG	Diacylglycerol
DMEM	Dubecco's modified Eagle's medium

DMSO	Dimethyl sulphoxide
DPCK	Dephospho-CoA Kinase
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	Escherichia coli
EB	Extraction Buffer
ECL	Enhanced chemiluminescence
EDTA	Ethelenediaminetetraacetic acid
FA	Fatty Acid
$FADH_2$	Flavin adenine dinucleotide (reduced form)
FadR	Fatty acid metabolism regulator protein
FAS	Fatty Acid Synthase
FBS	Foetal bovine serum
GFP	Green fluorescent protein
GSH	Glutathione
HAT	Histone acetyltransferase
HCl	Hydrocloric acid
HDAC	Histone deacetyase
Hek293	Human embryonic kidney 293 cells
HepG2	Human hepatocellular carcinoma cells
HMG CoA	3-Hydroxy-3-methyl-glutaryl Coenzyme A
Hrs	Hours
IMM	Inner mitochondrial membrane
IP	Immunoprecipitation
IPP	3-Isopentenyl pyrophosphate

$K_{0.5}$	Substrate concentration at which the reaction rate is a half of V_{max}
K_i	Dissociation constant for an enzyme inhibitor
K_d	Dissociation constant
LB	Luria-Bertani Broth
MAPK	Mitogens-activated protein kinase
MCAD	Medium chain acyl-CoA dehydrogenase deficiency
MCD	Malonyl CoA decarboxylase
MEF	Mouse embryonic fibroblast
Min	Minute
MOM	Mitochondrial outer membrane
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NEM	*N*-ethylmaleimide
Nudt	nucleoside diphosphate linked to another moiety (X) hydrolasase
O_2	Oxygen
PAGE	Polyacrylamide gel electropheresis
PanK	Pantothenate Kinase
PBS	Phosphate-buffered saline
PC	Pyruvate carboxylase
PCA	Perchloric acid
PCR	Polymerase chain reaction

PDH	Pyruvate dehydrogenase
PDHK	Pyruvate dehydrogenase kinase
PDK1	Phosphoinositide-dependent kinase 1
PI3-K	Phosphatidylinositol 3-Kinase
PKAN	Pantothenate kinase-associated neurodegeneration
PKB	Protein Kinase B
PPAR	Peroxisome proliferator activated receptor
PPAT	Phosphopantetheine adenylyltransferase
PPCDC	Phosphopantothenoylcysteine decarboxylase
PPCS	Phosphopantothenoylcysteine synthase
RNA	Ribonucleic acid
RPM	Revolutions per minute
S6K	S6 Kinase
SDS	Sodium n-dodecyl sulphate
SH	Sulfhydryl
S	seconds
siRNA	Small interfering ribonucleic acid
SOC	Super Optimal broth with Catabolite repression
TAE	Tris Acetate EDTA
TAG	Triacylglycerol
TCEP	*Tris*(2-carboxyethyl)phosphine
U	Units
UV	Ultraviolet
V	Volts
V_{max}	Maximum rate of the system

Chapter 1:

General Introduction

Chapter 1: General Introduction

1.1 General Introduction

Amino acids, carbohydrates and lipids are essential for maintaining life in all organisms. Metabolic reactions either use these organic compounds as structural components to build cells and tissues, or to break them down for use as a source of energy. Most of these compounds require activation to Coenzyme A thioesters to participate in these metabolic reactions.

1.2 Coenzyme A

1.2.1 Coenzymes

Coenzymes are small, organic compounds that are often regarded as cofactors, which are non-protein compounds that bind to proteins in order to assist in biochemical transformations. They are often vitamins, or vitamin derivatives, and generally conjugate loosely with the enzyme's active site. Coenzymes which bind tightly are regarded as prosthetic groups of the enzyme. Coenzymes usually function as activated carriers, aiding in the transfer of electrons, specific atoms, or functional groups. *Table 1.1* gives an overview on some of the more common coenzymes (Friedman 1995; Palmer 1995). This thesis focuses on Coenzyme A (CoA).

1.2.2 Coenzyme A

The existence of CoA was first described by Fritz Lipmann in 1945, during his studies on acetyl transfer in mammals (Lipmann 1945). Lipmann noticed that

Coenzyme	Abbreviation	Vitamin	Entity transferred	Metabolic Role
Nicotine adenine dinucleotide (phosphate)	$NAD(P)^+$	Niacin	Electron (hydrogen atom)	Numerous oxidation-reduction reactions
Flavin Mononucleotide	FMN	Riboflavin (vitamin B_2)	Electron (hydrogen atom)	Oxidative deamination of amino acids, β-Oxidation of amino acids, Oxidative phosphorylation, Purine catabolism
Flavin-Adenine Dinucleotide	FAD			
Thiamine pyrophosphate	TPP	Thiamine (vitamin B_1)	aldehydes	Decarboxylation of α-keto acids and α-keto sugars, Transketolase reaction
Coenzyme A	CoA	Pantothenic Acid (vitamin B_5)	Activated acyl groups	Multitude of reactions involving Acyl group transfer
Coenzyme Q (ubiquinone)	CoQ	Not a vitamin	Electron (hydrogen atom)	
Pyridoxal Phosphate	-	Pyridoxine (vitamin B_6)	Amino groups	Transfer of amino groups, Decarboxylate amino acids
Biotin	-	Biotin	Carbon dioxide	Carry activated CO_2
Tetrahydrofolic Acid	THFA	Folic Acid	One-carbon groups	Carry activated one-carbon groups
Coenzyme B_{12} (cobalamin derivatives)	-	Vitamin B_{12}	One-carbon groups	Carry activated one-carbon groups

Table 1.1: Common coenzymes

acetylation was dependent on a heat-stable factor, which was present in boiled extracts of all organs, and could not be replaced by any other known cofactor. Lipmann suspected he had found a new coenzyme, and eventually managed to purify the coenzyme from pork liver, where he found it to be active in choline acetylation with dialyzed brain extracts (Lipmann 1946). He named the factor coenzyme A, in which "A" stood for "activation of acetate."

1.2.3 CoA structure

The structure of CoA was determined by subjecting the molecule to a variety of selective enzymatic degradation studies and analysing the products. The molecule consists of 3' phosphoadenosine, coupled through the 5' link of the ribose to pantothenate (vitamin B_5) via a pyrophosphate linkage. The carboxy end of the pantothenate is linked to β-mercaptoethylamine via a peptide linkage (*Figure 1.1)*. The free sulfhydryl (SH) group at the end of the CoA molecule is extremely reactive and has enormous potential to be oxidised to disulphides during reactions involving other thiols (Srinivas and Mamidi 2003).

1.2.4 CoA derivatives

In addition to its free form (CoASH), CoA can readily form thioesters through its reactive sulfhydryl group (*Figure 1.2)*. The most abundant thioester of CoA is acetyl CoA. Other acyl CoA esters consist of various length carbon chains extended from acetyl CoA, including malonyl CoA, succinyl CoA, and long chain fatty acyl CoA (e.g. palmitoyl CoA). Many CoA esters have specific biological functions (e.g. membrane trafficking and modulation of ion channel activities) and consequently their levels are tightly regulated through processes such as gene expression (Black et al. 2000; Pfanner 1989; Rohács et al. 2003).

Figure 1.1: The CoA molecule is composed of a phosphoadenosine residue (green), linked via a pyrophosphate group to a pantothenic acid residue (blue) and a β-mercaptoethylamine moiety (pink).

Figure 1.2: Different forms of CoA. In addition to the free CoA-SH form, CoA can also be found as Acetyl CoA, which can be extended to Acyl CoA, with carbon chains of varying length represented by R, and HMG CoA. Images adapted from Chemspider: **http://tinyurl.com/2waxjyr**

1.3 Cellular Functions of CoA

1.3.1 Overview of CoA in metabolism

CoA and its thioesters are essential cofactors in over 100 reactions in intermediary metabolism. It plays a central role in the oxidation of all the major energy producing carbon substrates, as well as numerous synthetic reactions. In addition to forming thiol esters with carboxylic acids, the reactive SH group of CoA can also activate carbonyl groups. Furthermore, the 4'-phosphopantetheine (4'PP) component of the CoA molecule has been identified as a cofactor in many biosynthetic processes, such as biosynthesis of fatty acids, polyketides and peptides (Kleinkauf 2000). The ratio of free CoASH to acyl-CoA is consequently extremely important in controlling the rate of significant metabolic pathways (Robishaw and Neely 1985).

1.3.2 The role of CoA in energy generation

In nearly all organisms, most carbohydrates are broken down to and absorbed as glucose, which is then metabolised into CO_2, generating energy in the form of ATP during the process. Glycolysis involves the breakdown of glucose (C6) into two molecules of pyruvate (C3). Other sugars, such as fructose and galactose are funnelled into the glycolytic pathway at different stages, but still result in the formation of pyruvate. Pyruvate enters the citric acid cycle through its transformation to acetyl CoA.

Proteins are broken down to amino acids during digestion and once the α-amino group has been removed by the deamination reaction, the carbon skeleton of some amino acids can be degraded to acetyl CoA. This can occur directly or via pyruvate or acetoacetyl CoA. Amino acids that are not broken down into acetyl CoA are degraded to other intermediates in the citric acid cycle, including succinyl CoA and methylmalonyl CoA.

Acetyl CoA is the final product for oxidation of almost all the major carbon substrates (*Figure 1.3)*. It is formed inside the mitochondria by the irreversible oxidative decarboxylation of pyruvate, by pyruvate dehydrogenase, where CoA is chemically linked to the acetyl group of pyruvate (2C) in the presence of NAD^+. This acetyl group is combined with oxaloacetate (4C) to form citrate (6C), regenerating free CoASH. Citrate is converted back to oxaloacetate via a series of chemical transformations, which make up the citric acid cycle (*Figure 1.4)*. This process generates ATP as well as reduced cofactors (NADH, $FADH_2$), which are used in the electron transport chain to produce more ATP. The two carbons added through acetyl CoA in the citric acid cycle are released as two molecules of CO_2. As well as for the formation of acetyl CoA, CoA is required by α-ketoglutarate, an intermediate in the citric acid cycle, in the formation of succinate via succinyl-CoA (Stryer 2002).

1.3.3 The role of CoA in Fatty Acid Oxidation

Fatty acids play a number of important physiological roles, for example they are components of biological membranes in the form of phospholipids and glycolipids; they are often used to covalently modify proteins in order to target them to membrane locations; and they can serve as hormones (e.g. prostaglandins) and intracellular messengers (e.g. DAG). Fatty acids also act as energy stores and fatty acid oxidation is required for peripheral tissues to access the lipid energy stored in adipose tissue. The process involves three steps; mobilisation of triacylglycerols (TAG) to fatty acids and glycerol, activation of fatty acids by CoA, and oxidation of the fatty acid to acetyl CoA (which is then processed in the citric acid cycle). In the activation step, CoA uses ATP to form a thioester linkage with the carboxyl group of the fatty acid. This reaction occurs on the outer mitochondrial membrane and is catalysed by acyl CoA synthetase. These long chain acyl-CoA molecules are transported across the inner mitochondrial membrane to the mitochondrial matrix via the carnitine cycle. In the oxidation step, acyl-CoA is degraded in a recurring four-step

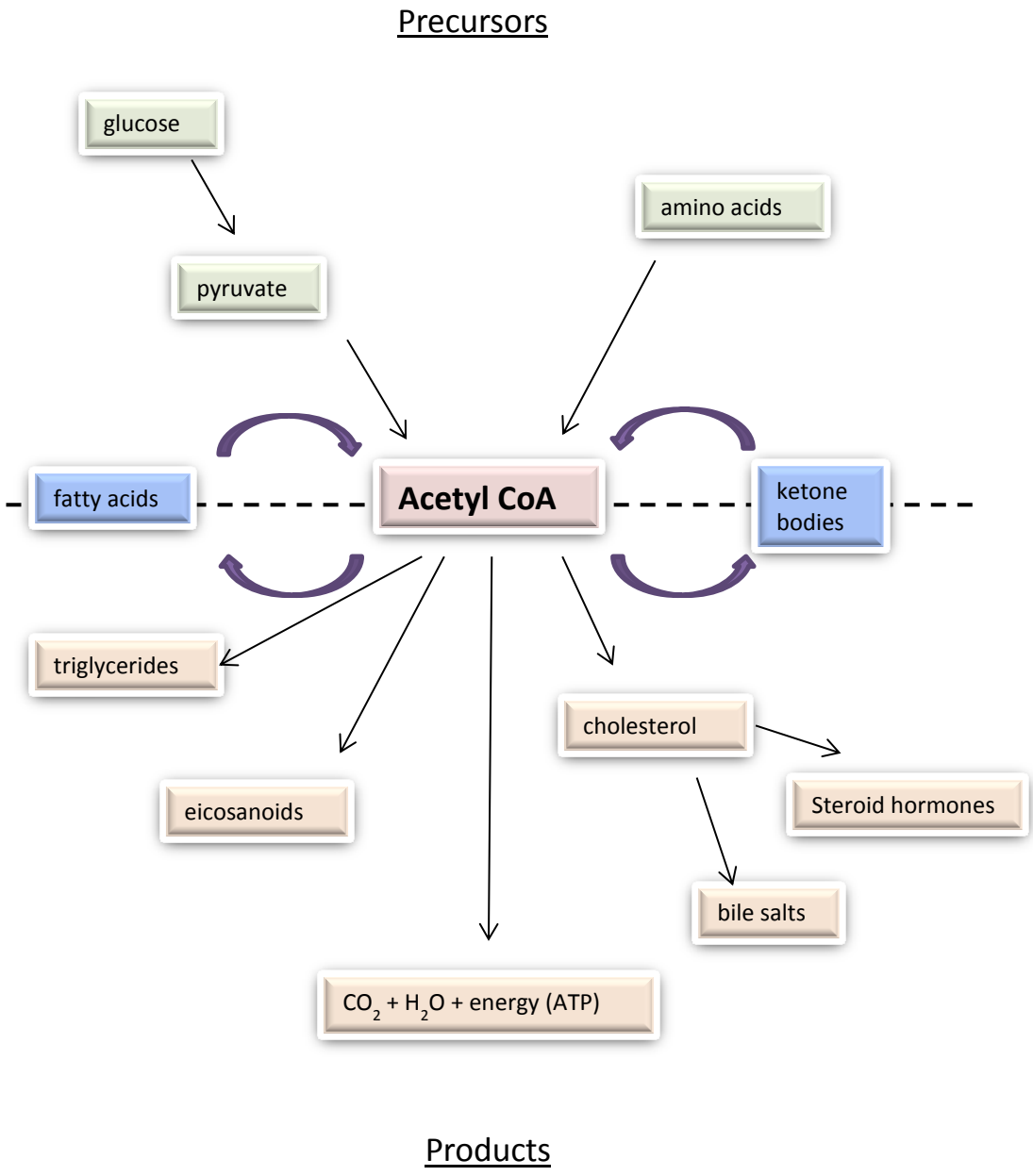


Figure 1.3: Acetyl CoA is a central metabolite in metabolism.

Figure 1.4: Acetyl CoA is essential for progress in the citric acid cycle. Acetyl CoA enters the citric acid cycle after the acetyl group of pyruvate from glycolysis is linked to CoA. The intermediates in the cycle are labelled in purple. The products of the cycle are labelled in purple. The products of the cycle are the four reduced electron carriers, in red and ATP. Image taken from Biology 230, https://wikispaces.psu.edu/display/230/Glycolysis%2C+Fermentation+and+the+Citric+Acid+Cycle

reaction, where the acyl-CoA is shortened by two carbon atoms at the end of each cycle *(Figure 1.5*). CoA is required again in the final thiolysis step of this pathway, which is mediated by β-ketothiolase. Acetyl CoA is the final product, however NADH and $FADH_2$ are also created. Acetyl CoA enters the citric acid cycle, whereas NADH and $FADH_2$ feed into the respiratory chain (Stryer 2002).

1.3.4 The role of CoA in Biosynthetic pathways

CoA functions as an essential cofactor in the multistep processes that lead to the formation of complex metabolites. These reactions usually require the assistance of a carrier protein, which along with CoA, help to stabilise the acyl groups. This method enables the formation of complex biosynthetic components by keeping intermediates within the carrier protein complexes until the final product is formed. Products are released from the carrier protein complex by the action of thioesterases (Kleinkauf 2000).

Mammals are able to make fatty acids *de novo*, although most are obtained through diet. Fatty acids are linked to CoA in order to alter the length of their carbon chains, or to alter the level of unsaturation by adding or removing double bonds (Sugiura 1995). If the energy demands of the cell are low yet nutrients are readily available, excess acetyl CoA is used for the synthesis of fatty acids. Fatty acid synthesis takes place in the cytosol, whereas acetyl CoA generated from oxidative metabolism is located in the mitochondria. Acetyl CoA cannot permeate the inner mitochondrial membrane, so acetyl CoA is condensed into citrate which is transported into the cytosol and converted back into acetyl CoA through the action of ATP citrate lyase (ACL). Acetyl CoA is then committed into the fatty acid synthesis pathway once it is converted into malonyl CoA through the action of acetyl CoA carboxylase (ACC), which is an essential regulatory enzyme for fatty acid metabolism (Kim et al. 1989). Malonyl CoA, as well as the intermediates in fatty acid synthesis is linked to an acyl carrier protein (ACP). *De novo* fatty acid synthesis starts with

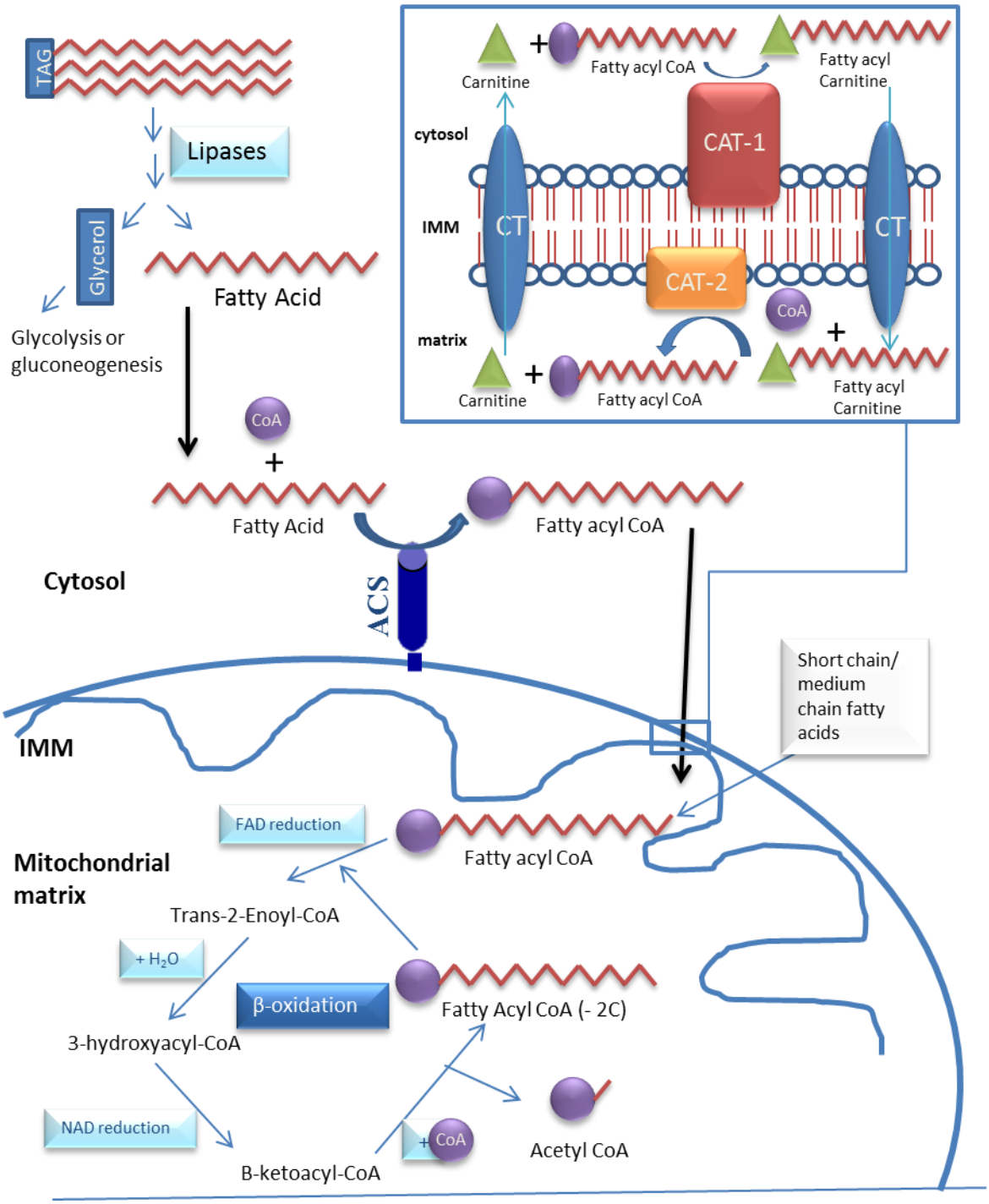


Figure 1.5: The role of CoA in Fatty acid oxidation. The breakdown of fatty acids occurs in three sterps. 1) Triacylglycerol is broken down into fatty acids and glycerol. 2) Free fatty acids are activated by CoA through the action of the enzyme Acyl CoA Synthase (ACS). The fatty acyl CoA is then converted into fatty acyl carnitine by carnitine acyltransferase 1 (CAT-1), also known as carnitine palmitoyltransferase 1 (CPT-1), and then shuttled across the inner mitochondrial membrane via the carnitine transporter. On the mitochondrial matrix side of the membrane, fatty acyl carnitine is converted back into fatty acyl CoA by carnitine acyltransferase 2 (CAT-2), or carnitine palmitoyltransferase 2 (CPT-2). This is known as the carnitine cycle. 3) Fatty acyl CoA is oxidised in the mitochondrial matrix in multiple rounds, removing Acetyl CoA until the fatty acid is depleted (β-oxidation). Fatty acids with 10 carbons or less can freely diffuse through the inner mitochondrial membrane.

acetyl-ACP, which is formed from acetyl CoA using acetyl transacylase. Malonyl-ACP adds two carbon units in each four-step elongation cycle to the lengthening acyl-ACP chain, which occurs on the enzyme Fatty Acid Synthase (FAS) (*Figure 1.6)*. This continues until 16-carbon palmitoyl-ACP is formed, as the 1st enzyme in the elongation cycle can no longer accept molecules of this size. Palmitoyl-ACP is hydrolysed by a thioesterase, releasing ACP and palmitate. Synthesis of fatty acids with a carbon chain longer than C16 palmitate is mediated by enzymes located on the cytosolic side of the smooth endoplasmic reticulum. Malonyl CoA is used as the two carbon donor and the fatty acid is elongated as its CoA derivative.

Polyketides are compounds made up of alternating carbonyl and methylene groups. They are synthesised in a similar process to fatty acids by polyketide synthases, which contain multiple enzymatic domains. Acetyl CoA, malonyl CoA or methylmalonyl CoA is loaded onto the ACP domain on the starter molecule and the growing chain is transferred along thiol groups of the elongation domains via trans-acylation reactions. Products are released at the end by hydrolysis. Polyketides form a vital class of naturally occurring compounds that are often used in industry, and include rapamycin (inhibitor of the mammalian target of rapamycin), several antibiotics (tetracycline, erythromycin, macrolides), anti-cholesterol drugs (lovastatin) and anti-cancer drugs (epothilone) (Koehn 2005; Wawrik et al. 2005).

Cholesterol modulates the fluidity of cellular membranes and is a precursor of many steroid hormones. The committed step in cholesterol synthesis is the formation of mevalonate from the reduction of HMG CoA by HMG CoA reductase. HMG CoA results from the combination of acetyl CoA and acetoacetyl CoA. Mevalonate is converted into 3-isopentenyl pyrophosphate (IPP) in a series of three reactions involving ATP and six molecules of IPP are combined in several condensation reactions to produce a C30

Figure 1.6: Fatty Acid synthesis. Image taken from:
http://www.agron.iastate.edu/courses/agron317/Lipid_Inhibitors.htm

molecule called squalene. Cholesterol is then formed from the cyclisation of squalene in a multistep process involving O_2 and NADPH.

1.4 Regulation of Proteins and Cellular processes by CoA and CoA thioesters

1.4.1 The role of CoA in the regulation of cellular processes

CoA can regulate proteins and processes through a number of mechanisms. These include the availability of CoA as a substrate or cofactor; the regulation of proteins allosterically or through other non-covalent mechanisms; and covalently through forming di-sulphide bonds with its SH group and by transferring acyl groups.

1.4.2 Availability as a cofactor or substrate

The rate of some biochemical reactions in the cell is dependent on the supply of CoASH or CoA thioesters. This is related to the K_m for the enzyme. If the K_m is well below the range of concentration in which fluctuations in CoASH or CoA thioesters occur, then the rate of the reaction catalysed by the enzyme is unlikely to be affected by any changes in CoASH or CoA thioester levels. Examples of reactions affected by CoASH/CoA ester supply are presented below.

Substrate availability of CoASH has the potential to regulate fatty acid oxidation at the point of fatty acid activation. Acyl CoA Synthase (ACS) activates fatty acids to fatty acyl CoA, enabling transport across the mitochondrial membrane. The cytosolic concentration of CoASH is equal to or less than the K_m of ACS, therefore the rate at which ACS activates fatty acids could depend on CoASH availability (Oram et al. 1975). Inhibition of ACS activity with troglitazone in hepatocytes affects the flux through fatty acid oxidation, further

supporting the notion that fatty acid oxidation could be regulated at this point (Fulgencio et al. 1996).

The enzyme ACC catalyses the rate-limiting step in fatty acid synthesis involving the conversion of acetyl CoA to malonyl CoA. Although its enzymatic activity is well known to be regulated allosterically by citrate (activator) and palmitoyl CoA (inhibitor), ACC could also potentially be regulated by substrate availability of acetyl CoA. Regulation by substrate availability is thought to occur in the liver (K_m=25 μM), adipose tissue (K_m=67 μM), heart (K_m=117 μM) and skeletal muscle (K_m=109 μM) since the K_m of ACC for acetyl CoA in these tissues is similar to or higher than the concentration of acetyl CoA present in the cytosol (Saddik et al. 1993; Tong 2005).

1.4.3 Regulation through non-covalent interaction

1.4.3.1 Balance between CoASH/CoA thioesters

Acetyl CoA is a product of the reaction mediated by PDH. When fatty acids are oxidised as the primary fuel, it is essential that the activity of PDH is reduced to conserve carbohydrate reserves. Therefore the activity of PDH is tightly controlled. The increased use of fatty acids for metabolism is registered through increases in the mitochondrial acetyl CoA to CoASH ratio (as well as the NADH to NAD^+ ratio), which enhance PDHK catalysed inactivation of PDH. The mammalian PDH complex consists of three enzymes, pyruvate dehydrogenase (E1), dihydrolipoamide transacetylase (E2) and dihydrolipoamide dehydrogenase (E3). PDHK is thought to associate with the PDH complex through the E2 core, and requires its lipoyl prosthetic group. Stimulation of the kinase activity of PDHK results from reduction (E3 reaction) and acetylation (E2 reaction) of the lipoamide domains of the E2 oligomer, consequently favouring a high acetyl CoA/ CoA ratio (*Figure 1.7*). PDHK has four isoforms (1-4) and acetyl CoA was shown to stimulate isoforms 1 and 2 by nearly 200 and 300% respectively, in the presence of NADH (Bowker-Kinley et al. 1998; Stryer

Figure 1.7: Summary of molecular mechanisms regulating PDK2 activity. E2 and E3 reactions are acting in the reverse directions to make reduced and reductively acetylated L2, resulting in the stimulation of PDK2. Taken from Roche and Hiromasa (2007).

2002). Activated PDHK, then phosphorylates the pyruvate dehydrogenase component (E1), consequently inactivating PDH. Increased levels of CoASH have been shown to reverse the activation of PDHK by acetyl CoA in rat liver and heart mitochondria (Batenburg and Olson 1976; Hansford 1976; Kerbey et al. 1976; Roche and Hiromasa 2007).

1.4.3.2 Allosteric and other non-covalent interactions

Allosteric regulation by CoA has been implicated for the enzymes Pantothenate Kinase (PanK). PanK mediates the first step in CoA biosynthesis and is feedback inhibited by CoA and is competitive with respect to ATP. Structural studies involving *E. coli* PanK indicate that CoA occupies the ATP active site in a bent conformation. Although both CoA and ATP both have a similar adenine base structure, they bind to the active site in different orientations to each other. Both molecules do however occupy a portion of the same space where they interact with a lysine residue of bPanK to neutralise the charge on their respective phosphodiesters. CoA also forms tight interactions with the side chains of several aromatic residues, which might explain why other CoA esters do not inhibit PanK as strongly (Yun et al. 2000).

As mentioned in Section 1.4.3.1, PDHK negatively regulates pyruvate dehydrogenase. There have been several reports stating that CoASH directly inhibits PDHK activity (Bao et al. 2004; Pettit et al. 1975). Conversely, CoASH has also been shown to increase the activity of PDHK, potentially by forming interactions with the enzyme through its thiol group. This stimulates PDH phosphorylation and consequent inactivation, preventing the conversion of pyruvate into acetyl CoA (Siess and Wieland 1982).

ADP/ATP exchange across the inner mitochondrial membrane is an important process which CoASH regulates in a non-covalent manner. This process is carried out by adenine nucleotide translocator (ANT) and is generally accepted to be the overall rate-limiting step in energy metabolism. Incubation of rat liver mitochondria with CoASH

resulted in the increased exchange of ATP by ANT, indicating that CoASH facilitates the interaction with ATP. Molecular docking simulation implied that CoASH binds independently of the ATP binding site, possibly through the accessory site of ANT. CoASH was also shown to increase ATP exchange in ANT isolated from mitochondria in heart, skeletal muscle and brain (Cione et al. 2010).

Acetyl CoA also has been shown to act as a positive allosteric regulator of pyruvate carboxylase (PC) in many species. PC mediates the carboxylation of pyruvate to oxaloacetate, which can be used to replenish the citric acid cycle, or alternatively is a substrate for several biosynthetic pathways (e.g. gluconeogenesis, adipogenesis, glutamine synthesis in astrocytes and insulin secretion). Binding of acetyl CoA in the allosteric domain of PC results in conformational changes, which enhance the rate of carboxylation of biotin (the moiety involved in the transfer of the carboxyl group) (Jitrapakdee et al. 2008).

The citric acid cycle is regulated through feedback mechanisms at several points to ensure the amount of products (ATP, NADH, $FADH_2$, biosynthetic precursors) meet the metabolic requirements of the cell. A-ketoglutarate dehydrogenase is one of the control points within the citric acid cycle, and an inverse relationship between α-ketoglutarate accumulation and succinyl CoA indicated that succinyl CoA inhibited this enzyme. Inhibition of α-ketoglutarate dehydrogenase by succinyl CoA in rat and guinea pig liver mitochondria is independent of NAD^+: NADH ratio, and competitive with CoASH, with a K_i of 6.9 µM (LaNoue et al. 1972; Smith et al. 1974; Stryer 2002).

Feedback regulation mechanisms involving the CoA ester, malonyl CoA, take place in fatty acid metabolism. It is important that fatty acid synthesis and degradation are reciprocally regulated in lipogenic tissues such as liver, so that both processes are not active at the same time. In skeletal and cardiac muscle, malonyl CoA acts as a signalling

molecule to control fatty acid oxidation. Malonyl CoA is rapidly turned over in mammalian cells, where it is formed through the carboxylation of acetyl CoA by ACC and broken down back to acetyl CoA by malonyl CoA decarboxylase (MCD). Production of malonyl CoA by ACC is a highly regulated step in fatty acid synthesis, and malonyl CoA was shown to be a potent inhibitor of carnitine palmitoyl transferase 1 (CPT1) at concentrations typical of those measured in rat liver ($K_{0.5}$ ~ 1-2 μM). CPT1 transports fatty acyl CoA across the mitochondrial outer membrane into the mitochondrial matrix, and is often referred to as the "rate-limiting step" of fatty acid oxidation. High levels of malonyl CoA therefore stimulate fatty acid synthesis and inhibit fatty acid oxidation (Saggerson 2008).

Malonyl CoA inhibits CPT1 by binding to two sites within the catalytic domain. One site has low affinity for malonyl CoA, where it competes with fatty acyl CoA, and the other site has high affinity for malonyl CoA. The N-terminal region of CPT1 is important for maintaining the structure of the high affinity malonyl CoA binding site. Desensitisation of liver CPT1 to malonyl CoA occurs during fasting and diabetes, and corresponds with increased fluidity of the membrane. In addition to the outer mitochondrial membrane, CPT1 inhibitable malonyl CoA has been detected in liver microsomal and peroxisomal fractions. CPT1, which is not inhibited by malonyl CoA was also detected in hepatic endoplasmic reticulum lumen and peroxisomal matrix fractions (Saggerson 2008). Furthermore, malonyl CoA has been implicated as a signal in glucose mediated insulin secretion in β-islet cells, whereby glucose metabolism results in an increase in malonyl CoA and consequently increases cytosolic levels of fatty acyl CoA, which is a coupling factor for insulin secretion (Herrero et al. 2005; Roduit et al. 2004).

Long chain acyl-CoA esters have been implicated in the regulation of a large number of proteins, including enzymes involved in energy metabolism (e.g. ANT, glucokinase, PDH), lipid metabolism (e.g. ACC, LKB1 (AMP activated kinase kinase), CPT1,

HMG CoA reductase, hormone sensitive lipase), Signal transduction (e.g. Protein Kinase C subtypes, ion channels/pumps), as well as cellular processes such as membrane fusion and gene regulation. Most of the reported effects of long chain fatty acyl CoAs were obtained by direct addition of acyl CoA to the system in question, without the presence of acyl CoA binding protein (ACBP). ACBP is a cytosolic protein present in a wide range of tissues and has been found in all eukaryotes tested. It binds medium and long chain acyl CoA esters with a K_d of about 0.5 nM. ACBP protects acyl CoA against hydrolysis and there is strong evidence that it also participates in acyl CoA transport. Furthermore, ACBP sequesters acyl CoA and has been shown to reduce the inhibitory effect of long chain acyl CoA on ACC and ANT *in vitro*. Sensitive methods for estimating the distribution of acyl CoA esters in different compartments are not currently available and the actual free concentration of cytosolic long chain CoA is not known for any tissue. Fatty acid synthesis still occurs even though long chain fatty acyl CoA inhibits ACC (K_i is equal to 5.5 nM), implying that liver cytosolic long chain acyl CoA is below 5.5 nM under normal conditions. If this is the case, the physiological role of long chain acyl CoA would be limited to ACC, LKB1 and the *E. coli* transcription factor FadR, which all have K_is in the nanomolar range, whereas all other mentioned proteins require µM concentrations before an effect is seen. ACBP is not found in the mitochondria and peroxisomes in yeast, so it is possible that the free concentration of long chain acyl CoA could increase to levels that could inhibit fatty acid oxidation, ANT, PDH and the citrate transporter. Acyl CoA hydrolyases present in these compartments could still prevent regulation of these proteins by acyl CoAs from occurring. ACBPs also could potentially donate long chain acyl CoAs directly to proteins for the regulation of specific processes (Faergeman and Knudsen 1997).

1.4.4 Regulation through covalent modifications

CoASH has been shown to modify a number of proteins *in vitro*, including the mutant β-subunit of F1-ATPase of *E. coli* (Odaka et al. 1993) and flavodoxin of *K. pneumonia* (Thorneley et al. 1992). To date, the only examples for *in vivo* covalent modifications of proteins by CoASH have been described in studies in Huth's laboratory (Huth et al. 1996; Huth et al. 2002; Schwerdt et al. 1991; Schwerdt and Huth 1993). Immunological and radiochemical evidence was provided for the novel post translational modification of several rat liver mitochondrial proteins (acetyl CoA acetyltransferase, 3-oxoacyl-CoA thiolase) by CoASH. This was demonstrated through immunoblotting total cellular lysates and purified enzymes with affinity purified anti-CoA antibodies. They proposed that CoASH modification may control the selective degradation of several mitochondrial proteins by protecting them from degradation, however no further studies on other cellular proteins or CoA's role in cellular processes have been carried out. It is important to note that during sample preparation, no alkylating agent was used to block the thiol group, therefore it is possible that CoASH modification may have occurred during sample preparation due to the presence of reactive oxygen species released in the mitochondria.

As well as CoASH, cysteine residues also contain this highly reactive thiol group and consequently have been conserved in the active sites of several proteins, including enzymes involved in both central and peripheral metabolism, signal transduction and even enzymes of protein catabolism (Cotgreave and Gerdes 1998). The thiol group enables cysteine to form intra-molecular or intermolecular disulphide bonds with other proteins, or with glutathione (γ-glutamyl-L-cysteine-glycine), which is the most prominent low-molecular weight thiol in most eukaryotic and prokaryotic cells. Glutathione plays an essential role during conditions of oxidative stress through detoxifying reactive oxygen

species as well as transmitting redox signals. S-glutathionylation of a number of proteins undergoing oxidative stress has been observed, including protein chaperones, cytoskeletal proteins, cell cycle regulators and enzymes in intermediary metabolism (Lind et al. 2002) Sulfhydryl modifications often cause significant conformational changes to the protein, altering its structural and catalytic properties. This can either lead to the activation or inactivation of the respective protein's function and ultimately triggers downstream processes (Leichert et al. 2005). The reactive thiol group on CoASH could potentially modify proteins with a conserved cysteine by reversible covalent modification, and possibly regulate a number of cellular processes in this way.

If covalent modification of proteins by CoASH is physiological, CoASH has to compete with glutathione, which is present in much higher concentrations in the cytosol (Hwang 1992). This suggests that proteins modified by CoASH need to contain a binding site that can specifically recognise CoASH, or there is a specific enzyme that transfers and attaches CoASH via a disulphide linkage to a particular protein. Indeed, *in vitro* experiments indicated that some enzymes, including phosphofructokinase and fructose 1,6-bisphosphate, showed preference for CoASH over glutathione for modulation of the enzyme activity (Gilbert 1982; Horecker 1969).

During protein synthesis many polypeptides undergo post-translational modifications (including phosphorylation, methylation and ubiquitination), which are common mechanisms for controlling the behaviour of the protein. These modifications can regulate the protein's catalytic properties, their ability to interact with other proteins, alter their localisation, or target them for degradation. Acetyl CoA is required for the acetyl CoA dependent acetylation of histones and non-histone proteins (e.g. transcription factors). These acetylation reactions are mediated by a family of enzymes called acetyltransferases and the removal of the acetyl group is controlled by deacetylases. Histone

acetyltransferases (HATs) catalyse the transfer of acetyl CoA onto the lysine residues of histone proteins. The primary effect of acetylation is to neutralise the positive charge on the lysine, consequently altering the biochemical properties of the protein. As the acetylation modification is specific to lysine residues, it has great regulatory potential with the ability to interfere with cellular functions that rely on other lysine modifications, such as methylation and ubiquitination. Also, lysine acetylation also creates a platform that either enables the formation of new protein-protein interactions or prevents specific partners from binding. It is also important to note that acetylation can also occur on the N-terminal α amine of proteins through the action of N-acetyltransferases (Sadoul et al. 2008).

Palmitoylation is a reversible covalent attachment of the long chain fatty acids, such as palmitate, to cysteine, and occasionally threonine and serine residues of proteins. Addition of palmitate increases the hydrophobicity of proteins and facilitates membrane interactions and membrane targeting. In addition, palmitoylation has been shown to play a role in trafficking proteins between different subcellular compartments, modulate protein: protein interactions and also enzymatic activity through altering the binding capacity of proteins, consequently affecting numerous signalling pathways. The reversible modification of proteins by palmitoylation is regulated through the actions of acyltransferases and acylthioesterases (Resh 2006).

Myristoylation is an irreversible covalent attachment of the 14-carbon saturated fatty acid, myristate, usually onto the N-terminal glycine of a protein via an amide bond. The substrate for the transfer of myristate is myristoyl CoA. This modification can occur co-translationally as well as post-translationally, and is important in many signalling cascades (Farazi et al. 2001).

Succinylation of lysine residues is a post-translational modification that occurs on a large number of cellular proteins and has been evolutionary conserved. It has been proposed that succinyl CoA is the cofactor for this reaction, however the enzyme mediating this reaction is currently unknown. Succinylation of a lysine residue completely reverses the charge from positive to negative and also adds a much larger structural moiety than acetylation or methylation. Succinylation is therefore likely to lead to significant changes in the protein's structure and function. Due to the abundance of lysine succinylation detected in whole cell lysates when using western blot analysis with an anti-succinyl lysine antibody, alongside its induced chemical changes, it has been predicted that lysine succinylation could have important cellular functions (Zhang et al. 2011). Indeed, succinyl CoA has been implicated in the succinylation of ox liver mitochondrial HMG-CoA synthase, an important enzyme in the mevalonate and ketogenesis pathways. Succinyl CoA inactivated the enzyme through succinylation, probably through a covalent thioester linkage at the same cysteine residue that is acetylated in the normal enzymatic reaction (Lowe 1985).

1.5 Control of CoA levels

The level of CoA depends on three factors; the rate at which CoA is synthesised; the rate at which it is degraded; and the rate at which it is converted into CoA derivatives.

1.5.1 CoA Biosynthetic Pathway

Biosynthesis of CoA is conserved in prokaryotes and eukaryotes and involves five enzymatic steps. CoA is derived from three substrates; pantothenate (vitamin B_5), cysteine and ATP. Prokaryotes, fungi and plants are able to synthesise pantothenate, however animals must obtain it from their diet (Smith and Song 1996). There is no evidence for any CoA transport between cells and it is assumed that the CoA biosynthetic pathway is

sufficient to generate an independent pool of CoA (Robishaw and Neely 1985). The cloning of the *E. coli* genes encoding the proteins involved in CoA biosynthesis was completed in 2001 (Kupke et al. 2000; Strauss et al. 2001), and the mammalian genes were cloned by 2002 by several groups.

In the first step of CoA biosynthesis, pantothenate is phosphorylated to form 4'-phosphopantothenate. This step is mediated by PanK and is rate-limiting. Unlike the other enzymes in the biosynthetic pathway, the bacterial and eukaryotic versions of the PanK enzyme appear to be unrelated (Calder et al. 1999). PanK, therefore served as a prime target for the identification of novel antibacterial drugs (Gerdes et al. 2002). Phosphopantothenoylcysteine synthetase (PPCS) conjugates cysteine with the product of the first reaction to form 4'-phosphopantothenoylcysteine, which is then decarboxylated by phosphopantothenoyl cysteine decarboxylase (PPCDC). The 4'-phosphopantetheine produced is adenylated by phosphopantetheine adenylyltransferase (PPAT), forming dephospho-CoA. Dephospho-CoA is then phosphorylated by dephospho-CoA kinase (DPCK) to produce CoA as a final product *(Figure 1.8)*. The bacterial equivalents of these enzymes are often labelled CoaA-E. The activities of PPAT and DPCK are associated with two different enzymes in prokaryotes and plants but are fused as a bifunctional enzyme, also termed CoA synthase (CoASy), in mammals (Zhyvoloup et al. 2002).

The two rate-limiting steps of the CoA biosynthetic pathway were determined by metabolic labelling experiments in *E. coli* and mammalian COS-7 cells (Jackowski and Rock 1981; Rock et al. 2000). The data demonstrated that both pantothenate and 4'-PP could accumulate in significant amounts under limiting conditions for CoA biosynthesis, indicating that PanK and PPAT activities were rate-limiting (Jackowski and Rock 1984; Rock et al. 2000). Similar studies in perfused rat hearts showed an accumulation of just pantothenate in normal conditions, and only during artificial stimulation of CoA Synthesis was an increase

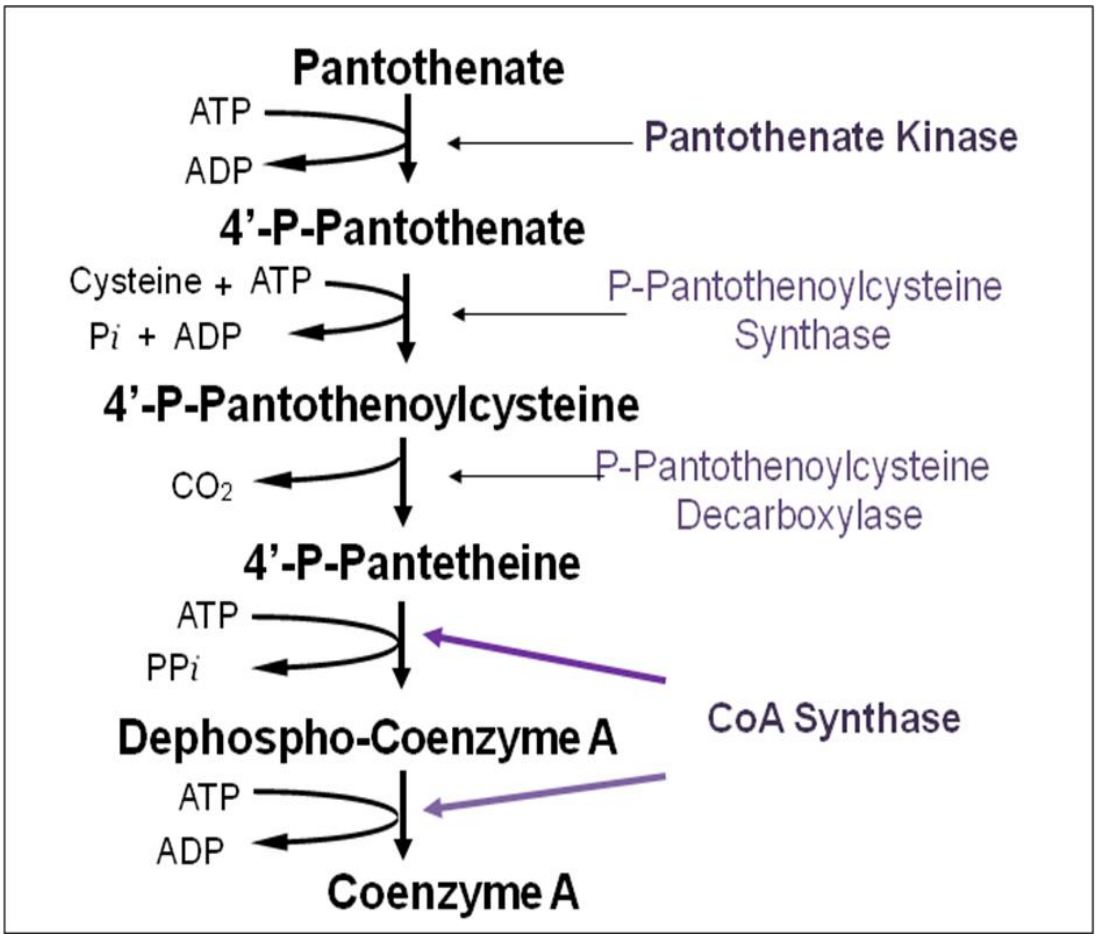


Figure 1.8: CoA Biosynthetic Pathway

in 4'PP also seen (Robishaw et al. 1982; Robishaw and Neely 1984). In *Arabidopsis thaliana*, over-expression of a mutant form of PPAT resulted in 60-80% reduction in CoA levels, whereas over-expression of PPAT resulted in a 1.6 fold increase in free CoASH + acetyl CoA levels (Rubio 2008). PanK is consequently widely accepted as the rate-limiting enzyme in CoA biosynthesis in all organisms, however the rate-limiting activity of CoASy may not be as widespread.

1.5.2 Regulatory enzymes in CoA Biosynthesis: Pantothenate Kinase

PanK is the primary regulatory enzyme in the CoA biosynthetic pathway for both bacteria and mammals, and consequently is the most widely studied of the CoA biosynthetic enzymes (Jackowski & Rock 1981; Robishaw et al. 1982). It is feedback regulated through inhibition by CoASH and/or CoA thioesters and is consequently the main control point of CoA synthesis in response to the cell's metabolic demand. Bacterial PanK is competitively inhibited by CoASH and its thioesters binding to the ATP site, with free CoASH acting as the most potent inhibitor. Thus, in bacteria the CoA biosynthetic activity is also linked to the energy state of the cell (Rock et al. 2000). In mammalian cells, feedback regulation of PanK is more complex due to its multiple isoforms, which are encoded by four genes in humans and mice, some of which express splicing variants (Zhang et al. 2005). PanK1 has 1α and 1β isoforms; PanK2 has 3 isoforms; PanK3 and PanK4 have one isoform known to date. The various isoforms of PanK have different enzymatic qualities and different isoforms are expressed by different tissues. All the PanK isoforms share a >80% identical common catalytic core attached to various unrelated extensions. PanK1α and PanK2 possess long N-terminal extensions (100-200 amino acids), whereas the N-terminal extensions for PanK1β and PanK3 are only a few amino acids long. PanK4 has a long C-terminal extension, however its function is unknown.

The catalytic core is intrinsically insensitive to inhibition by free CoASH and is weakly inhibited by acetyl CoA, however, the addition of the long N-terminal chain in the PanK1α isoform results in inhibition by free CoASH and long-chain acyl-CoA and further increases the inhibition by acetyl CoA and malonyl CoA. Similarly, PanK2 is very sensitive to inhibition by acetyl CoA, long chain acyl-CoA and malonyl CoA, but less sensitive to free CoASH. The N-terminal extension of PanK2 also directs it to the mitochondria, while PanK1α and PanK1β and PanK3 are located in the cytoplasm. PanK3 is more sensitive to CoA thioester regulation than the PanK1 isoforms, with acetyl CoA and palmitoyl CoA acting as the main regulators. The variation in PanK3's regulatory pattern compared to PanK1β is due to differences in their amino termini. It is thought that the ranging sensitivities of the various PanK isoforms to feedback inhibition by CoA thioesters allows CoA biosynthesis to receive regulatory signals from different branches of intermediary metabolism (Leonardi et al. 2005; Leonardi et al. 2007; Zhang et al. 2005).

In addition to feedback inhibition, PanK can also be regulated by gene expression in mammalian cells to modify long-term CoA levels in response to diet and disease. The mechanisms by which these responses are governed are largely unknown. Tissues that have high levels of PanK expression or in conditions where over-expression of PanK has been enforced, correspond with high intracellular CoA levels (Leonardi et al. 2007; Ramaswamy et al. 2004; Rock et al. 2000). This implies that pantothenate supply and transport would also play a role in determining cellular CoA concentration. Pantothenate deficiency has not been reported in humans, presumably due to its ubiquitous nature (Leonardi et al. 2005).

One of few known mechanisms underlying the regulation of PanK1 expression is through the peroxisome proliferator activated receptor transcription factor α (PPARα). PPARα-regulated PanK1 expression is consistent with the requirement to increase the

availability of CoA to support the accelerated processing of its thioesters through the fatty acid oxidation pathway. Analysis of the PanK1α promoter sequence reveals the presence of binding sites for transcription factors involved in the regulation of carbohydrate and lipid metabolism.

1.5.3 Regulatory enzymes in CoA Biosynthesis: CoA Synthase

The secondary rate-limiting step of CoA biosynthesis is mediated by the PPAT activity of CoASy, although this enzyme has not been studied to the same extent as PanK. CoASy was first identified in mammals in 1983 from pig liver extract (Worrall 1983), however it was not until 2002 that the gene was cloned (Aghajanian and Worrall 2002; Daugherty et al. 2002; Zhyvoloup et al. 2002).

Fluorescent microscopy of GFP-tagged CoASy indicated that CoASy localises on the outer mitochondrial membrane, whereas the other enzymes in the pathway are located in the cytosol. Additionally, subcellular fractionation studies revealed that CoASy was associated in the mitochondrial fractions. It appears to have a mitochondrial localisation signal in its N-terminal sequence since CoASy missing the N-terminal region mislocates to the cytosol (Zhyvoloup et al. 2003). Furthermore, both activities of CoASy are potently activated by phosphatidylcholine and phosphatidylethanolamine, which are the main components of the outer mitochondrial membrane (Zhyvoloup et al. 2003).

There is some evidence that CoASy is feedback inhibited either by CoA or its thioesters, yet to date very little direct assessment on CoASy regulation has been carried out (Izard 2003). Members of our group have further revealed interactions between CoASy and members of the mTOR (mammalian target of rapamycin) and PI3 Kinase (PI3K) pathways, providing a link between signalling pathways and energy metabolism.

mTOR is a central mediator of metabolism and growth, integrating various metabolic signals exerted by hormonal and stress factors, nutrient availability, and energy status. The mTOR pathway regulates a range of physiological functions including gene transcription, protein metabolism, cell cycle and cytoskeletal organisation. PI3K signalling also influences a wide range of cellular functions including cell growth, differentiation and survival, glucose metabolism and cytoskeletal organization (Sarbassov et al. 2005).

CoASy was shown to interact with the ribosomal protein S6 Kinase 1 (S6K1) in a yeast two-hybrid screen (Nemazanyy et al. 2004; Zhyvoloup et al. 2002). S6K is activated in response to mitogenic stimuli and nutrients via the PI3K and mTOR pathways. CoASy was not found to be a substrate of S6K. CoASy also appears to form a direct *in vitro* complex with the regulatory p85α domain of PI3K. This complex formation involves both SH2 and SH3 domains of p85α and only forms once CoASy tyrosines have been phosphorylated. Further *in vivo* interactions were shown through siRNA mediated knockdown of CoASy, which brought about changes in the phosphorylation patterns of the immediate downstream targets of PI3K, PDK1 and PKB (Breus et al. 2009; Nemazanyy et al. 2004). What's more, small fractions of S6K1, mTOR and both regulatory and catalytic subunits of PI3K were identified in the mitochondrial fraction. As CoASy is located on the outer mitochondrial membrane, this leads to speculation that CoASy may form a binding platform for the formation of a multi-enzyme complex, which may include these signalling molecules (Breus et al. 2009; Nemazanyy et al. 2004).

1.5.4 Degradation of CoA

Although the CoA degradation pathway has been determined to be nearly the reverse of the biosynthetic pathway, actual measurements of CoA turnover rates have not been done. This is because degradation of CoA proceeds so slowly, consequently making it difficult to study. How CoA is actually degraded *in vivo* is still under question, since the

majority of CoA is mitochondrial, whereas the first enzyme in the degradative pathway is lysosomal. The inner mitochondrial membrane appears to be impermeable to CoA, so how CoA comes into contact with the lysosomal enzymes is difficult to explain. It is also important to note that CoA-degrading enzymes have low substrate specificities, therefore it is unlikely that degradation of CoA is that important in the regulation of CoA levels (Robishaw and Neely 1985).

In peroxisomes, CoA is broken down into 4'-PP and ADP by the nudix (nucleoside diphosphate linked to another moiety X) hydrolases (Nudts). Nudt7 and Nudt19 are the two nudix hydrolases which are active on CoASH or CoA derivatives. During fasting, an increase in CoA levels in liver correlates with down regulation of Nudt7 expression, suggesting that Nudt7 plays a central role in CoA homeostasis (Leonardi et al. 2010; Reilly 2008).

An alternative to direct CoA degradation can occur through the transfer of the 4'phosphopantetheine moiety of CoA to carrier proteins such as the fatty acid synthase (FAS), mediated by 4'phosphopantetheinyl transferase. The phosphopantetheinyl prosthetic group is then able to re-enter the CoA biosynthetic pathway or exit from the cell and consequently can regulate intracellular CoA concentration (Joshi et al. 2003).

1.5.5 Conversion of CoASH to other esters

Total tissue levels of CoA normally remain fairly constant, although the distribution of free CoASH and its acyl esters may vary over a wide range. The ratio of CoASH: acyl CoA is important in the control of a number of key metabolic reactions. For example, treatment of hearts with high levels of fatty acids, pyruvate or ketone bodies, alters the composition of CoA from 80% free CoASH to 80% acyl esters, primarily acetyl CoA (Olson 1978; Oram 1973).

1.5.6 Compartmentalisation of CoA

Intracellular CoA is segregated in pools within eukaryotic cells, limited by the membranes of the mitochondria and peroxisomes. Cytosolic concentrations of CoA in mammalian tissues range from 0.02-0.14 mM and processes such as lipid synthesis, microsomal fatty acid oxidation, protein modifications and membrane trafficking (fatty acid synthesis) are affected by these concentrations. Mitochondrial CoA levels are much higher, ranging from 2.2 to over 5 mM and mediate processes such as mitochondrial fatty acids oxidation, the citric acid cycle, and mitochondrial fatty acid synthesis. Peroxisomes also contain a large proportion of CoA (0.7 mM) where it plays a major role in very long chain fatty acid oxidation. The transfer of CoA activated acyl moieties across organelle membranes is achieved through the carnitine system. The method of free CoASH transport into mitochondria and peroxisomes has not fully been characterised, however, although it is believed to be a specific, energy dependent uptake process which is sensitive to the electrochemical and pH gradient across the mitochondrial membrane (Tahiliani and Neely 1987). The exchange between these compartments appears to be very slow (Idell-Wenger et al. 1978). The intracellular distribution of CoA in tissues such as heart and liver are thought to be important in determining whether fatty acids are oxidised in the mitochondria or converted into lipids in the cytosol (Idell-Wenger and Neely 1978).

1.6 CoA homeostasis

CoASH, acetyl CoA and other acyl-CoA esters play important roles in numerous biosynthetic, degradative and energy yielding processes, therefore it is essential that their levels and ratio in cellular compartments are tightly regulated.

1.6.1 Basal levels of CoA

CoA is present in every mammalian tissue that has been studied and levels of CoA differ corresponding to the metabolic activity of the tissue. In rats, liver contains the highest levels of CoA, ranging from 22 to 368 nmol/g tissue (Tahiliani 1991; Tokutake et al. 2010). Heart/cardiac muscle, which carries out a high rate of substrate oxidation to meet energy requirements, contains the next most abundant levels (approximately 100 nmol/g tissue), followed by kidney, testes, diaphragm, adrenal and gastrocnemius muscle/white skeletal muscle in that order (Leonardi et al. 2005; Reibel et al. 1982; Tahiliani 1991).

1.6.2 Physiological changes in CoA levels

CoA levels in animal tissue, particularly liver, have been shown to change in response to various extracellular stimuli, including hormones, nutrients and cellular metabolites. Insulin, glucose, fatty acids, pyruvate and ketone bodies reduce the level of CoA, whereas glucocorticoids and glucagon increase CoA levels. Fibrate drugs (bezafibrate, clofibrate), and diet (starving) can also increase CoA levels, whereas feeding reduces CoA levels (Brass et al. 1990; Corkey et al. 1988; Halvorsen and Skrede 1982; Mcallister et al. 1988; Rapp 1973; Reibel et al. 1981a; Robishaw et al. 1982; Smith and Savage 1980; Smith 1978; Smith et al. 1978; Voltti et al. 1979). In these studies, it is not known in which cellular compartments these changes occur. If they occur in the cytosol (where the level of CoA is normally very low), significant effects on fatty acid metabolism among other pathways utilising CoA, and cytosolic proteins that CoA potentially regulates, might be expected.

1.6.3 Pathological conditions which change CoA levels

Deregulation of CoA homeostasis can be seen in a number of pathological conditions. CoA levels are particularly high in diseases such as diabetes and Vitamin B_{12} deficiency, whereas the levels are abnormally low in certain tumours, Reye syndrome, and medium chain acyl-CoA dehydrogenase deficiency (Brass et al. 1990; Corkey et al. 1988;

Halvorsen & Skrede 1982; Mcallister et al. 1988; Rapp 1973; Reibel et al. 1981a; Robishaw et al. 1982; Smith & Savage 1980; Smith 1978; Smith et al. 1978; Voltti et al. 1979). Most of the data regarding CoA levels is not recent, yet the mechanisms behind how CoA levels are changed still have not been characterised.

More recently, genetic analysis linked the neurological disorder, Hallervorden-Spatz syndrome or Pantothenate Kinase-associated neurodegeneration (PKAN) to mutations in the human PanK2 gene. PKAN patients accumulate iron in the globus pallidus of the brain and symptoms include progressive rigidity, frequent intellectual disability and convulsions. This is thought to be because the deficient PanK2 no longer produces phosphopantothenate, which usually condenses with cysteine, resulting in the accumulation of cysteine. In the presence of iron, cysteine is hypothesised to undergo auto oxidation, which results in the production of reactive oxygen species and leads to neurodegeneration (Zhou et al. 2001).

To obtain a better understanding of the cellular alterations that occur during the onset and progression of the disease, PanK2 knockout mice were generated (Kuo et al. 2005). These studies did not show any neurological abnormalities, and it was later discovered that murine and human PanK2 have different subcellular localisation patterns and expression levels in the brain (Kuo et al. 2005; Leonardi et al. 2007). Further genetic studies involving *Drosophila melanogaster* were carried out, where hypomorphic mutant alleles of the enzymes dPanK, dPPCS, dPPAT-DPCK of the CoA biosynthetic pathway were created (Bosveld et al. 2008). The main phenotype of mutant flies was abnormal locomotor function, which was progressive with time and exemplified by abnormal wing position, reduced flight performance, and impaired ability to climb against gravity. It was also observed that mutant flies also had a reduced life span, altered lipid homeostasis and elevated DNA damage since they were hypersensitive to infra-red radiation. Also, dPPCS

and dPanK/fbl, but not PPAT-DPCK mutants were hypersensitive to cysteine, which was consistent with the previous hypothesis. Additionally, it was proposed that changes in lipid homeostasis, resulting from the inhibition of CoA biosynthesis, would disrupt the integrity of membranes. This would consequently disrupt the integrity of tissues, including the central nervous system, leading to neurodegeneration. It was also thought that altered membrane lipid composition might disturb the dynamic interplay between cytoskeletal components, membrane structures and lipid signalling, causing division errors during central nervous system development and impaired DNA integrity (Bosveld et al. 2008).

1.7 Aim of thesis

Although cellular CoA levels are normally kept within a narrow range, they have been shown to change under various conditions such as nutritional state as well as in pathological conditions. These changes in CoA levels can influence cellular processes regulated by CoA through the mechanisms discussed in section 1.4. Regulatory roles of CoA have mainly been studied previously in the context of feedback/feedforward regulation of metabolic pathways or enzymes by free CoASH, CoA thioesters or by changes in the CoASH: acetyl CoA ratio in the mitochondria. There have been a few reports suggesting other pathways and processes, such as cell growth, may be regulated by changes in CoASH and/or CoA thioesters, as well as reports of enzymes which are regulated directly by CoASH through allosteric or covalent interaction, suggesting wider regulatory roles of CoA (Huth et al. 1988, 1991, 2002; Thorneley et al. 1992; Odaka et al. 1993; Charlier et al. 1997; Cai et al. 2011).

Abnormal CoA levels are observed in a number of diseases, however the role of CoA in the pathogenesis of these diseases has not been studied in detail. The aim of this thesis is to gain a greater understanding of the function of CoA as a regulator of cellular

processes. For this, it is necessary to develop methods to reliably and routinely measure CoA levels in tissues and cells (Chapter 3). Chapter 4 will use a "top down approach" to focus on understanding how cellular CoA levels are regulated and identify cellular processes and pathways which are influenced by changes in CoA levels. In Chapter 5 a "bottom-up" approach shall be used to identify individual proteins directly interact with CoA and establish how they are regulated.

Chapter 2:

Materials and Methods

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Common chemicals and Reagents

All the general purpose chemicals were purchased from Sigma-Aldrich, Thermo Fisher Scientific UK limited, BDH AnalaR®, and Melford Laboratories Ltd. UK unless otherwise stated. General cell culture reagents were purchased from PAA Laboratories GmbH. Cell inhibitors were purchased from Calbiochem and LC Laboratories. Enzymes were purchased from Sigma-Aldrich. Pre-stained protein molecular weight markers, DNA markers and restriction enzymes were obtained from Fermentas.

2.1.2 Antibodies and sera

Antibodies against CoA Synthase and S6K were previously generated in the laboratory for other applications. Anti-PanK1 was acquired from AbCam® (ab69354). Antibodies against PKB, phospho-PKB, AMPK, phospho-AMPK and acetyl lysine were from Cell Signalling Technology. Anti-phospho S6K was acquired from Millipore and anti-β-actin from Sigma-Aldrich. Horseradish peroxidase-linked (HRP) secondary antibodies (anti-rabbit and anti-mouse) were purchased from Promega.

2.1.3 Mammalian cells

Hek293 (human kidney embryonic cells) and HepG2 (hepatocellular carcinoma) were obtained from the American Type Culture Collection (ATCC). MEF, K562 and MOLT cells lines were a gift from Professor M. O'Hare.

2.1.4 Plasmids and Primers

The Pet28a plasmid containing PanK1β insert was obtained from Addgene (plasmid #25517), pet30a and pcDNA3.1(+) plasmids were purchased from Invitrogen and Novagen respectively. Amplification primers used in this thesis were ordered from MWG.

2.2 Nucleic Acid Manipulation

2.2.1 Polymerise Chain Reaction

The polymerase chain reaction (PCR) was used to amplify specific DNA sequences from DNA templates, using specific DNA primer oligonucleotides. PCR was performed using 2.5 ng DNA template in a 25 µL total volume containing 0.2 mM dNTPs, 2 µM of each primer, and 0.5 U Phusion DNA polymerase (Thermo Scientific) in Phusion 1x HF reaction buffer as supplied. The reaction was carried out for 30 cycles, consisting of a denaturing stage (98°C for 30s), a primer annealing stage (56-59°C for 30s), and finally a polymerase extension stage (72°C for 1 min). After completion of the cycling amplification, a final extension stage (72°C for 3 mins) was applied. Reactions were carried out in a thermal cycler (Labnet Multigene II).

2.2.1.1 Primer Design

Primers were designed using the known DNA sequence of the template (PanK1β sequence from ADDGENE), with restriction enzyme sites created at the ends of primers to facilitate subsequent subcloning of fragments. Additional bases missing from the PanK1β template supplied were also included in the primer design. Annealing temperature (T_m) was calculated using the equation T_m (°C) = 2(A+T) + 4(C+G). The primers used in this study, written 5' to 3' are shown below (*Figure 2.1*). To check primers were correctly designed and also whether DNA had been correctly inserted into a vector, 10-50 ng/µL DNA samples in 30 µL H_2O were sent off for sequencing analysis at GATC-biotech.

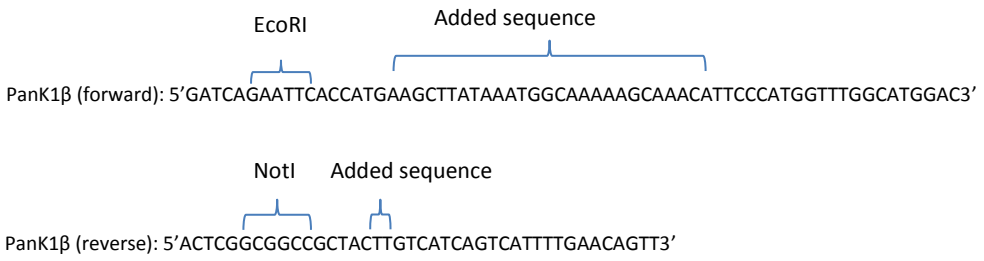


Figure 2.1: PCR primers for PanK1β.

2.2.2 DNA Digestion using Specific Restriction Endonucleases

Typically, 1 µg DNA was digested with 4 U of restriction enzyme in the appropriate 1X buffer as recommended by the manufacturer in a total volume of 20 µL for 1 hr at 37°C. Resultant restricted DNA fragments were then separated and analysed by DNA gel electrophoresis and gel purified when necessary.

2.2.3 DNA Electropheresis and Purification of DNA Fragments

DNA separation through an agarose-based gel can be used to allow visualisation and analysis of DNA fragments. The elecropheretic mobility of DNA molecules depends upon their size and the concentration of agarose gel used. Here, 0.9% agarose gels were used for DNA analysis and purification. The appropriate weight of agarose was added to TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 7.0) and heated to allow the agarose to dissolve. The solution was cooled to approximately 60°C and 1X gel red dye (DNA visualisation) or 1X gel green dye (gel purification) was added. The melted agarose solution was poured into a mould with a well-forming comb inserted, and allowed to solidify at room temperature. DNA samples were mixed with 6X DNA loading buffer (Fermentas), loaded into the wells and separation was achieved by electrophoresis in TAE buffer at a constant voltage of 100 V. Standard molecular weight markers (1 kb Gene Ruler,

Fermentas) were applied alongside the samples to determine relative sample DNA sizes. DNA was visualised and photographed under exposure to long-wave UV light.

When required, samples run in agarose containing gel green dye could be purified using the Wizard DNA Cleanup system (Promega) according to kit instructions.

2.2.4 DNA Ligation

The Fermentas T4 DNA ligase kit was used to clone DNA fragments into linearised plasmids. To ensure the DNA insert was ligated, and to minimise vector self-ligation, a 1:3 molar ratio of vector: insert was used. Conversion of molar to mass ratios was calculated using the following equation:

$$\frac{ng\ of\ vector \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times molar\ ratio\ of\ \frac{insert}{vector} = ng\ insert$$

The reaction was carried out in the presence of 1 µL T4 DNA ligase, 1X ligation buffer in a total volume of 20 µL at room temperature for 15 mins.

2.2.5 Transformation and Growth of Bacteria

XL10-Gold *E. coli* competent cells, initially stored at -70°C were thawed on ice and 50 µL bacterial suspension was mixed with 1 µL ligation mixture. After 20 mins incubation on ice, the cells were induced to take up the ligated plasmid DNA by heat shock treatment at 42°C for 45 s, followed by immediate cooling on ice for 3 mins. 1 mL of warm SOC (Super Optimal broth with Catabolite repression) medium was added to the bacteria, which were allowed to recover for 1 hr at 37°C in a shaking incubator (200 RPM). Bacterial cells were either taken directly from the SOC medium (5– 100 µL) or were collected by centrifugation at 3000 RPM for 5 mins and resuspended in 100 µL SOC medium were spread onto prewarmed Luria Bertani (LB) agar plates containing the appropriate selection antibiotic. Plates were incubated overnight at 37°C.

2.2.5.1 Preparing Chemical Competent Cells

To prepare chemically competent cells, a single colony of a bacterial strain was incubated overnight in 2 mL LB medium with shaking (225 RPM). The overnight culture was diluted 1:100 by inoculating 2 mL with 200 mL LB supplemented with 20 mM $MgSO_4$. Cells were grown in a 1 L flask at 37°C with shaking until the OD_{600} reached between 0.4 and 0.6. The cells were pelleted by centrifugation at 4000 RPM for 5 mins at 4°C. The cell pellet was gently resuspended in 80 mL ice-cold TFB1 (30 mM potassium acetate, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% (v/v) glycerol). The resuspended cells were incubated on ice for 5 mins before repelleting by centrifugation at 4000 RPM for 5 mins, 4°C. The bacterial cells were then resuspended in 8 mL ice-cold TFB2 (10 mM NaMOPs, pH 7.0, 75 mM $CaCl_2$, 10 mM KCl, 15% (v/v) glycerol). Cells were incubated on ice for 15 mins before snap-freezing on dry ice in pre-chilled microcentrifuge tubes and storing at -70°C.

2.2.6 Purification of Plasmid DNA

Plasmid DNA was purified from bacterial cultures using the QIAprep miniprep kit (Qiagen). The procedure is based on alkaline lysis of the bacterial cells, followed by adsorption of DNA onto silica in the presence of high salt. Briefly, a bacterial pellet from a 3 mL overnight culture of XL10 *E. coli* was resuspended in the suspension buffer P1 provided. The suspension was subsequently lysed and neutralised with buffers P2 and N3 respectively. The lysed mixture was centrifuged and the supernatant containing the circular plasmid DNA was collected and transferred to a QIAprep spin column. Further centrifugation through the column results in the plasmid DNA binding to the column. The plasmid DNA was then washed by centrifugation with PB and PE buffers and eluted using ddH_2O. The purified plasmid DNA was checked for purity and concentration by loading onto a DNA agarose gel alongside a comparative DNA ladder (1 kb GeneRuler, Fermentas)

as well as a plasmid with a known DNA concentration. DNA concentration was also determined by nanodrop analysis.

2.3 Mammalian Cell Culture and Methodology

2.3.1 Maintenance of Cell lines

Cell lines (Hek293 and HepG2) were cultured in Dubecco's Modified Eagles Medium (DMEM) with high glucose (obtained from PAA). All media were supplemented with filtered foetal bovine serum (FBS) (10% v/v) (Hyclone), 2 mM L-glutamine, 50 U/mL penicillin and 0.25 µg/mL streptomycin (PAA). Cells were grown in humidified 37˚C incubators at 10% CO_2 to a confluency of 70-80%. The media was removed and the cells were washed once in pre-warmed sterile Dubecco's phosphate buffered saline (PBS), and then pre-warmed sterile Trypsin-EDTA was added. Cells were incubated at 37˚C in Trypsin-EDTA until cells began to detach from the plastic plate. Typically this takes 1-2 mins, however it can vary between cell lines. Cells were re-suspended in fresh DMEM to neutralise the trypsin and pipetted up and down to disperse any clumps of cells. The resulting cell suspension was diluted at the desired ratio (usually 1:10) into new plates, which contained fresh medium. Subculturing procedures were carried out in a laminar flow hood in a sterile environment using media/reagents that were all pre-warmed to 37°C.

2.3.2 Freezing Cell lines

Healthy cells of a low passage number (passage 2-5) were grown to about 80% confluency in DMEM, supplemented with 10% FBS and 5% penicillin/streptomycin at 37˚C, 10% CO_2. After media was removed, the cells were washed with PBS and treated with Trypsin-EDTA as described for the maintenance of cell lines. Cells were resuspended in fresh DMEM and spun for 5 mins at 1000 RPM. The medium was removed and cells were re-suspended in 90% FBS, 10% Dimethyl Sulphoxide (DMSO). The cell suspension was

placed in freezing vials and stored at -80˚C overnight in a box containing isopropanol, which allows the vials containing the cells to slowly come to temperature, preventing cell death. Cells were then placed in liquid nitrogen storage tanks for future use.

2.3.3 Defrosting Cell lines

To maintain cell lines, the vial containing cells was thawed quickly in a 37˚C water bath. The outside of the vial was decontaminated with 70% ethanol just before cells were completely thawed, and the contents were transferred to DMEM. The cells were spun at 1000 RPM for 5 mins and the pellet of cells were re-suspended in fresh DMEM and transferred to a new plate, which also contained fresh DMEM. The cells were incubated in a humidified, 37˚C, 10% CO_2 incubator and checked daily until they reached 80-90% confluence (2-3 days). The cells were further maintained, or harvested for subsequent experiments.

2.3.4 Cell counting

DMEM was removed from plated cells, which were then washed with 1X PBS and trypsin as described for maintenance of cell lines (2.2.1.1). Un-detached cells were mixed with DMEM and spun at 1000 RPM for 5 mins. The cell pellet was re-suspended in fresh PBS, a 2 µL aliquot of which was added to a vial containing 10 mL CASYTON Buffer solution (Innovatis). The cells were thoroughly mixed with the buffer and then analysed using the CASY Model TT cell counter (Innovatis), which relies on electronic pulse area analysis for measurements. The CASY machine enables the measurement of total and viable cell numbers, cell size, percentage viability and cell aggregation factors.

2.3.5 Inducible gene expression in cell lines

Inducible cell lines were generated using the Invitrogen T-REX system. Parental Hek293 cells which stably express the tetracycline (tet) repressor were purchased from

Clonetech, whereas Hek293 cell lines over-expressing CoA Synthase wild type (CoASy WT) and a CoA Synthase that had been mutated at position 203 from Histidine to Alanine (CoASy*203) were previously generated in the laboratory. To induce protein expression, a final concentration of 5 mg/µl tetracycline was added to cells in DMEM once they had reached a confluency of 50% and cells were incubated for 24 hrs at 37˚C, 10% CO_2.

2.3.6 Transient transfection

24 hrs prior to transfection, HepG2 or Hek293 cells were seeded into 6 cm plates containing fresh DMEM (+ 10% FBS) with a starting confluency of 25%. After 24 hrs incubation at 37°C, 10% CO_2, medium was removed and replaced with fresh DMEM (+ 10% FBS) prior to the addition of the transfection mixture. 5 µg of the required DNA was added to 200 µL sterile 150 mM NaCl solultion. 16.5 µL of ExGen500 reagent (Fermentas) was subsequently added and the mixture was immediately mixed for 10 s. The resultant transfection mixture was incubated at room temperature for 10 mins, followed by drop-wise addition to the appropriate plates of cells. Transfected cells were incubated for a further 24 hrs at 37°C, 10% CO_2 before analysis.

2.3.7 Generation of Stable cell lines

Stable cell lines were generated using the pcDNA3.1™(+) vector, which contains a neomycin (Geneticin®) resistance gene for selection. The gene of interest was cloned into pcDNA3.1(+) and the resulting vector constructs were used for transfection into HepG2 and Hek293 cells. 24 hrs after transfection, cells were washed and fresh medium was added. 48 hrs after transfection, cells were split so that the cells were no more than 25% confluent into 6 well plates containing fresh medium supplemented with 800 µg/mL Geneticin®. Selective medium was replaced every 3-4 days of exponential growth to remove dead cells and to provide fresh media to encourage positively-selected cell growth. Once non-transfected control cells were completely dead, the resultant cells that survived selection

were amplified in media supplemented with 200 µg/mL Geneticin®, and checked for protein over-expression.

2.3.8 Serum deprivation and stimulation of cells

Hek293 cells were starved at approximately 70% confluency using DMEM high glucose medium with L-glutamine and penicillin/streptomycin (5% v/v), but without FBS for 24 hrs. Serum stimulation is achieved by the addition of 10% fetal bovine serum for 1-6 hrs followed by harvesting the cells.

2.3.9 Treatment of Cells with Extracellular Stimuli

In this thesis, HepG2 and Hek293 cells were treated with a number of extracellular stimuli including nutrients, hormones, cell signalling inhibitors and cell permeable peptides. All incubations were carried out in 6 or 10 cm tissue culture plates at 37°C, 10% CO_2. HepG2 cells grown to 70% confluency were used for nutrient studies with glucose and fatty acids. Before nutrient treatment, cells were incubated for 16 hrs in DMEM, containing 5 mM glucose and 1% BSA. HepG2 cells were then stimulated with glucose concentrations ranging from 0-20 mM, 0.5 mM palmitate, 1 mM carnitine or 0.5 mM palmitate + 1 mM carnitine for either 6 or 24 hrs. For insulin studies, HepG2 and Hek293 cell lines at 60-70% confluency were starved of FBS for 24 hrs, and then stimulated with 10 nM (HepG2) or 100 nM (Hek293) insulin for times ranging between 1 and 24 hrs. In studies involving cell signalling inhibitors and other small molecule treatments, after reaching a confluency of 60-70%, HepG2 or Hek293 cells were incubated in DMEM + 10% FBS in the presence 100 nM Rapamycin, 2.5 µM PP242, 50 µM LY294002, 20 µM PD98059, 0.5 mM Bezafibrate, or 100 µM pantethine for 24 hrs at 37˚C, 10% CO_2. All these small molecular compounds were made up fresh in DMSO, immediately before treatment. For studies involving pantothenate and Hopantenate (Hopan), HepG2 cells were seeded at a confluency of 30% and incubated for 4 hrs in DMEM. DMEM was then removed and cells were washed with

PBS before incubating cells in pantothenate free DMEM with 0, 1 or 16 µM pantothenate +/- 200 µM Hopan, for 48 hrs. For studies with cell penetrating peptides, HepG2 cells were grown to 70% confluency, and then washed in PBS. Cells were subsequently incubated in FBS free DMEM with 10 µM of the TAT peptide, or poly-arginine for 1 hr.

2.3.10 Growth Assay

Growth analysis was carried out for HepG2 cells in pantothenate free media; Hek293 cells incubated with the HDAC inhibitor, trichostatin A (TSA) (0.1-2 µM); and Hek293 cells over-expressing PanK1β. Cells were seeded into 96 well plates at either 100 cells/well (pantothenate free media) or 2000 cells/well (all treatments) in 100 µL medium and the number of viable cells was measured using the CellTitre-Blue® Viability Assay (Promega). This assay uses the indicator dye resazurin to measure the metabolic capability of cells. Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent at $579_{Ex}/584_{Em}$. 20 µL of the CellTitre-Blue® reagent was added to the medium and incubated for 2 hrs at 37°C, 10% CO_2 before analysis using the Pherastar plate reader system with a $560_{Ex}/590_{Em}$ filter set (BMG Labtech).

2.4 CoA Isolation

2.4.1 Harvesting Mammalian cells

Harvesting of Hek293 and HepG2 cells took place once cells reached a confluency of about 90% or after the required treatment time. Growth medium was removed from the cells, while plates were on ice and immediately 5-10 mL ice cold PBS was carefully added to wash the cells and remove any residual growth medium. As soon as PBS was removed, 0.5 mL ice cold 5% Perchloric Acid (PCA) (VWR) was added to the plate of cells on ice and cells were detached by scraping. It is important to lyse the cells in 5% PCA as quickly as possible to reduce any changes in CoASH: CoA ester levels that may occur due to

the stressful change of conditions. The lysate was pipetted up and down several times to disperse any clumps of cells. Lysates were transferred into a pre-chilled 1.5 mL tube and kept on ice before collection for CoASH extraction. Alternatively for protein analysis, see section 2.7.1.

2.4.2 CoA extraction

Lysed cells were centrifuged at 4˚C for 5 mins, 13 000 RPM. The supernatant containing CoASH and short chain acyl CoA esters was transferred into a pre-weighed mirocentrifuge tube and placed on ice. The PCA pellet containing DNA and long chain acyl-CoA was weighed for later standardisation. 0.1 mL 1M Triethylamine (TEA)/mL PCA extract was added before neutralising the sample with 1M potassium carbonate (K_2CO_3) to pH 6.5. The extract was spun for 5 mins, 10000 RPM and the supernatant was transferred into a fresh, pre-weighed microcentrifuge tube. The PCA extract was then concentrated for about 1.5 hrs using a speed vac concentrator, until 100-200 µL remained. The cell extract was then analysed by the enzymatic recycling assay (section 2.5.1) or HPLC (section 2.5.2).

2.4.3 Alkaline Hydrolysis

In order to measure total CoA esters present in a cell sample, alkaline hydrolysis of the acid soluble supernatant (short chain CoA esters) or the acid precipitated pellet (long chain acyl CoA esters) was carried out. For analysis of short chain esters, 50 µL of the supernatant collected from PCA treatment of cells was mixed with 50 µL KOH to give a final concentration of 0.2 M. The rest of the supernatant was analysed normally for CoASH and acetyl CoA. Acid soluble KOH treated extracts were then incubated at 30°C for 30 mins before cooling on ice and neutralising with 10 µL 10% PCA. The extract was spun for 10 mins at 13000 RPM and the pH of the supernatant was adjusted to pH 6.5, followed by sample concentration as described in section 2.4.2. Long chain acyl CoA esters were analysed by washing the PCA pellet once with 2% PCA and once with 0.2 mL H_2O. The pellet

was then resuspended in 90 µl 0.5 M KOH + 5 mM DTT and incubated at room temperature for 15 min. The CoA released from this hydrolysis procedure was analysed by the recycling assay following neutralisation with 100 mM TEA and 2M HCl.

2.4.4 *N*-ethylmaleimide treatment

Treatment of neutralised PCA extracts with *N*-ethylmaleimide (NEM) enables CoASH and acetyl CoA to be measured separately using the recycling assay (section 2.5.1). NEM reacts with the sulfhydryl group of CoASH, so that acetyl CoA alone is determined. Neutralised cell extracts were divided in two so half could be used to measure combined CoASH and acetyl CoA and the other half for the measurement of just acetyl CoA. Free CoASH can be calculated from the difference between these two measurements. For acetyl CoA determination, the extract was incubated with 8 mM NEM for 5 mins, 30°C. Excess NEM was removed following a further incubation of the mixture with 8 mM DTT for 5 mins, 30°C. The NEM treated extract was then analysed by the recycling assay (section 2.5.1).

2.5 CoA analysis

2.5.1 Recycling Assay

The concentration of CoA in tissue and cultured cell extracts can be determined using the following enzymatic assay where CoASH is recycled through two enzyme catalysed reactions, originally described by Allred and Guy (Allred and Guy 1969). The rate of NADH formation produced during this reaction is proportional to CoA concentration in both its free and acetylated form. CoASH is recycled through the following reaction sequence:

1) Acetyl phosphate + CoASH $\xrightarrow{\text{Phosphotransacetylase}}$ acetyl CoA + phosphate

2) Acetyl CoA + oxaloacetate $\xrightarrow{\text{Citrate Synthase}}$ citrate + CoASH

3) Malate + NAD^+ $\xrightarrow{\text{Malate Dehydrogenase}}$ oxaloacetate + NADH

Reaction 1 is catalysed by phosphotransacetylase, reaction 2 is catalysed by citrate synthase and reaction 3 is catalysed by malate dehydrogenase. The overall reaction is shown below:

$$\text{Malate + acetyl phosphate + } NAD^{+} \xrightarrow{\text{(CoASH + acetyl CoA)}} \text{citrate + phosphate + NADH}$$

When all the necessary substrates and enzymes are supplied in excess, the rate of the over-all reaction is limited by the CoASH concentration. NADH production can be measured spectrophotometrically. The analysis of CoA by the recycling assay was carried out using a 96-well plate reader (Magellan), with a final reaction volume of 200 µL. Solutions of reagents were combined in a premix, just prior to use in the proportions shown in *Table 2.1*.

Component	Amount per reaction (200 µl reaction volume)
Tris Buffer (1M, pH 7.2)	60 µl
Potassium Chloride (1M)	12 µl
Malate (0.2M)	12 µl
Acetyl Phosphate (0.08M)	12 µl
NAD^{+} (0.2M)	12 µl
Malate dehydrogenase	1 unit
Citrate synthase	2 units

Table 2.1: Components of the recycling assay

50 µL PCA cell extracts were combined with 4 mM DTT to prevent the oxidation CoASH into dimers, or to form heterodimeric complexes with other thiols, such as glutathione. 50 µL CoASH standards ranging from 0-200 pmol were made up in a final concentration of 4 mM DTT to ensure all components of the assay were working correctly. The extract/standards were combined with 110 µl of the premix in a 96-well plate and incubated for 5 mins at 30˚C. 40 µL Phosphotransacetylase (3.5 units/reaction) that had

also been pre-incubated at 30˚C was added to start the reaction. The change in NADH (Δ340) was measured for 10 mins at 30°C. As the light path travels through the reaction solution, it is essential that the reaction volume is the same in each well.

2.5.2 HPLC

High performance liquid chromatography (HPLC) is a form of chromatography used to separate, identify and quantify compounds based on their different polarities and interactions with the stationary phase. During this process, a solvent (mobile phase) is forced through a column (stationary phase) under high pressure.

CoA and acetyl CoA were measured by reverse phase HPLC. The HPLC system used consisted of Jasco PU-980 pump and UV-975 UV/visible detector. The column used was a 100 X 4.60 mm Kinetex C18 column (Phenomenex), with a silica particle size of 2.6 µM and 100 Å pore size. The column temperature was maintained at 40°C using a column heater. The mobile phase initially consisted of 150 mM NaH_2PO_4 and 8.5% methanol filtered and degassed through a 0.22 µM filter and the flow rate was 1 mL/min. Following a 20 min isocratic run under these conditions, methanol was gradually increased to 30% and flow reduced to 0.5 mL/min over 5 min. This was followed by a linear gradient back to the initial conditions over 5 min and the column was reequilibrated under initial conditions for 11 min. Each run lasted for 41 min and CoASH and CoA esters were detected by absorbance at 254 nm which are emitted as a series of peaks. The elution profile was stored and processed using Bowing software. Amounts of CoA compounds were determined by the comparison of peak areas with those of standards.

2.5.2.1 Sample preparation

50 µL neutralised PCA extract was incubated with 10 mM TCEP (*tris*(2-carboxyethyl)phosphine) for 10 mins, 30°C, to prevent the oxidation of CoASH (for example, to glutathione CoA). Before injection, the total volume was adjusted to 100 µL

(for a 50 µL injection) with HPLC quality H_2O, 1.5M NaH_2PO_4 (sodium phosphate), and methanol so that the concentration of NaH_2PO_4/methanol is the same as that of the mobile phase (150 mM NaH_2PO_4 in 8.5% methanol).

2.5.2.2 Standard preparation

It is important to run standards of CoASH/acetyl CoA to determine the correct peaks for CoASH and acetyl CoA. Standards were made up as shown in *Table 2.2*:

Component	Volume required (µL)
HPLC quality H_2O	61.5
TCEP (100 mM)	10
20 µM CoA/acetyl CoASH standard	5 (each)
1 M NaH_2PO_4	10
Methanol	8.5

Table 2.2: Standard preparation for HPLC

Once prepared, 100 µL of the sample or standard was injected into the HPLC machine for a 50 µL injection volume and a flow rate of 1 mL/min. The peaks formed on the chromatogram were integrated to calculate the amount of CoASH/acetyl CoA present in the sample.

2.6 CoA binding Partners

2.6.1 CoA Sepharose preparation

CoA Sepharose is prepared by covalently coupling CoA to sepharose previously activated with cyanogen bromide (CNBr) (Sigma). The sepharose beads were treated with 1 mM HCl and allowed to swell for 15 mins, before being washed twice with 1 mM HCl and 100 mM phosphate buffer (pH 6). The washed beads were then incubated with 100 mM phosphate buffer, pH 6, containing 6 mM CoA for 3 hrs at room temperature with gentle

mixing. After washing once with 100 mM phosphate buffer followed by 100 mM Tris/HCl, pH 6.8, the beads were incubated with 100 mM Tris/HCl, pH 6.8, for 2 hrs at room temperature to block unreacted CNBr. Beads were stored as a 50:50 mix in storage buffer (50 mM Tris/HCl pH 5.6, 250 mM NaCl, 10 mM DTT) at 4˚C until required for affinity binding analysis.

2.6.2 Determination of coupling efficiency by Ellman's test

Small samples from the beginning and end of the CoA coupling reaction (2.6.1) were incubated with 5, 5'-dithiobis (2-nitrobenzoate) (DTNB) reagent (0.1 mM DTNB + 100 mM Tris/HCl pH 8 + 2.5 mM sodium acetate) in a 96 well microtitre plate for 30 mins at room temperature. During this reaction, DTNB readily undergoes thiol-disulphide exchange with free thiols to produce 2-nitro-5-thiobenzoate, which absorbs light at 412 nm. The coupling efficiency of CoA was calculated by reading the absorbance at 412 nm to determine the amount of CoASH that was still present in the solution after the coupling reaction.

2.6.3 Preparation of Rat Liver homogenates

Frozen, powdered liver was homogenised in 1 mL/0.1 g liver tissue ice cold Homogenisation Buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 10% glycerol, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF and 0.2 µl/mL protease inhibitor cocktail (Sigma P8340)) using a tissue disintegrator (Ultra Turrax). Triton X-100 (final concentration of 1%) was added to the homogenate and this was left on ice for 30 mins. The homogenate was centrifuged for 10 mins, 13000 RPM, 0˚C, and the supernatant was collected for analysis of CoA binding proteins.

2.6.4 Preparation of mammalian cell lysates

Cells (Hek293/HepG2) were washed twice with ice-cold PBS and extracted with ice cold Homogenisation Buffer and 1% Triton X-100. Cells were scraped from the dishes and transferred to micro centrifuge tubes. Tubes were incubated on ice for 30 mins prior to centrifugation at 13,000 g at 4°C for 20 mins to remove insoluble material. Supernatants were transferred into fresh tubes and the total protein concentrations of the lysates were determined by Bradford protein assay (2.7.2).

2.6.5 Affinity binding with CoA Sepharose

Cell lysates/liver homogenate (200 µL) containing approximately 0.6 mg protein were incubated with either 200 µL CoA coupled Sepharose beads or control beads (CNBr Sepharose blocked with Tris) for 2 hrs at 4˚C with gentle mixing. For experiments determining the orientation of CoA binding, de-sulpho CoA, or CoA pre-treated with NEM or maleimide were coupled to Sepharose. Beads were washed once and then incubated with Homogenisation Buffer containing 1% Triton X-100 for 15 mins at 4˚C with gentle mixing. After washing twice with 50 mM Hepes pH 7.4 and 1 mM DTT, 1X SDS loading buffer was added to the beads to release bound proteins. The mixture was heated for 10 mins at 99˚C before proteins were separated on 4-12% Bis-Tris gel (2.2.5.3). For more specific analysis of CoA binding proteins, proteins were eluted with increasing concentrations of CoA (10, 50, 100 mM) and increasing concentrations of LiCl (30, 50, 300 mM) as a control, before resolving proteins by SDS-PAGE analysis. In experiments investigating CoA binding to the Sepharose, CoA Sepharose beads were pre-treated with 100 mM maleimide before resolving proteins by SDS-PAGE analysis. When checking the specificity of proteins binding to CoA Sepharose, proteins were eluted with 100 mM CoA, ADP or pantothenate before resolving by SDS-PAGE analysis. Following SDS-PAGE analysis, proteins were visualised by silver or Coomassie staining (2.7.4).

Proteins from liver lysate that were to be analysed by mass spectrometry were eluted from Tris and CoA Sepharose with 100 mM CoA, boiled in SDS loading buffer, resolved by SDS-PAGE analysis and Coomassie-stained. Each-lane was sliced into 30 pieces and then digested with trypsin. The identity of the proteins in these samples was analysed by mass-spectrometry in collaboration with N. Totty, Cancer Research UK.

CoA binding assays were also carried out with the individual proteins, lactate dehydrogenase (LDH), aldolase and glucose-6-phosphate dehydrogenase (G6PDH). 20 µL CoA and Tris Sepharose beads were washed in 1 mL TBS (50 mM Tris HCl pH 7.5, 150 mM NaCl) before incubating with 0.5 mL TBS containing 50 µg LDH, aldolase or G6PDH for 2 hrs, 4°C with mixing. Beads were washed again in TBS and proteins that bound to the beads were released by boiling in SDS loading buffer. Proteins were resolved by SDS-PAGE analysis and Coomassie staining.

2.7 Isolation and Analysis of Cellular Protein

2.7.1 Preparation of mammalian cell lysates

Cells were washed twice with ice-cold PBS and extracted with EB buffer (20 mM Tris HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, EDTA-free protease inhibitor cocktail (Roche)). Cells were scraped from the dishes and transferred to micro centrifuge tubes. Tubes were incubated on ice for 30 mins prior to centrifugation at 13,000 g at 4°C for 20 mins to remove insoluble material. Supernatants were transferred into fresh tubes and the total protein concentrations of the lysates were determined by Bradford protein assay (section 2.7.2).

2.7.2 Determination of protein concentration in solution

The total protein concentration in cell lysates was determined using the Coomassie (Bradford) protein reagent (Pierce). The reagent was diluted 1:1 with ddH_2O to give the working solution. Samples were made up by adding 1 µL of each lysate sample to 1 mL of reagent solution and standards were prepared by diluting appropriate amounts of 2 mg/mL BSA (Pierce) to give a range from 0.5 – 8 µg/mL. All absorbance measurements were taken on an Eppendorf BioPhotometer at 595 nm using plastic 1 mL cuvettes.

2.7.3 SDS-PAGE

Cellular proteins were resolved using a modification of the discontinuous SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) system, described by Laemmli (Laemmli 1970). When proteins are denatured in SDS (an anionic detergent) they become coated in detergent molecules resulting in a uniform negative charge to mass ratio. This allows separation of the denatured proteins based solely upon their size, as charge differences present in the native state are negated. In SDS-PAGE, a potential is applied across a polyacrylamide gel. As the proteins migrate from the cathode to the anode, the larger proteins are impeded by the matrix, while smaller proteins are able to move more easily through the gel.

In this study, mini-PROTEAN® TGX™ (Tris-Glycine eXtended) precast gels were used to resolve proteins. These gels retain Laemmli-like separation characteristics, however have an increased gel matrix stability. Gels with 4-20% polyacrylamide concentration were typically used and were run using the Mini-PROTEAN Tetra Cell electrophoresis system (BioRad).

2.7.3.1 Protein sample preparation

Samples were prepared for SDS-PAGE analysis by adding the appropriate amount of 5X Laemmli sample buffer (250 mM Tris pH 6.8, 50% glycerol, 0.5% bromophenol blue,

500 mM DTT, 10% SDS) to a final concentration of 1X. All samples were boiled for 5 mins at 99°C and briefly centrifuged at room temperature prior to loading onto the polyacrylamide gel.

2.7.3.2 Gel Electropheresis

Samples were loaded into the appropriate wells of the mini-PROTEAN precast gels using a Hamilton syringe (Hamilton, Reno, Nevada). Gels were run in 1X Tris/glycine/SDS buffer (25 mM Tris, 192 mM glycine, 0.1% [w/v] SDS) at 300 V constant voltage until the dye reached the end of the gel. Ice packs were added to the running buffer in the tank to prevent the temperature rising due to the high voltage. Visualisation and analysis of separated proteins were performed as described below.

2.7.4 Visualization of proteins

2.7.4.1 Coomassie Stain

Following electrophoresis, gels were stained by immersion in Coomassie Blue stain (0.5% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid) with gentle agitation for 20 min, followed by de-staining in 20% (v/v) methanol, 10% (v/v) acetic acid for several hrs. The gel was then dried under vacuum at 80°C for 1 hr. Coomassie brilliant blue binds to proteins stoichiometrically, so this staining method is preferable when relative amounts of protein are to be determined by densitometry.

2.7.4.2 Silver staining

When more sensitive staining was required, gels were silver stained using a silver staining kit from Fermentas (PageSilver). The gel was first fixed in a fixative solution (50% ethanol, 10% acetic acid) for 10 mins. The fixed gel was washed two times in 30% ethanol for 10 mins, followed by two washing cycles with ddH_2O for 20 s. The gel was agitated with sensitizing solution (0.4% sensitizer concentrate) for 1 min, and after the sensitizing

solution was removed, it was washed twice in ddH_2O for 20 s. The gel was stained with staining solution (4% stainer, 0.054% formaldehyde) for 20 mins and after two wash cycles with ddH_2O, developing solution (10% sensitizer concentrate, 10% developing reagent, 0.027% formaldehyde) was added. The gel was left to develop for 4 mins, or until the required intensity of bands was reached. Staining was stopped by the addition of stopping solution (8% stop reagent), with gentle agitation for 5 mins. After the stopping solution was discarded, the gel was stored in ddH_2O.

2.7.5 Immunoblot analysis (Western Blot)

2.7.5.1 Transfer to Nitrocellulose membrane

After separation using SDS-PAGE, proteins were transferred to nitrocellulose membranes using the Trans-Blot® Turbo™ transfer system (BioRad) for analysis by western blotting. Pre-cut Trans-Blot Turbo transfer packs containing filter paper, transfer buffer and a nitrocellulose membrane were generally used in this study. The gel was removed from the cast and briefly rinsed in H_2O before placing in between the turbo transfer pack, directly above the membrane. This stack was placed onto the Trans-Blot® Turbo™ transfer system with the membrane closest to the anode (bottom). Air bubbles were expelled by rolling over the stack with a blot roller. The transfer process was run constantly at 1.3 A for 7 mins.

2.7.5.2 Immunoblotting of transferred membranes

Membranes with transferred proteins were washed briefly in TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) before incubation in Western blocking buffer (TBST containing 5% (w/v) non-fat dried milk powder or 5% BSA for phosphospecific antibodies) for 45 mins at room temperature to avoid non-specific binding of antibodies to the membrane. Membranes were washed 4 times for 5 mins in TBST before probing overnight at 4°C with the appropriate primary antibody diluted (as recommended by manufacturer)

in primary antibody buffer (2% BSA and 0.02% sodium azide). The membrane was transferred to fresh TBST buffer and washed 3 times for 10 mins. The membranes were then probed with the appropriate diluted HRP-linked secondary antibody in TBST, 5% milk for 1 hr at room temperature and were then washed 3 times for 10 mins before development by enhanced chemiluminescence (ECL).

2.7.5.3 Developing Immunoblots

An equal volume of ECL reagent 1 (50 mM Tris-HCl pH 8.5, 1% luminol, 0.44% coumaric acid) and ECL reagent 2 (50 mM Tris-HCl pH 8.5, 0.02% H_2O_2) were mixed and incubated with the membrane for 1 min at room temperature. Excess ECL reagent was removed by blotting and the membrane wrapped in Saran wrap. Bands were detected using the Fujifilm LAS-1000 imaging system and densitometric analysis was performed using the Quantity One™ image processing software (BioRad). In some instances Immobilon western chemiluminescent HRP substrate (Millipore) was used to increase the sensitivity. This required extensive washing before development to reduce background staining and was incubated with the membrane for 5 mins.

2.7.6 Stripping and re-probing western blots

In some instances, membranes were required for re-probing with a different antibody. Antibodies were first stripped from the membrane by incubating in freshly prepared Stripping buffer (62.5 mM Tris-HCl, pH 6.7; 2% SDS; 100 mM 2-mercaptoethanol) at 50°C for 30 mins. Extensive washes in TBST buffer were required before blocking and then re-blotting with another primary antibody.

2.7.7 LDH assay

The LDH assay was carried out as a measure of standardisation and also when determining the effect of CoA on the activity of LDH. For standardisation studies 190 µL

reaction mix (65 mM Tris HCl, pH 7.5, 10 mM NADH, 40 mM pyruvate) was incubated with 10 µL of lysed sample (see 2.7.1) for 10 mins, 25°C and ΔA_{340} was measured spectrophotometrically using a 96-well plate reader (Magellan). For experiments investing LDH activity with CoA, 170 µL reaction mixture was incubated with 20 µL CoA (10, 50 or 100 µM) for 5 mins at 25°C to achieve temperature equilibrium. The solution was then quickly mixed with 10 µL LDH in Tris HCl, pH 7.5 (0.5 U) and ΔA_{340} was measured for 10 mins using a 96-well plate reader (Magellan).

2.7.8 G-6-PDH assay

To determine the effect of CoA on G-6-PDH activity, 170 µL of the reaction mix (50 mM Tris HCl, pH 7.5, 2 mM glucose-6-phosphate, 0.5 mM NADP, 10 mM $MgCl_2$) was incubated with 10 µL G-6-PDH (0.0175 U) alone or in the presence of 20 µL CoA (5, 50 or 500 µM). The reaction was started with the quick mixing of G-6-PDH with the rest of the components of the assay and ΔA_{340} was measured for 10 mins at 25°C using a 96-well plate reader (Magellan).

2.8 Metabolic Studies

Metabolic studies were carried out in HepG2 cells that had reached a confluency of 50%. Culture medium was removed and cells were washed in PBS. Glucose oxidation was measured by incubating cells for 0-120 mins at 37°C in 1.5 mL PBS containing 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 5 mM glucose, 2% BSA and D-[^{14}C(U)] glucose (5 µL/mL)(0.1 µCi/mL) (PerkinElmer). After the required incubation time, the incubation medium was acidified with a final concentration of 5% PCA to release [^{14}C]CO_2, which was trapped through further incubation with 0.5 mL benzethonium hydroxide for 1 hr at 37°C in a sealed container. [^{14}C]CO_2 containing benzethonium hydroxide was mixed with 10 mL Ecoscint A and [^{14}C]CO_2 was analysed by scintillation counting.

Palmitate oxidation was measured following incubation of cells for 0-120 mins at 37°C in 1.5 mL PBS containing 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 5 mM glucose, 1% BSA, 0.1 mM [9,10 – ^{3}H] palmitate bound to 10% BSA (1 µCi/mL) (PerkinElmer). After the required incubation time, the incubation medium was acidified with a final concentration of 5% PCA and ^3H_2O produced from palmitate oxidation was measured following extraction with organic solvents. Initially the acidified incubation medium was mixed with 1.88 mL chloroform: methanol (1:2 v/v), followed by 0.625 mL chloroform and 0.625 mL 2M HCl, which was then mixed by vortexing. The upper aqueous phase was collected after centrifuging for 5 mins at 4000 RPM, and treated with 0.3 mL chloroform, 0.3 mL methanol and 0.27 mL 2M KCl: 2M HCl. The mixture was centrifuged again for 5 mins at 4000 RPM and 1 mL of the aqueous phase was added to 10 mL Ecoscint A for analysis by scintillation counting.

Lactate production was measured by incubating cells in 1.5 mL PBS containing 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 5 mM glucose for 0-6 hrs. Cells were lysed by adding a final concentration of 5% PCA to the incubation media at the required time point. The lysed extracts were neutralised with K_2CO_3 before incubating 50 µL with 130 µL of the reaction mix (0.65 M glycine, 0.25 M hydrazine, 5 mM EDTA) for 5 mins, 30°C. 20 µL LDH (2U/mL) was added to start the reaction and the total difference in 340 nm absorbance was measured when the reaction was complete.

2.9 Statistical Analysis

Statistical comparisons were performed by Student's t-test (using Microsoft Excel). Values were expressed as mean ± standard error of the mean (SEM). n designates the number of independent experiments.

Chapter 3: Results

Developing methods for measuring CoA in mammalian tissues and cultured cells

Chapter 3: Developing methods for measuring CoA in mammalian tissues and cultured cells

3.1 Introduction

Due to the functional imperative of CoA and its acyl derivatives in many metabolic processes, a large number of methods have been developed to separate, detect and quantify these molecules from biological samples. Enzymatic assays were the first methods used to measure tissue contents of CoA and its derivatives. Several non-enzymatic methods were also available, including adenine absorption, polarography and Ellman's reagent, however these were generally non-specific (Stadtman 1957; Ellman 1959; Weitzman 1966). Initially, end point enzymatic assays were commonly preferred as they were simple, convenient and the enzymes were often commercially available. They involved measuring the equilibrium concentration of a final product in a reaction that required CoA. Examples include spectrophotometric or fluorometric measurement of sorboyl CoA using purified acyl CoA synthetase, of NAD^+ production following reactions involving ATP citrate lyase and malate dehydrogenase, of NAD^+ production following the reaction involving 3-hydroxyacyl CoA dehydrogenase and by following the molar extinction change of Phosphotransacetylase (Bergmeyer 1963; Michal and Bergmeyer 1963; Pearson 1967). The main issue with these stoichiometric assays was the sensitivity, which often was in the range of μmoles, whereas CoA levels in most tissues are in the nanomolar range. Catalytic enzymatic reactions tend to be more sensitive, in which the rate of the formation of a product is measured spectrophotometrically or fluorometrically. Reactions involving arsenolysis of acetyl phosphate by phosphotransacetylase, acetylation of arylamine by arylamine acetyltransferase and oxidation of 2-oxoglutarate by oxoglutarate

dehydrogenase with succinyl CoA breakdown are examples of catalytic methods (Stadtman et al. 1951; Von Korff 1953; Bergmeyer 1963; Garland 1964). Although the sensitivity is improved with the latter approaches, there are still some shortcomings with regards to specificity, sensitivity and linearity. These issues have greatly been improved with enzymatic recycling methods, where CoASH is continuously cycled around several enzymatic reactions; the product formed for a given quantity of CoASH is larger than in equilibrium methods and becomes greater as the reaction time increases (Allred and Guy 1969; Kato 1975).

A general issue when measuring CoA levels using chemical or enzymatic assays is that these methods are indirect and assay components can potentially be affected by unknown compounds present in the CoA sample extract. Alternative chemical methods were consequently developed for direct measurement of CoA and it was found that high-performance liquid chromatography (HPLC) was a very useful analytical tool. Furthermore, HPLC enables the measurement of a much wider range of CoA compounds than previously possible by enzymatic assays. In the appropriate conditions, CoA is retained on a strong anionic exchange resin, which makes up the stationary phase of the HPLC column. The negative charge on CoA enables it to bind to the stationary phase and different compounds bind with different strengths, allowing separation and measurement of these components. A mobile phase is pumped through the column along with all the analytes within the sample, which are then detected according to a characteristic retention time. HPLC of acyl CoA compounds was first described by Baker and Schooley, where reverse phase paired-ion chromatography was used to separate acyl CoA thioesters of different length chains (Baker 1979). Subsequently, HPLC methods were developed to measure free CoASH in biological samples (Ingebretsen and Farstad 1980).

HPLC can be coupled to different detection methods for the analysis of CoA. HPLC coupled to spectrophotometric detectors which measure UV or fluorometric absorption of the CoA adenine moiety are commonly used methods for measuring CoASH and CoA esters (King and Reiss 1985; Patel and Walt 1987; Valentin and Steinbüchel 1994). Spectrophotometric detection cannot provide molecular mass or structural information that can positively identify CoASH or its thioesters, and also is restricted by the availability of pure standards. Methods coupling mass spectrometry to HPLC have been reported and overcome the limitations of HPLC coupled to UV/fluorometric detection and have very high specificity and selectivity. Mass spectrometry involves the ionization of chemicals, often through continuous fast atom bombardment with an electron beam, generating charged molecules which can be identified by measuring their mass-to-charge ratio. Although effective, this method depends on complex dedicated instrumentation, which is expensive, unreliable and requires expertise. HPLC coupled to radioactive CoA is another technique that has been used to measure CoASH and its thioesters. This approach removes the 3' phosphate from CoA and replaces it with ^{33}P by initially treating with phosphatase and then with DPCK and [γ-^{33}P]ATP. The resulting ^{33}P-labelled CoA compounds are then separated by HPLC and quantitated by scintillation counting. Radioactive labelling only occurs on CoA and its esters due to the specificity of DPCK. Other than the fact radioactivity is used in this method, the main drawback of this procedure is that it relies on the activities of the phosphatase and DPCK enzymes where sample components could potentially affect their enzymatic activity.

3.2 Detection Methods

Methods for CoA extraction and measurement in tissues are well-established. However, there are a limited number of studies reporting CoA levels in cultured cells. Therefore, the first objective was to establish a reliable method for measuring the levels of

CoASH and CoA esters in cultured cells. The process by which CoA levels are determined from cultured cells occurs in several steps (*Figure 3.1)*. The detection methods which have been explored here are an enzymatic recycling assay (Allred and Guy 1969), HPLC (Corkey et al. 1981) and mass spectrometry.

3.2.1 Recycling Assay

The recycling assay is a well-established enzymatic method for measuring CoA in tissues (Allred & Guy 1969). The reactions are as follows:

$$\text{Acetyl phosphate + CoASH} \xrightarrow{\text{Phosphotransacetylase}} \text{acetyl CoA + phosphate}$$

$$\text{Acetyl CoA+ oxaloacetate} \xrightarrow{\text{Citrate Synthase}} \text{citrate + CoASH}$$

$$\text{Malate + } NAD^{+} \xrightarrow{\text{Malate Dehydrogenase}} \text{oxaloacetate + NADH}$$

CoASH is recycled and consequently amplified through two enzyme-catalysed reactions. The rate of NADH formation is proportional to CoA concentration, in both its free and acetylated form (*Figure 3.2*). Of the enzymatic assays reported, this assay was the most sensitive and since all the required enzymes were commercially available, it appeared to be a convenient method to use. To further increase sensitivity, 96 well plates and a plate reader was utilised.

3.2.1.1 Reaction time

It is necessary to measure the change in A_{340} over a time period where it is increasing linearly so that an accurate rate of the reaction can be determined. CoA standards in the range expected to be present in cell extracts (20-80 pmol) were incubated with all of the components of the recycling assay for an hr. A reaction time of 10 mins was decided upon, since this was a time period over which even the higher concentrations of CoA were linear, yet at the same time enough data points would be collected for

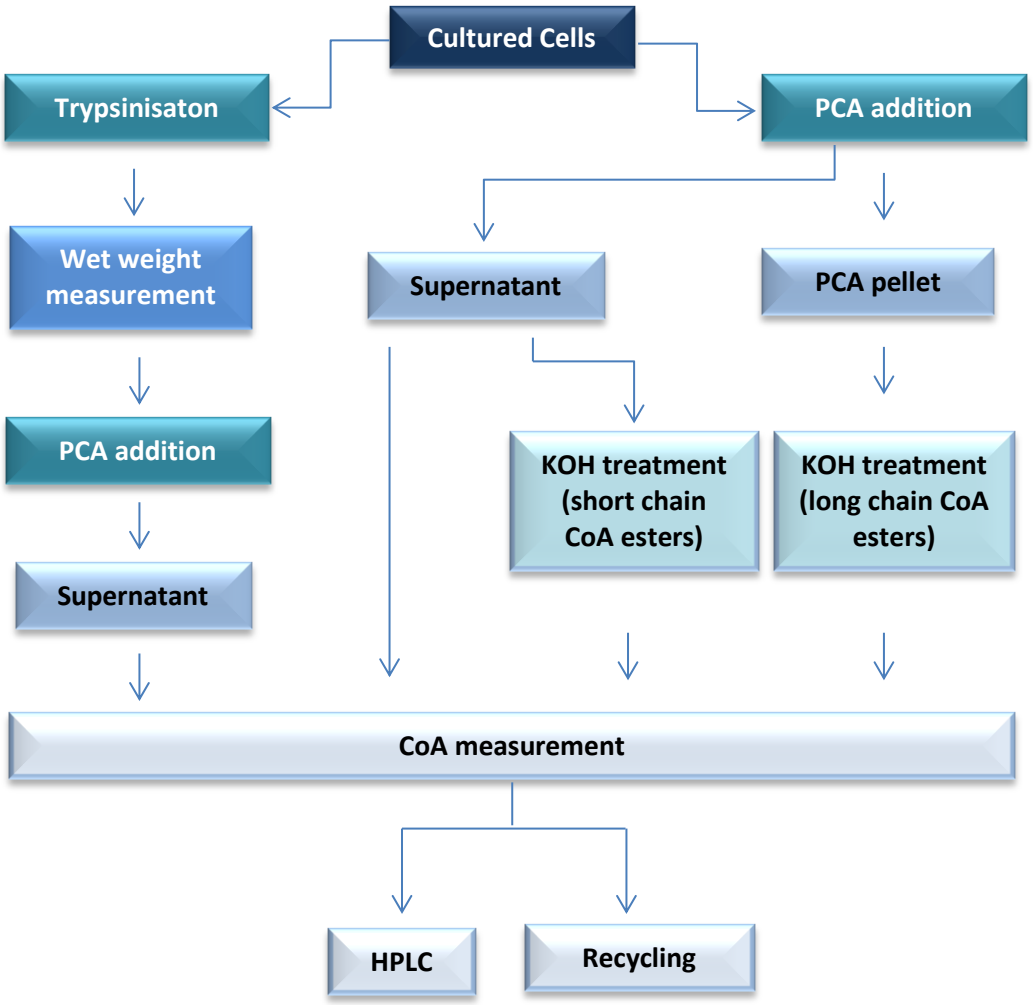


Figure 3.1 Overview of CoA measurement in cultured cells.

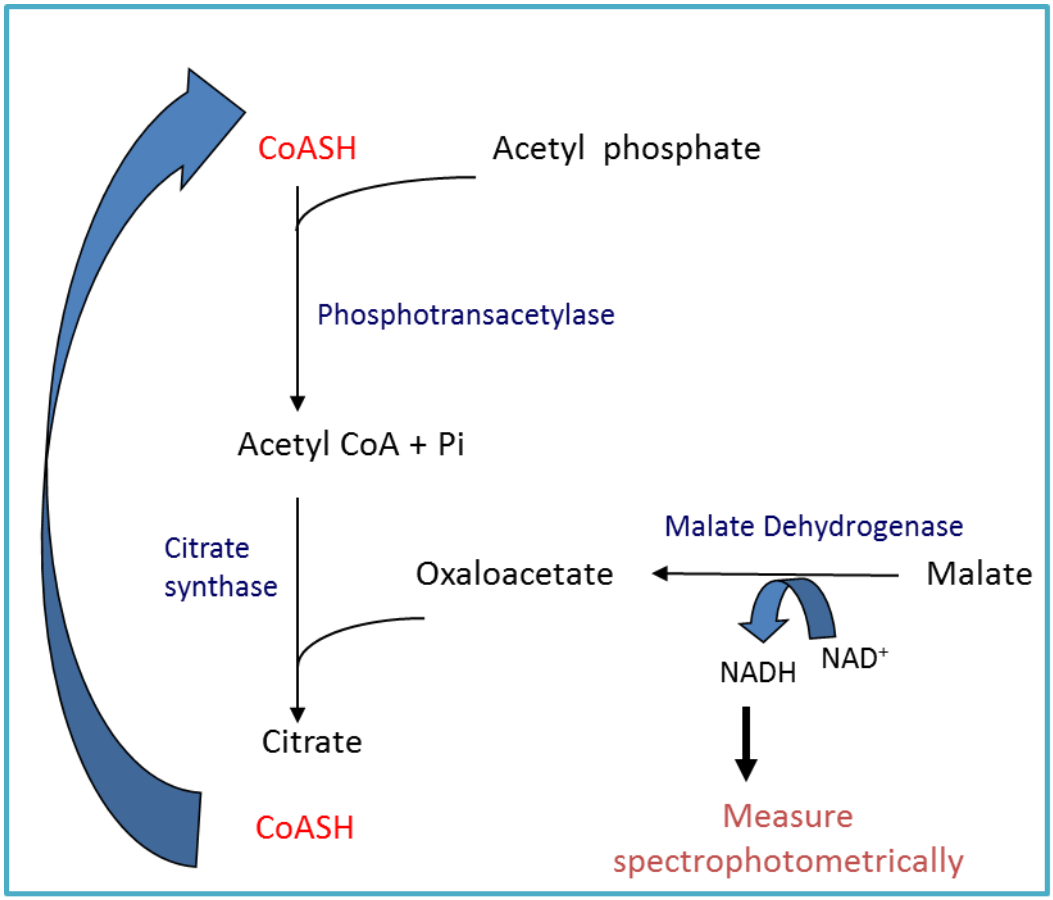


Figure 3.2: Overview of the recycling assay. CoA is recycled through two enzyme-catalysed reactions so that the rate of NADH formation is proportional to CoA concentration, in both its free and acetylated form. NADH concentration is calculated using its extinction coefficient of 6220 $M^{-1}cm^{-1}$ at 340 nm.

sufficient amplification of the signal (*Figure 3.3*). For higher CoA concentrations, under the conditions used, it is likely that the rate of reaction is limited by citrate synthase activity (Allred and Guy 1969).

3.2.1.2 Measuring CoASH and acetyl CoA separately

The most abundant form of the CoA thioesters reported in tissue extracts is acetyl CoA. The recycling assay measures the combined concentration of CoASH and acetyl CoA, however it is also possible to measure CoASH and acetyl CoA separately. This is achieved by incubating the sample with NEM; an alkene which is reactive towards thiols, which as a result removes any free CoASH present. To check whether acetyl CoA could be detected separately and accurately, 100 pmol CoASH and acetyl CoA standard in 1 mM DTT was incubated with 8 mM NEM, followed by 8 mM DTT to react with the excess NEM, before measuring ΔNADH by the recycling assay. The total value for combined CoASH and acetyl CoA was also measured so that free CoASH could be calculated by subtracting the acetyl CoA value from the total CoA value. As expected, all the acetyl CoA was fully recovered, while hardly any free CoASH could be detected by the recycling assay after NEM treatment *(Table 3.1)*. This indicates that, in these conditions, all the free CoASH reacts with the NEM and it is therefore possible to measure free CoASH and acetyl CoA separately. Experiments with standards show that the recycling assay can measure CoA accurately and reliably in the picomolar range expected of cultured cell extracts *(Figure 3.4)*.

3.2.2 HPLC

The HPLC method used here was developed from Corkey et al. (1981) (Corkey et al. 1981), and uses a C18 Kinetex column and sodium phosphate/methanol elution. CoASH and acetyl CoA were detected at an absorbance of 254 nm. A high concentration of sodium phosphate was used to separate other UV absorbing compounds, such as adenine

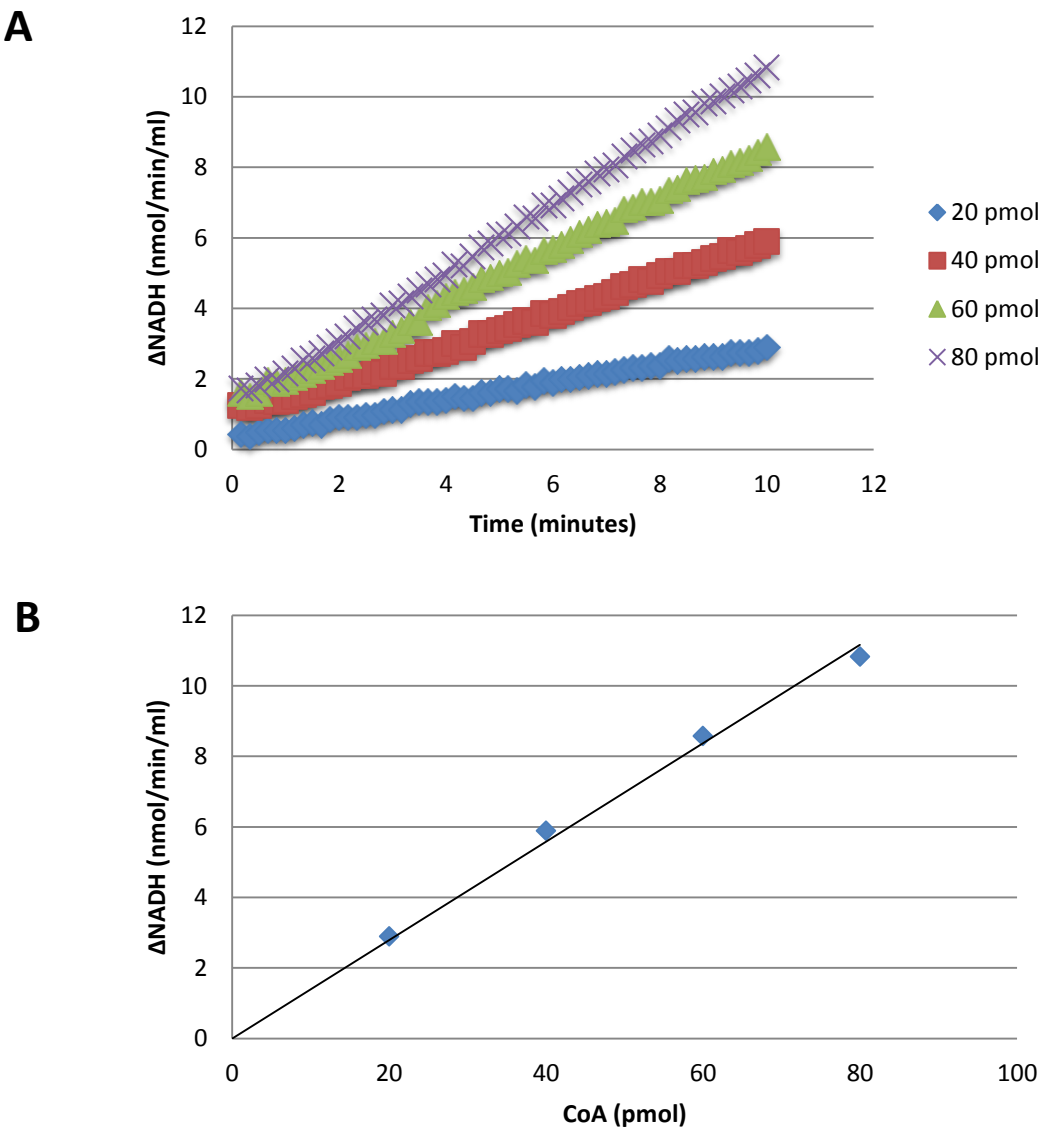


Figure 3.3: Determining the optimal assay time. A) CoA standards ranging from 20-80 pmol in 1 mM DTT were incubated with all of the components of the recycling assay for 60 mins at 30˚C. The NADH produced during the reaction was measured spectrophotometrically using a 96-well plate reader. **B)** ΔNADH values for the 10 min time point were taken for CoA standards ranging from 20-80 pmol.

Treatment	Sample	pmol in final sample	% recovery
None	Total CoA + acetyl CoA (3)	197.1 ± 12.8	98.5 ± 6.4
NEM treated	Acetyl CoA (3)	108.9 ± 5.7	108.9 ± 5.7
	CoA (3)	2.9 ± 0.6	2.9 ± 0.6

Table 3.1: Percentage recovery after NEM treatment of CoA standards. 100 pmol standards of CoA and acetyl CoA in 1 mM DTT were incubated with 8 mM N-ethylmaleimide (NEM) for 5 mins at 30˚C, followed by 8 mM DTT for 5 mins at 30˚C. Total CoA represents 100 pmol CoA and acetyl CoA standards that were not incubated with NEM or further DTT. Free CoA was calculated by subtracting the value obtained for acetyl CoA (incubated with NEM) from the total CoA value. The number of repeats is shown in parentheses.

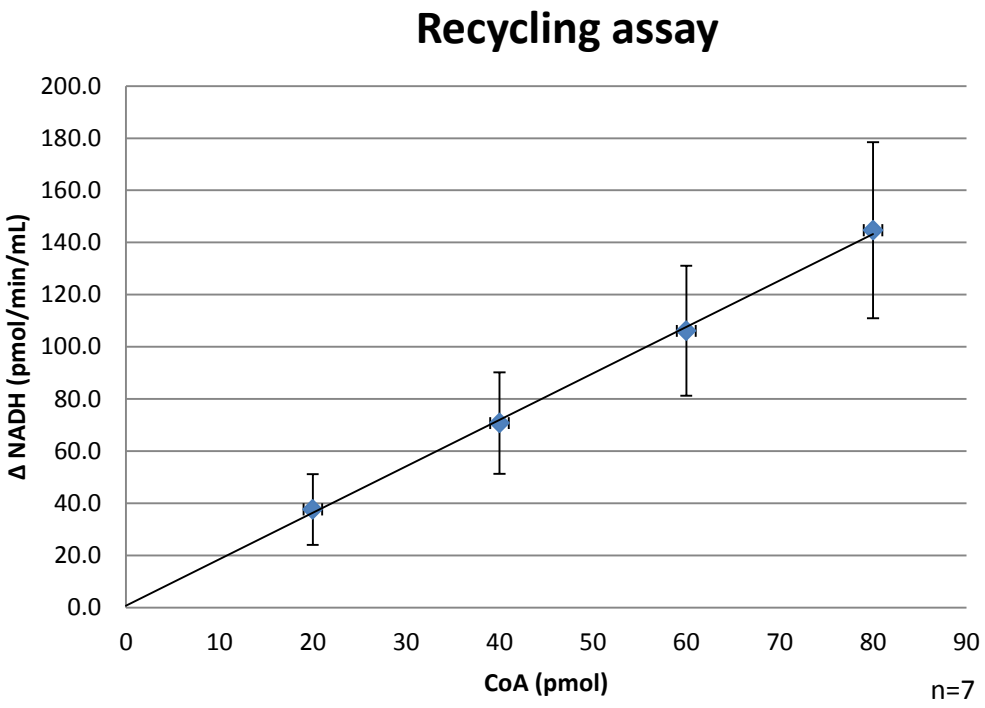


Figure 3.4: CoA standard curve measured by the recycling assay. Known CoA standards ranging from 20-80 pmol were incubated for 10 mins at 30˚C, with all of the components of the recycling assay and 1 mM DTT with a final reaction volume of 200 µL. NADH produced during the reaction was measured spectrophotometrically using a 96-well plate reader.

nucleotides, from CoASH and its derivatives (*Figure 3.5)*. Isocratic rather than a gradient elution conditions were also used to ensure a stable baseline and increase the sensitivity of the method. This makes it possible to measure very small concentrations of compounds (detection limit of 5 pmol) (*Figure 3.6*). CoASH and acetyl CoA levels can be measured with standards (*Figure 3.7A*), in tissues such as liver (*Figure 3.7B)*, kidney *(Figure 3.7C*), brain (*Figure 3.7D)* as well as in HepG2 cell and Hek293 extracts (*Figure 3.7E, Figure 3.8)*.

The CoASH/acetyl CoA peaks were determined in cell extracts by comparing retention time with authentic CoASH/acetyl CoA standards. Internal standards were also used to identify CoASH and acetyl CoA peaks by adding a known concentration of CoASH/acetyl CoA immediately prior to analysing the sample (*Figure 3.8*). This was compared with the same sample that had H_2O added instead of the CoASH/acetyl CoA standard. The identification of a particular compound is often more accurate with internal standards, since the peaks of external standards do not always correspond exactly with the relevant peaks in the samples. This is because the retention time of a compound can be affected by the sample environment.

To ensure there were no co-eluting peaks with the acetyl CoA peak, HepG2 PCA extracts were incubated in alkaline conditions (0.2 M KOH) for 30 mins at 30°C to hydrolyse CoA thioester bonds. Complete disappearance of the acetyl CoA peak (as well as the succinyl CoA and HMG CoA peaks) indicated it was completely hydrolysed to CoASH in these conditions, corresponding with an increase in the CoASH peak (*Figure 3.9)*. No other peaks remained after alkaline hydrolysis confirming that nothing elutes at the same time as acetyl CoA. It is not possible to tell whether this is the case for CoASH, and this method might lead to the over-estimation of CoASH levels. However, it is unlikely that there is anything contaminating the CoA peak, since both recycling assay and HPLC methods give

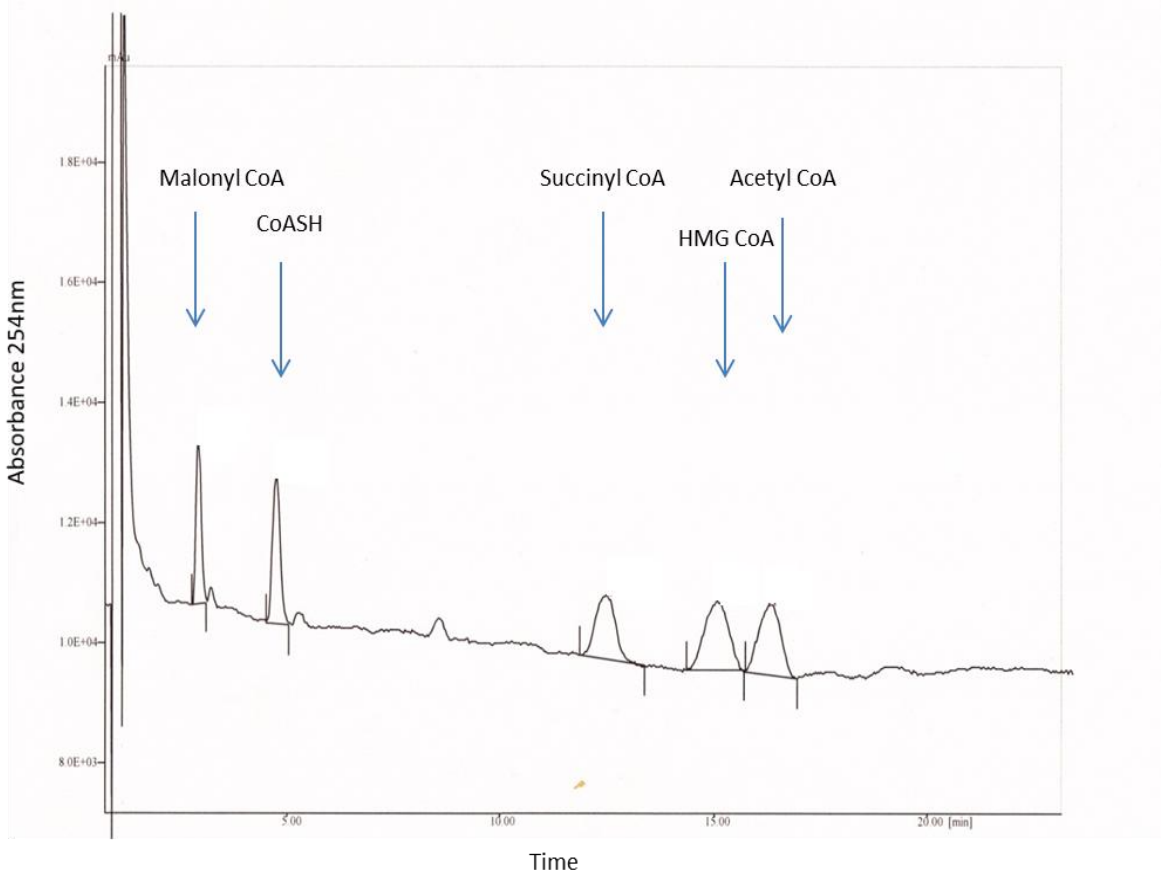


Figure 3.5: Chromatogram of CoASH and CoA esters. 50 pmol malonyl CoA, CoASH, succinyl CoA, HMG CoA and acetyl CoA standards were measured by HPLC using the conditions described in section 2.5.2.1.

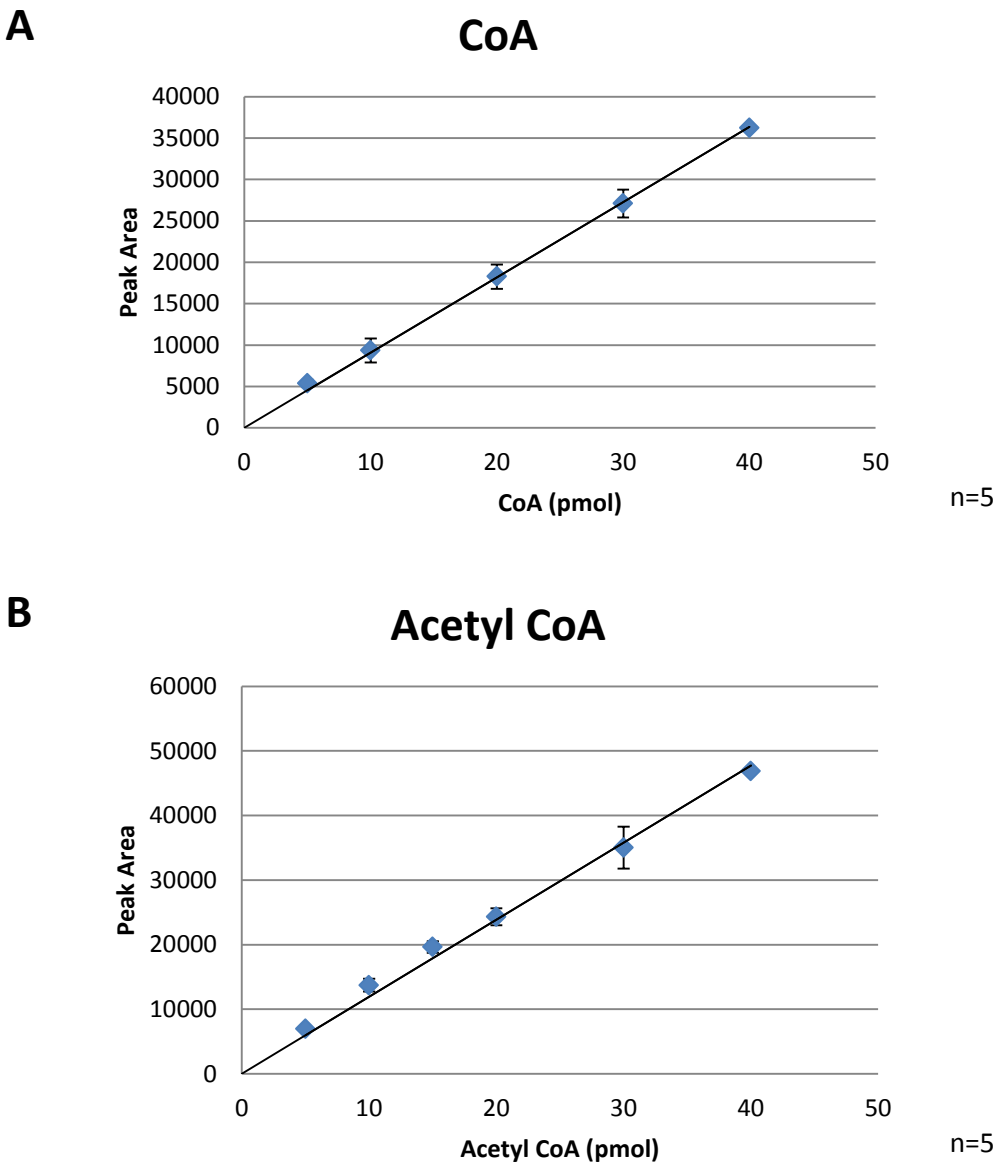


Figure 3.6: CoA standard curve measured by HPLC. Known CoA standards ranging from 20-80 pmol were measured by HPLC using the conditions described in section 2.5.2.1.

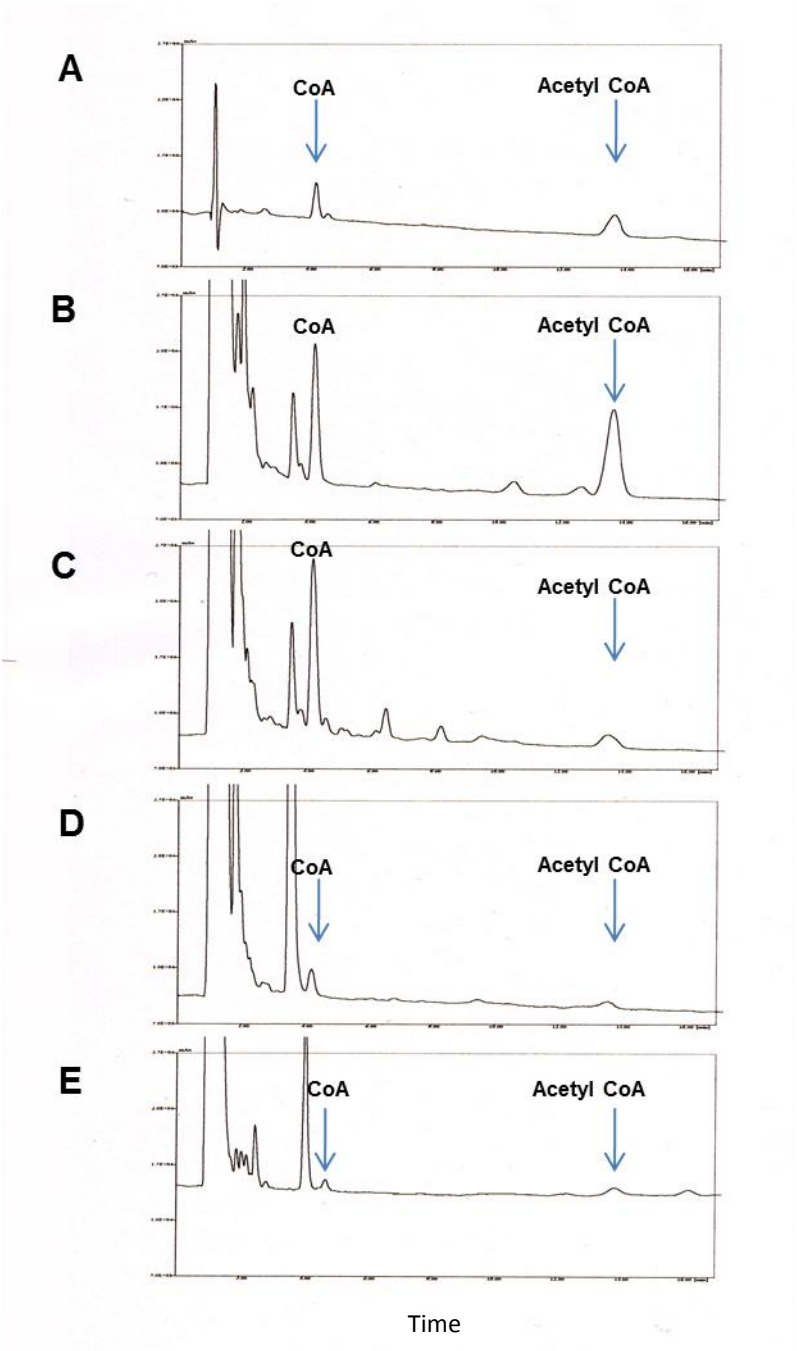


Figure 3.7: HPLC chromatograms of tissue and cultured cell PCA extracts. 50 pmol CoA/acetyl CoA standards **(A)**, liver extract **(B)**, kidney extract **(C)**, brain extract **(D)**, and HepG2 extract **(E)** were measured by HPLC.

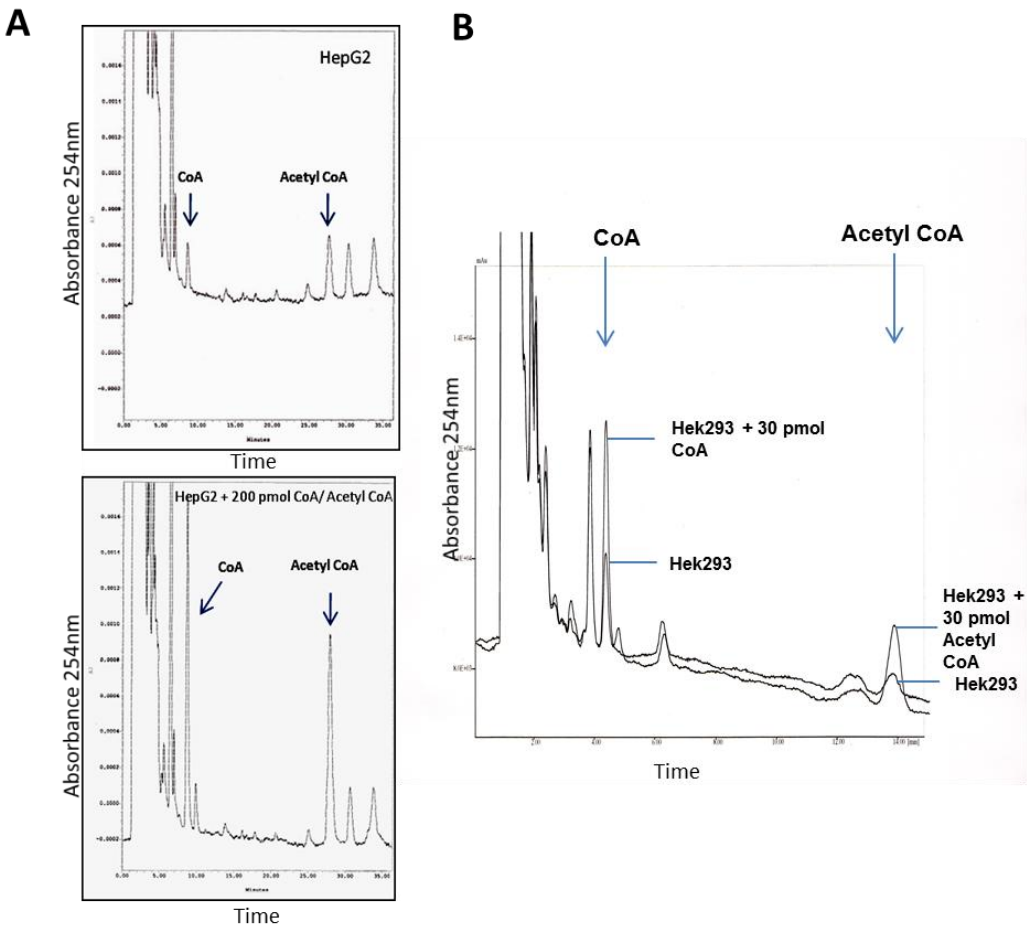


Figure 3.8: Chromatogram of cultured cell extracts alone or in the presence of internal standards. A) HepG2 extract with or without an added 200 pmol CoA/ 200 pmol acetyl CoA was measured by HPLC analysis. **B)** Hek293 extract with or without an added 30 pmol CoA/ 30 pmol acetyl CoA was measured by HPLC analysis.

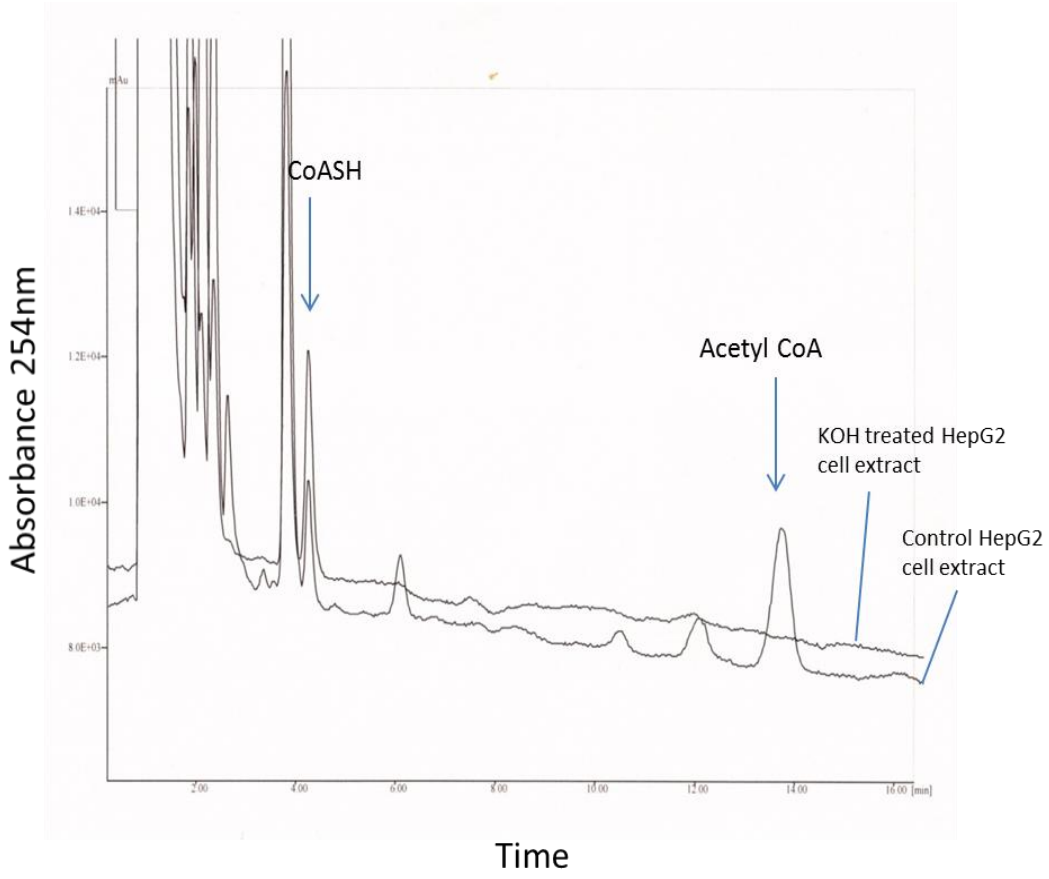


Figure 3.9: KOH treatment hydrolyses CoA thioesters to CoA in HepG2 cells. HepG2 PCA extracts were incubated with 0.2 M KOH for 30 mins, 30°C before HPLC analysis.

almost identical CoASH values for the same sample. Alkaline hydrolysis can also be used to determine the total acid soluble and acid insoluble CoA esters in a sample extract.

3.2.3 Mass Spectrometry

Mass spectrometry is a widely used technique for identifying and quantifying CoA thioesters. Here, mass spectrometry was performed with an ESI-dual hexapole MicroTOF mass spectrometer (Bruker-Daltonics). The spectrometer was operated in positive ion mode using Bruker-Daltonics "MicroTOF Control v1.1" software, with the following data collection parameters: capillary voltage, 4.5 kV; nebulizer, 0.8 Bar; dry gas, 4.0 l/min; dry temperature, 200˚C; capillary exit, 150 V; hexapole 1, 21 V; hexapole 2, 20.6 V. The mass spectrometer was programmed to collect data from a single full-scan MS (m/z 50-3000) scan event. M/z data was deconvoluted to yield molecular mass values, using Bruker-Daltonics "Data Analysis v.3.3" software.

The detection limit for CoA was approximately 100 pmol and the CoA lithium ions present made it difficult to calculate the total amount of free CoASH *(Figure 3.10)*. When Hek293 cell PCA extracts were analysed by mass spectrometry, the intensity of the CoASH peak was 1000 times lower than that of the standard, and consequently difficult to detect above the baseline noise *(Figure 3.11)*. This suggests that the levels of CoASH in cultured cells are too low to measure CoA accurately by mass spectrometry. The recycling assay and HPLC were therefore chosen as the main methods to measure CoA levels in future experiments.

3.3 Sample preparation

When preparing a sample for CoA analysis, it is essential that the method used ensures that 1) The CoA level in the sample is above the detection limit of the analysis method; 2) CoA esters are preserved; 3) oxidation is avoided; 4) maximum recovery of CoA is achieved.

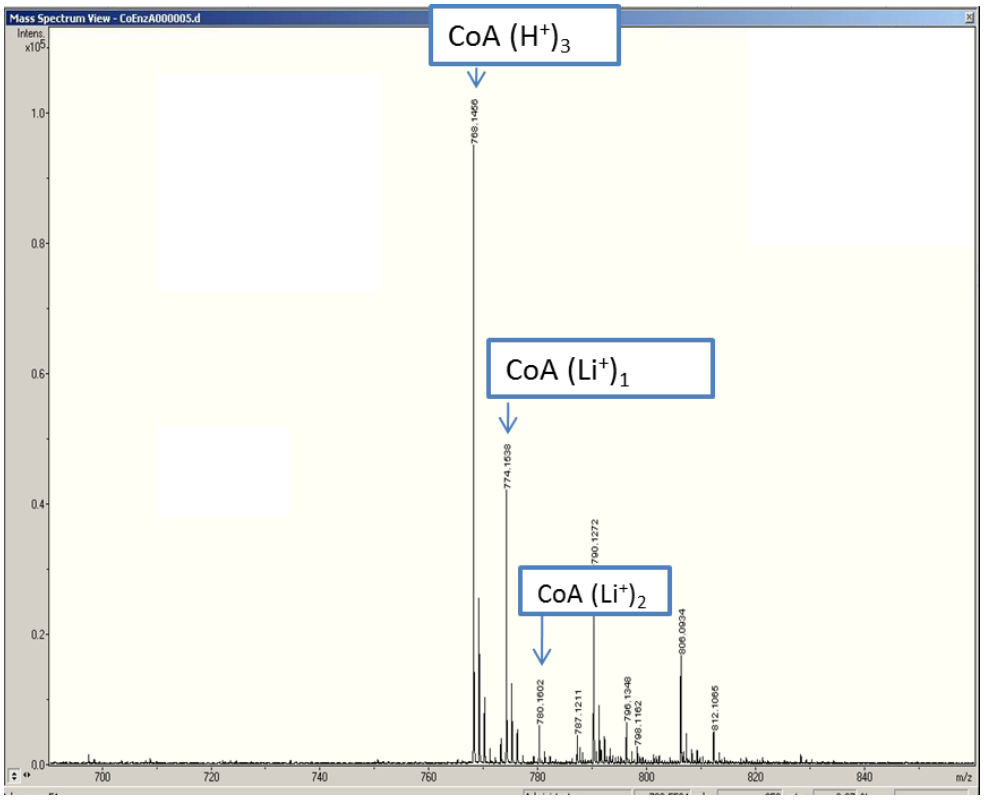


Figure 3.10: Mass spectrum of 100 pmol free CoASH standard (m/z 768). CoA was mixed with 50% acetonitrile and 0.05% formic acid before analysis. The other peaks correspond to $(Li^+)_1$, (m/z 774) and $(Li^+)_2$ (m/z 780) as indicated, or various contaminants of the CoA standard powder. The y axis is relative abundance.

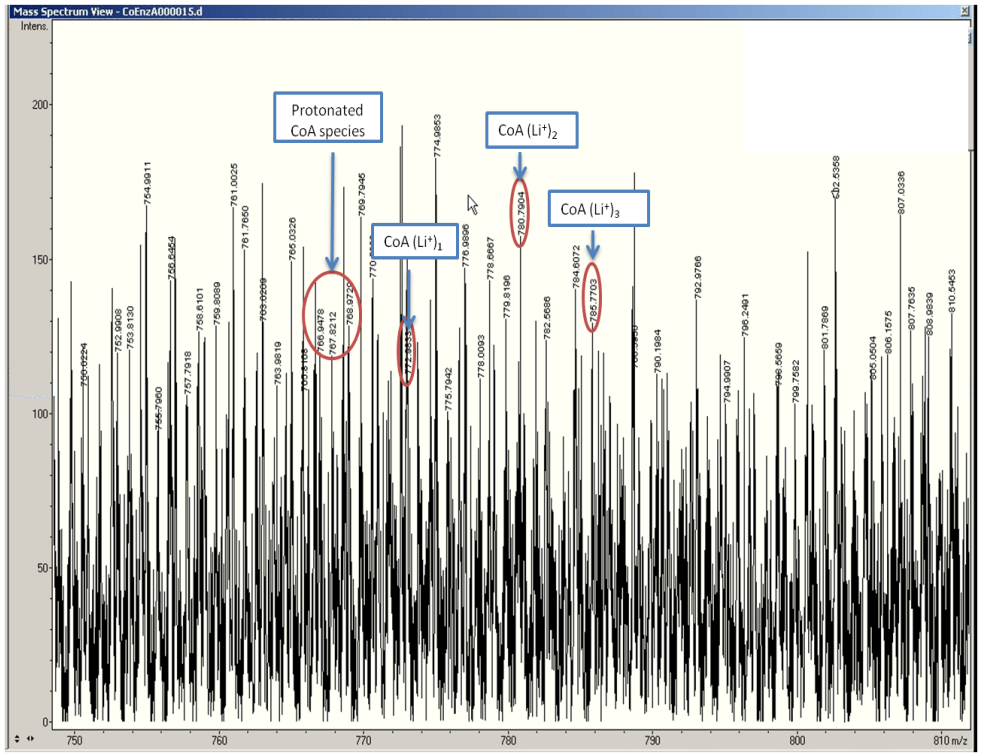


Figure 3.11: Hek293 mass spectrum. Hek293 cells were treated with 0.1% trifluoroacetic acid (TFA) before mixing with 50% acetonitrile and 0.05% formic acid for mass spectrometry analysis. The protonated CoA species (m/z 766, 767, 768) and lithium ions (m/z 780, 785) are indicated within the red circles. The intensity readings are 1000 times lower for protonated species of CoA compared to standard CoA readings.

3.3.1 Deproteinisation

Extraction with trichloroacetic acid (TCA) [10% (w/v), final concentration], followed by removal of excess TCA with ether, is often used to prepare acid-soluble acyl-CoA compounds (Corkey 1988), however here the results indicate that this method degrades CoA (*Figure 3.12A*). This degradation is not seen with perchloric acid (PCA) [5% (w/v), final concentration] extraction of CoASH and its esters, followed by careful neutralisation with 1M K_2CO_3 (*Figure 3.12B, Figure 3.13).*

3.3.2 Preserving CoA esters

CoA esters rapidly break down at pH values of 8-9 and free CoASH is often a product. To prevent free CoASH levels from being altered during the extraction procedure, it is desirable to adjust the pH of solutions containing CoA esters so the conditions maintain free CoASH levels *(Figure 3.13)*.

3.3.2.1 The effect of pH on the recycling assay

Due to the various enzymes involved in the enzymatic assay, it is necessary to adjust the pH of the PCA extracts near physiological pH (around pH 6-7). During the extraction procedure, the PCA extract was neutralised with K_2CO_3. As the volume of the extract is too small to allow the pH to be measured using a pH meter, 0.5 µl aliquots of the extract were used to measure the pH using indicator paper. This method can occasionally result in slight variations in pH, so it was necessary to test whether the recycling assay is affected by small pH fluctuations. There appeared to be little difference between the standards at various pHs ranging from pH 5 to pH 7 *(Figure 3.14)*, especially over the expected range of CoA levels in cultured cell extracts (20-100 pmol). This indicates that small fluctuations in pH that may have occured during the neutralisation step do not affect the assay, possibly because they were cancelled out by the Tris buffer in the reaction mixture.

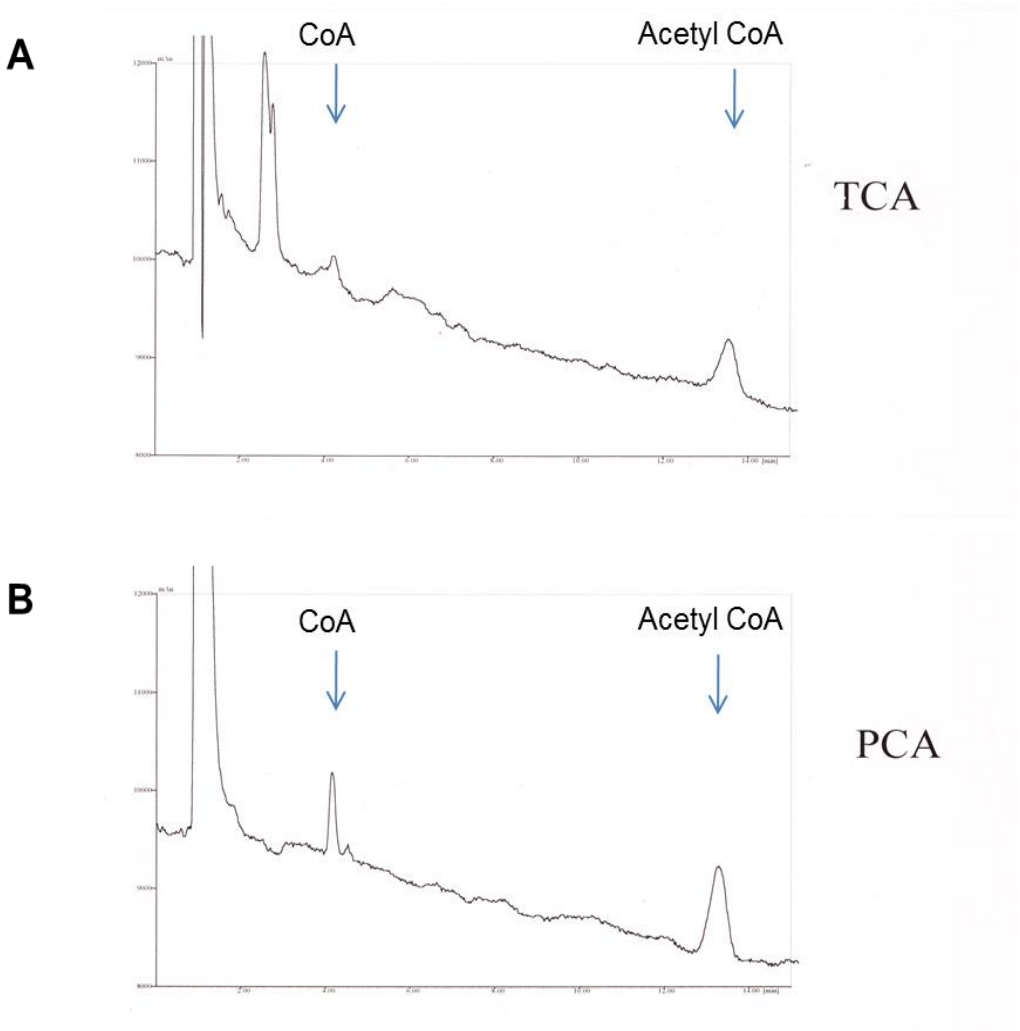


Figure 3.12: HPLC profiles of CoA/ acetyl CoA standards extracted with A) 10% TCA (final concentration) or **B**) 5% PCA (final concentration). Extracts were analysed by HPLC.

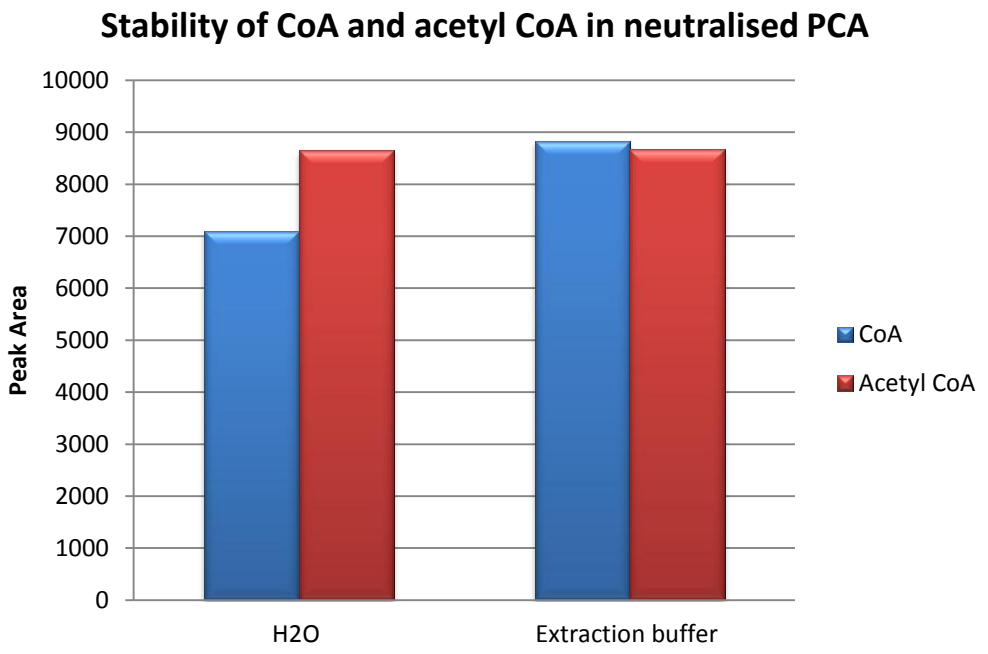


Figure 3.13: Stability of CoA/ acetyl CoA in neutralised PCA. The CoA/ acetyl CoA peak areas for 10 pmol CoA/ Acetyl CoA standards made up in H_2O or extraction buffer (5% PCA, neutralised with K_2CO_3). The standards were analysed by HPLC.

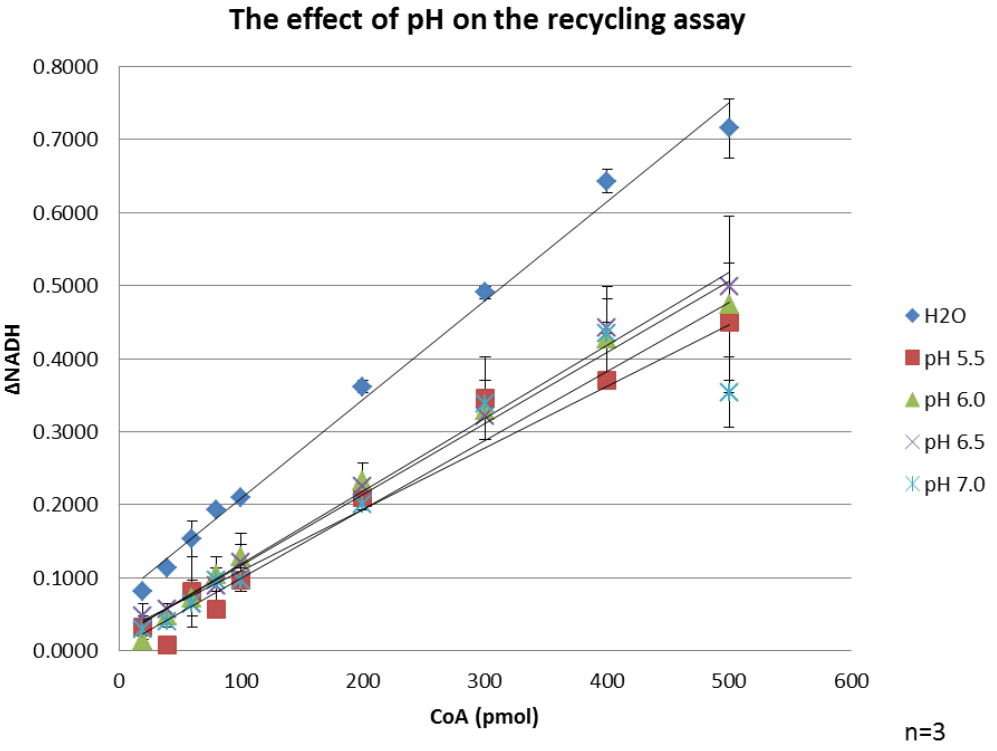


Figure 3.14: The effect of pH on the recycling assay. CoA standards ranging from 20-500 pmol were incubated with H_2O alone (control) or 5% perchloric acid that had been adjusted to pH 5.5, 6.0, 6.5 or 7.0, together with all of the components of the recycling assay and 1 mM DTT for 10 mins at 30˚C. NADH produced during the reaction was measured spectrophotometrically using a 96-well plate reader.

3.3.2.2 Compatibility with the Recycling Assay

As the recycling assay is an indirect method for measuring CoA, it is important to know whether the extraction procedure affects the assay in any way. When comparing the ΔNADH of increasing CoA standards (20-80 pmol) in water with HepG2 extracts, the increase in ΔNADH was slightly reduced for standards in the presence of cell extracts *(Figure 3.15).* This suggests that a component of the extraction buffer inhibits the activity of at least one of the enzymes in the recycling assay, or alternatively quenches the NADH absorbance. The increase in ΔNADH was still linear, so it is possible to measure CoA levels in cultured cell extracts accurately, providing an internal standard is used to correct for the ΔNADH decrease.

3.3.3 Concentration

Cell samples were initially collected by trypsinisation and then lysed with a relatively small volume of PCA (150 µl) to release CoASH and its short chain esters into solution. The main issue with this method is that there is a significant amount of time (~5-10 mins) from when the DMEM is removed to when the cells were lysed. Cells potentially become stressed without DMEM and during trypsinisation and this may cause an unwanted change in the ratio of CoASH: CoA esters.

The extraction method was consequently modified, so that the time between DMEM removal and PCA addition was kept as short as possible. This was achieved by adding PCA directly to the plate, after washing cells with PBS, and then scraping and collecting the cell lysates. The combined values for CoASH and acetyl CoA were very similar for both trypsinisation and scraping *(Figure 3.16).* This suggests that no significant loss of CoASH/acetyl CoA occurs during scraping. However, it is important to note that scraping dilutes the sample, making it even more difficult to accurately detect CoASH by the

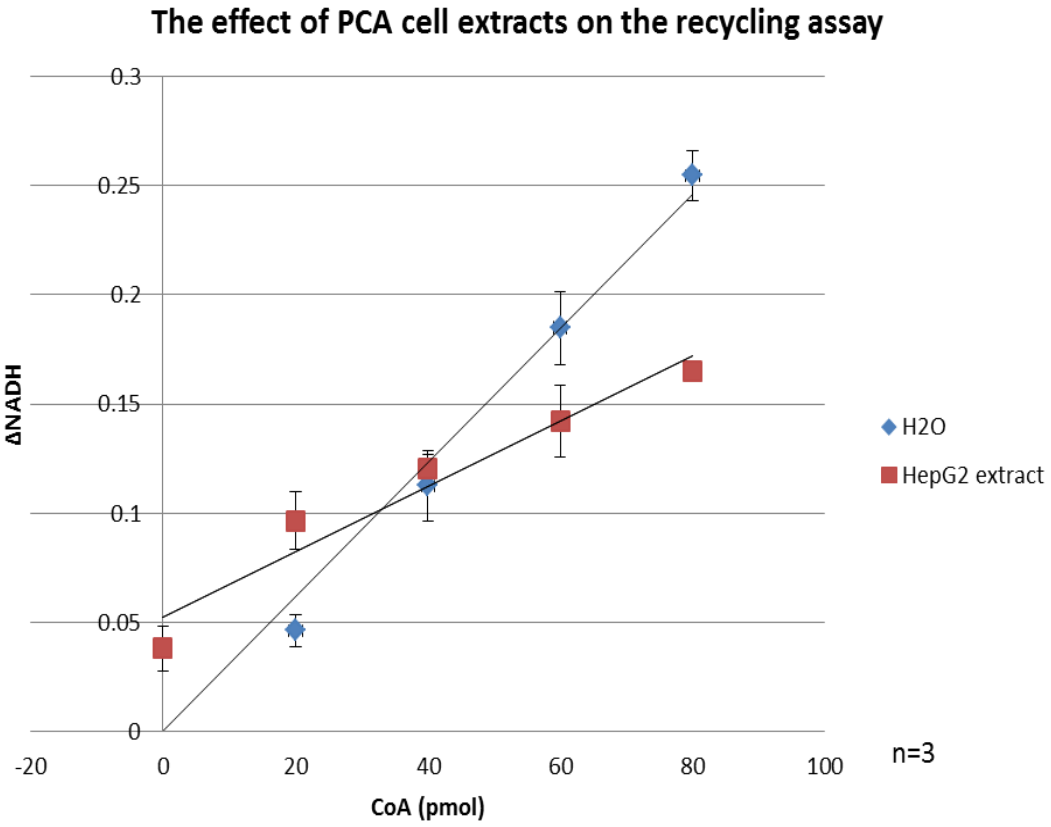


Figure 3.15: The effect of PCA cell extracts on the recycling assay: Known CoA standards ranging from 20-80 pmol were incubated for 10 mins at 30˚C, with all of the components of the recycling assay, 1 mM DTT and either H_2O or HepG2 extracts, with a final reaction volume of 200 µL. HepG2 cells were extracted as described in Chapter 2. NADH produced during the reaction was measured spectrophotometrically using a 96-well plate reader.

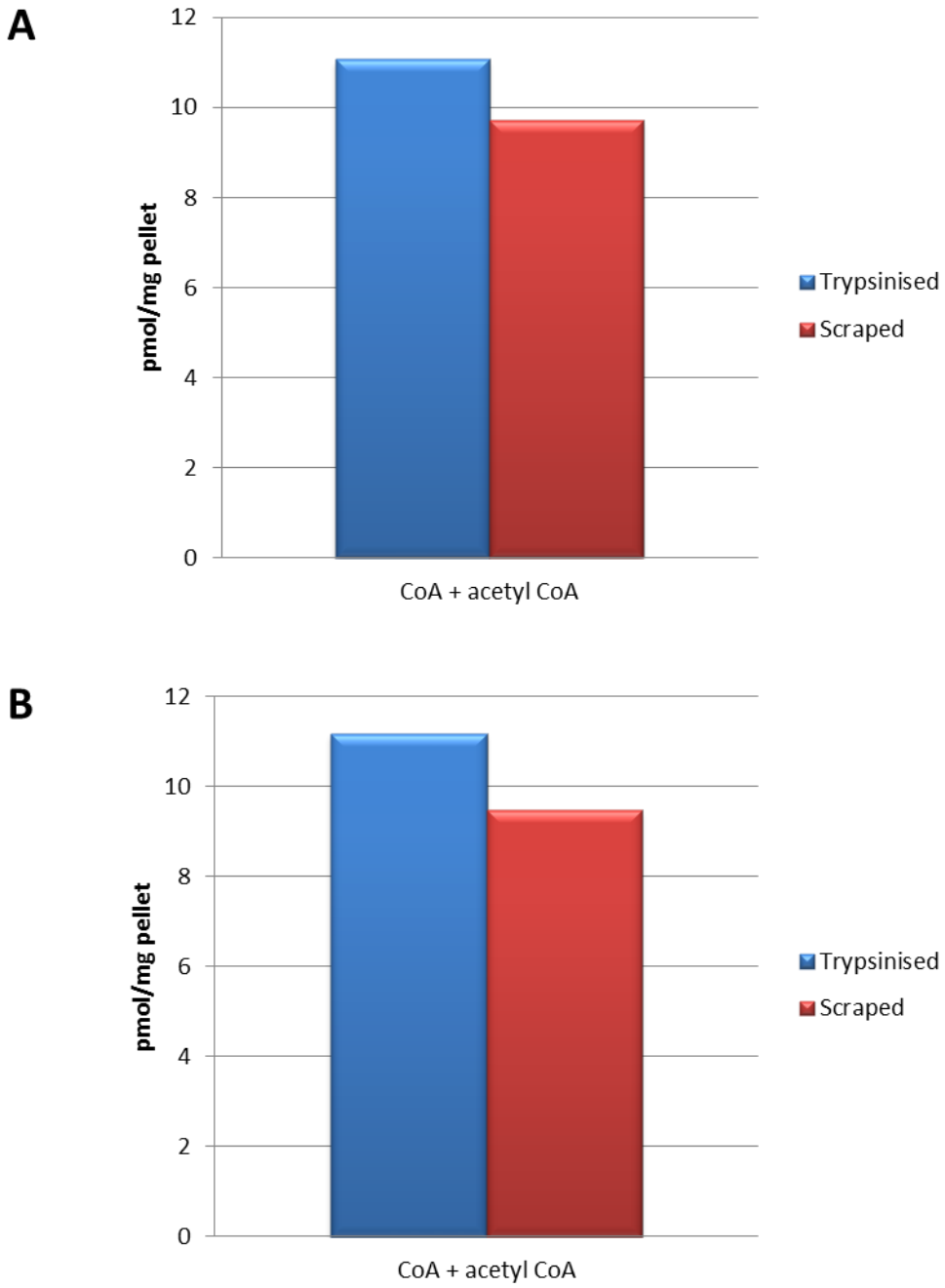


Figure 3.16: CoA levels in cultured cells collected by different methods. Combined CoA + acetyl CoA levels were measured in HepG2 **(A)** and Hek293 **(B)** extracts which were either trypsinised before PCA extraction or PCA was added directly to the cell culture plates and cells were collected by scraping. Extracts were measured by HPLC.

recycling assay or HPLC. Therefore it is necessary to concentrate the extract before analysis.

Lyophilisation is a well-documented method for concentrating samples, however during this process degradation, especially of acetyl CoA occurs *(Figure 3.17).* A speed vac concentrator is used to partially, but not completely reduce the extract volume.

3.3.4 Reducing Agent

The underestimation of free CoASH due to its oxidation to form glutathione CoA, CoA dimers, or other mixed dimers is a potential problem when measuring CoA levels. Dithiothreitol (DTT) or β-mercaptoethanol are the traditional reducing agents of choice for the recycling assay, however they cannot be used for HPLC as their peaks interfere with the CoASH peak. TCEP (tris(2-carboxyethyl)phosphine) is another effective reducing agent that is structurally very different from DTT and β-mercaptoethanol and has a very low absorbance at 254 nm. It was therefore used to reduce CoASH during HPLC instead.

Ideally the extraction procedure should be kept as similar as possible for all the methods used to measure CoA levels, so CoASH standards in the presence of TCEP were compared to standards in DTT using the recycling assay. TCEP drastically reduces the change in NADH absorbance in the recycling assay, especially at the range of CoA levels present in cell extracts (20-100 pmol) *(Figure 3.18)*. Consequently, it is not possible to use the same reducing agent for both methods of CoA detection.

3.4 Standardisation

Traditionally, CoA levels in tissues are standardised per gram wet weight. Standardising CoA levels using PCA pellet weight was found to be the most convenient for cultured cells with the extraction method used here, since other methods of

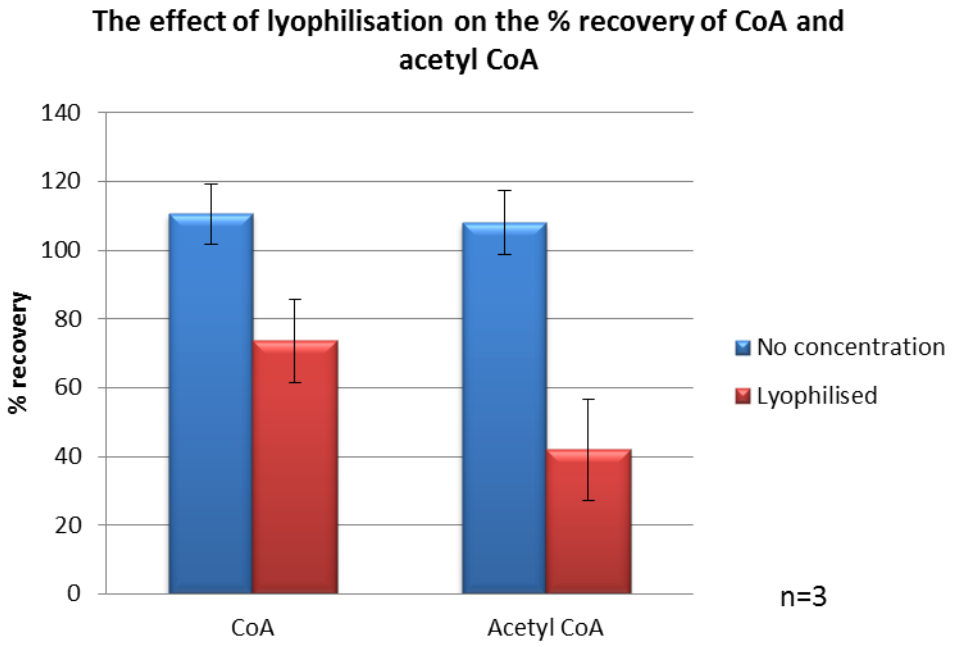


Figure 3.17: Lyophilisation affects % recovery of CoA and acetyl CoA. A known concentration of CoA/ acetyl CoA was added to liver tissue extracted with 7.5% PCA (0.5 mL/ 100 mg liver), then neutralised to a pH of 6.5. Tissue extracts were incubated with 10 mM TCEP (10 mins, 30˚C), and then made up to a final volume of 90 µL in mobile phase, with an injection volume 50 µL and analysed by HPLC straight away or after 2 hours lyophilisation. The amount of known CoA/ acetyl CoA recovered was measured by comparing with the same liver extract containing no additional CoA/ acetyl CoA.

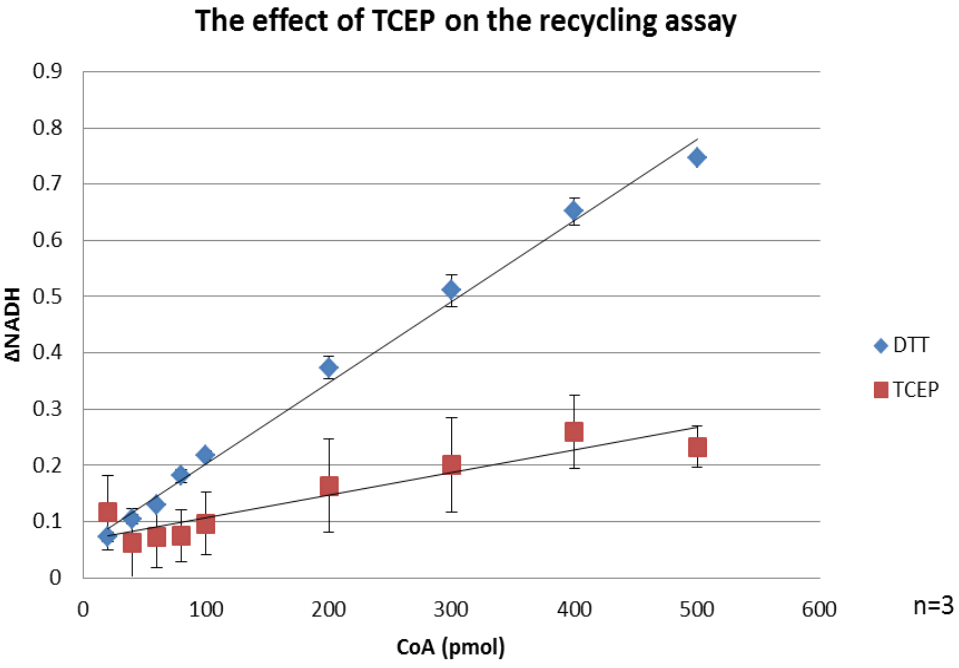
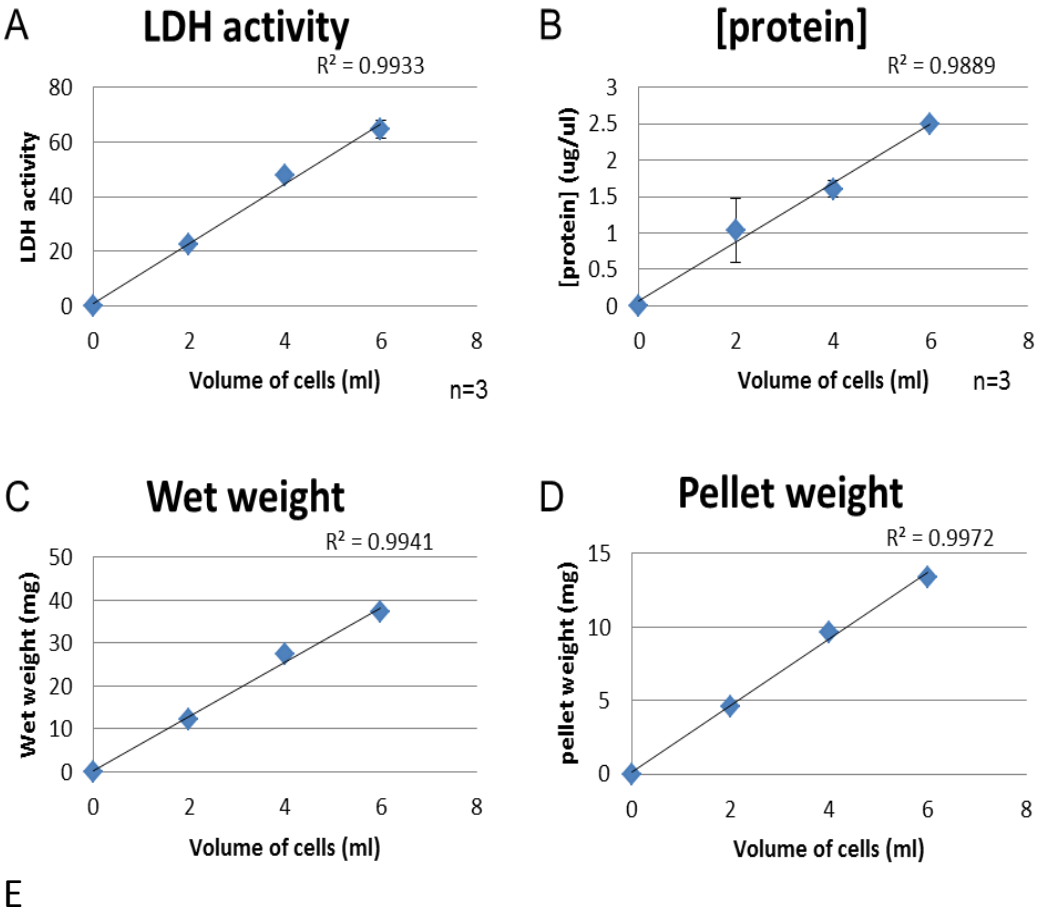


Figure 3.18: The effect of TCEP on the recycling assay. Known CoA standards ranging from 20-500 pmol were incubated for 10 mins at 30˚C with all the components of the recycling assay and either 1 mM DTT or TCEP. The final reaction volume was 200 µL and NADH produced during the reaction was measured spectrophotometrically using a 96-well plate reader.

standardisation (e.g. protein, wet weight, and cell number) require a second culture plate in which it cannot be guaranteed that cells will grow in the same way. The relationship between PCA pellet weight, wet weight, protein, and LDH content was established using increasing volumes of trypsinised cultured cells and this allowed CoA levels in cultured cells to be expressed using different standardisation methods *(Figure 3.19)*.

3.5 Percentage recovery

Method development studies show that the best extraction conditions for CoASH and CoA thioesters in cultured cells are with 5% PCA, followed by neutralisation with 1M K_2CO_3 (to pH 6). Samples should then be concentrated partially, using the speed vac concentrator. TCEP is the reducing agent of choice for HPLC, whereas DTT is used for the recycling assay. To examine the reliability of this extraction method, recovery experiments of standard CoASH or acetyl CoA added to HepG2 PCA extracts before neutralisation and concentration were analysed using both detection methods. Under the extraction conditions described, percentage recovery was nearly 100%, indicating that there was no oxidation or degradation of CoASH/acetyl CoA *(Figure 3.20).* It is also important to note that CoA levels measured by the recycling assay and HPLC are similar (Chapter 4), further supporting the reliability of these methods.



E

PCA pellet weight (mg)	wet weight (mg)	LDH activity	protein (mg)	Cell number ($x10^6$)
1	2.7	4.8	0.2	0.3

Figure 3.19: Methods of standardisation. Trypsinised HepG2 cells were pooled then divided into 2, 4 and 6 mL samples. 200 µL aliquots were lysed in buffer containing 1% trition, then used to measure **A)** LDH activity (spectrophotometric assay) **B)** protein concentration (Bradford assay) **C)** wet weight **D)** PCA pellet weight. **E)** Conversion table for all methods of standardisation. Cell number was calculated using measurements against LDH activity.

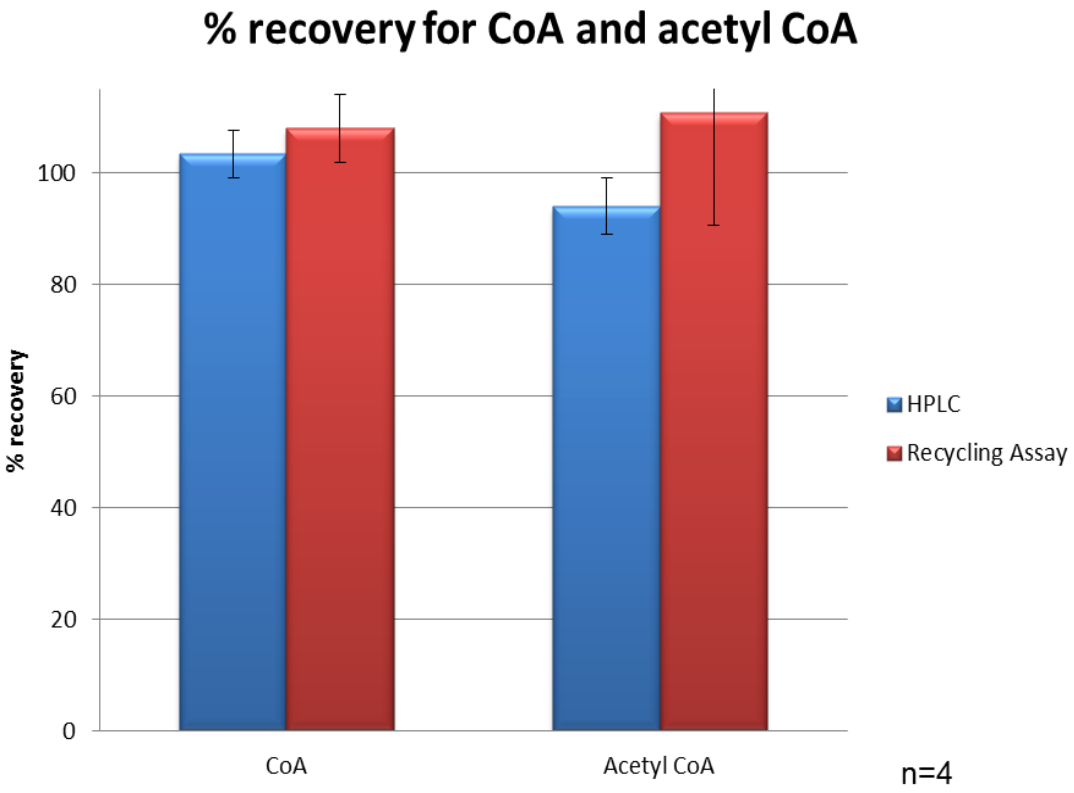


Figure 3.20: Percentage recovery of CoA and acetyl CoA in cell extracts is good. HepG2 cells grown in 10 cm plates were extracted with 5% PCA and 200 pmol of CoASH and acetyl CoA were added to the extracts before neutralisation and concentration. % recovery of added standards were analysed by HPLC and the recycling assay.

Chapter 4: Results

Studying the role of Coenzyme A as a potential regulator of cellular function

Chapter 4: Studying the role of Coenzyme A as a potential regulator of cellular function

4.1 Introduction

The levels of CoA and its thioesters vary over a wide range among different animal tissues, depending on the tissue's metabolic activity. Intracellular CoA levels also have been shown to change in response to a number of extracellular stimuli including nutrients, hormones, fasting and fibrate drugs (Berge 1983; Brass et al. 1990; Finlayson 1983; Horie et al. 1986; Kerbey 1977; Mcallister et al. 1988; Rapp 1973; Reibel et al. 1981a; Reibel et al. 1981b; Reibel et al. 1983; Robishaw et al. 1982; Shibata et al. 1983; Smith and Savage 1980). A study involving *PanK1*$^{-/-}$ knockout mice demonstrated that an increase in CoASH during fasting is required for the metabolic transition from the fed to the fasted state (Leonardi et al. 2010). Processes such as fatty acid oxidation and gluconeogenesis were impaired as a result of the lower levels of CoASH, illustrating the importance of CoASH as an essential cofactor. Apart from acting as a cofactor, CoASH and its thioesters are also known to bind non-covalently and regulate the activities of enzymes. At present, the established regulatory roles of CoASH and CoA thioesters appears to be mainly limited to feed forward/feedback regulation of metabolic enzymes as exemplified by the regulation of PDH and PanK. However, it is likely that changes in cellular CoA levels under various conditions can have wider regulatory consequences than currently recognised. Indeed, there are reports of processes other than metabolic pathways potentially regulated by CoASH and its thioesters. For example S-thiolation by CoASH has been implicated as a possible mechanism for degradation (Huth et al. 1996; Huth et al. 2002; Schwerdt et al. 1991; Schwerdt and Huth 1993); possible changes in acetylation and succinylation following

changes in CoASH levels both have the potential to regulate multiple cellular proteins and processes; and long chain acyl CoAs have been suggested to regulate signal transduction pathways, membrane fusion as well as gene regulation (Faergeman & Knudsen 1997). Alterations in CoA levels have also been observed in several pathological conditions such as diabetes, cancer, cardiac hypertrophy, Reye syndrome, MCAD deficiency and vitamin B12 deficiency (Brass et al. 1990; Kerbey 1977; Mcallister et al. 1988; Rapp 1973; Reibel et al. 1981a; Reibel et al. 1981b; Reibel et al. 1983). It is not known whether these alterations in CoA levels are due to the metabolic abnormalities associated with the pathologies, or whether abnormal CoA levels contribute to the pathologies. It is therefore important to gain a better understanding of the role of CoA as a regulator of cellular processes and consequences of altered CoA levels.

Little is known about how changes in CoA are brought about. Potential mechanisms are through alterations in biosynthesis and degradation of CoA. Most studies of CoA biosynthesis in the literature focus on PanK, which is the primary rate-limiting enzyme in the CoA biosynthetic pathway. The phosphopantetheine adenylyltransferase (PPAT) activity of CoASy (step 4) is thought to act as a secondary rate-limiting point during CoA biosynthesis, exemplified by the fact the 4'phosphopantetheine pool is almost as high as the pantothenate pool (Jackowski and Rock 1981; Jackowski and Rock 1984). Although CoASy has not been studied to the same degree as PanK, a few potential mechanisms for its regulation have been determined. CoASy activity appears to be inhibited through phosphorylation on its tyrosine residues and activated by phosphatidylcholine and phosphatidylethanolamine, which are the main phospholipids in the outer mitochondrial membrane where CoASy is localised (Breus et al. 2010; Zhyvoloup et al. 2003). There is also some evidence that CoASy is feedback inhibited either by CoA or its thioesters (Ivey 2004). Furthermore, interactions between CoASy and p85α subunit of PI3K as well as S6K1 have

previously been shown in this laboratory (Breus et al. 2009; Nemazanyy et al. 2004). Whether these signalling pathways regulate CoA biosynthesis *in vivo* is not known.

Cultured mammalian cells are used in a diverse range of biological research. Most studies on CoA metabolism and function have used freshly isolated organs/primary cells or whole animals. Surprisingly, very few studies on CoA function have used cell lines. Isolated cells are well suited for experimentally modulating intracellular CoA levels and studying its effect on cellular processes, as well as studying signalling pathways regulating CoA metabolism. In addition, long term effects such as growth can be studied in cell lines as opposed to freshly isolated primary cells which do not last for more than a few days.

Therefore, the first objective of this chapter is to characterise cell lines in terms of CoA metabolism and to assess the suitability of cultured cell lines as a model for studying the regulatory function of CoA. Secondly, the effects of mTOR and PI3K signalling on the levels of CoA shall be determined. The final objective is to establish a method to experimentally modulate CoA levels and consequently investigate the effects of these changes on cellular function.

4.2 Results

4.2.1 Levels of CoA in Tissues and Cultured cells

CoASH and acetyl CoA levels were measured in PCA extracts of freeze clamped adult rat tissues: liver, kidney, heart and brain as well as in various cultured cell lines including Hek293 and HepG2 by the enzymatic recycling assay and HPLC (*Table 4.1, Figure 4.1)*. Tissue CoA levels were standardised against wet weight, which is the most commonly used standardisation method in previously published reports. Cultured cells are normally standardised against the weight of the pellet precipitated after cells were lysed in PCA.

Tissue/ Cell Type	Additional information	Method of detection	nmol/g wet weight		pmol/mg PCA pellet weight[a]		nmol/g protein[b]		pmol/10^6 cells[c]		Reference
			CoA	Acetyl CoA	CoA	Acetyl CoA	CoA	Acetyl CoA	CoA	Acetyl CoA	
Rat											
Liver (4)	**Fed ad libitum**	**HPLC**	**62.3**	**27.9**					**560.9**	**250.8**	**This Study**
Liver (3)	**Fed ad libitum**	**Recycling Assay**	**144.2**						**1297.8**		**This Study**
Liver (7)	balanced diet	Enzymatic assay	368								Tubbs and Garland (1964)
Liver (8)/(6)	Fed	Recycling Assay	135	38							Allred & Guy (1969)
Liver (6)	fed	Enzymatic assay	44.7	35.6							Kondrup & Grunnet (1972)
Liver (6)	re-fed	Enzymatic assay	95.0	30.0							Guynn et al. (1972)
Liver (5)	solid chow	Enzymatic assay	49.8	37.6							Savolainen et al. (1976)
Liver (5)	fasted	Enzymatic assay (fluorometric)	117								Smith (1978)
Liver (16)	fed	Recycling Assay	110.0	70.0							Brass&Hoppel (1980)
Liver (12)	starved	HPLC	56.1	100.5							King & Reiss (1984)
Liver (6)		HPLC	70.3	35.6							Hosokawa et al. (1985)
Liver (5)	fed	HPLC	127.1	23.1							Corkey et al. (1988)
Liver (3)		HPLC	76.5	27.7							Demoz et al. (1995)
Liver (5)		HPLC	135								Jenniskens et al. (2002)
Liver (3)		Acyl-CoA cycling	22.3	7.2							Tokutake et al. (2010)
Liver (6)	High carbohydrate diet	Acyl-CoA cycling	97.1	5							Tokutake et al. (2012)
Kidney (2)	**Fed ad libitum**	**HPLC**	**43.5**	**6.3**					**391.6**	**57.1**	**This Study**
Kidney (1)	**Fed ad libitum**	**Recycling Assay**	**63.2**						**568.8**		**This Study**
Kidney (5)		Recycling Assay	68	11							Allred & Guy (1969)
Kidney (3)		Acyl-CoA cycling	1.9	5.1							Tokutake et al. (2010)
Kidney (6)	High carbohydrate diet	Acyl-CoA cycling	0.5	2.9							Tokutake et al. (2012)

Tissue/ Cell Type	Additional information	Method of detection	nmol/g wet weight		pmol/mg PCA pellet weight[a]		nmol/g protein[b]		pmol/10^6 cells[c]		Reference
			CoA	Acetyl CoA	CoA	Acetyl CoA	CoA	Acetyl CoA	CoA	Acetyl CoA	
Heart (1)	**Fed ad libitum**	**HPLC**	**13.8**	**50.8**					**124.3**	**457.3**	**This Study**
Heart (1)	**Fed ad libitum**	**Recycling Assay**	**71.9**						**647.1**		**This Study**
Heart (5)		Recycling Assay	61	38							Allred & Guy (1969)
Heart (3)		Acyl-CoA cycling	11.0	5.6							Tokutake et al. (2010)
Heart (6)	High carbohydrate diet	Acyl-CoA cycling	13.7	6.8							Tokutake et al. (2012)
Brain (2)	**Fed ad libitum**	**HPLC**	**7.5**	**2.2**					**67.3**	**19.6**	**This Study**
Brain (1)	**Fed ad libitum**	**Recycling Assay**	**20.7**						**186.6**		**This Study**
Brain (5)		Recycling Assay	23	8							Allred & Guy (1969)
Brain (7)		HPLC	68.7	7.6							Deutsch et al. (2002)
Cerebral Cortex (3)	Fasted, Male	Acyl-CoA cycling	1.5	1.9							Tokutake et al. (2010)
Cerebral Cortex (3)	High carbohydrate diet	Acyl-CoA cycling	0.6	2.1							Tokutake et al. (2012)
Skeletal muscle (5)		HPLC	4								Jenniskens et al. (2002)
Skeletal muscle (3)		Acyl-CoA cycling	1.6	1.5							Tokutake et al. (2010)
Skeletal muscle (6)	High carbohydrate diet	Acyl-CoA cycling	3.4	1.8							Tokutake et al. (2012)
Lung (5)		Recycling Assay	22.0	3.0							Allred & Guy (1969)
Lung (5)		HPLC	10.0								Jenniskens et al. (2002)
Spleen (5)		Recycling Assay	14.0	7.0							Allred & Guy (1969)
Spleen (3)		Acyl-CoA cycling	4.5	2.4							Tokutake et al. (2010)
Spleen (6)	High carbohydrate diet	Acyl-CoA cycling	2.2	1.2							Tokutake et al. (2012)
Testes (5)		Recycling Assay	19.0	5.0							Allred & Guy (1969)

Tissue/ Cell Type	Additional information	Method of detection	nmol/g wet weight		pmol/mg PCA pellet weight[a]		nmol/g protein[b]		pmol/10^6 cells[c]		Reference
			CoA	Acetyl CoA	CoA	Acetyl CoA	CoA	Acetyl CoA	CoA	Acetyl CoA	
Perirenal adipose tissue (3)		Acyl-CoA cycling	0.2	1.2							Tokutake et al. (2010)
Perirenal adipose tissue (6)	High carbohydrate diet	Acyl-CoA cycling	0.4	0.2							Tokutake et al. (2012)
Brown adipose tissue (3)		Acyl-CoA cycling	7.9	5.9							Tokutake et al. (2010)
Brown adipose tissue (6)	High carbohydrate diet	Acyl-CoA cycling	36.4	13.1							Tokutake et al. (2012)
Epididymal adipose tissue (3)		Acyl-CoA cycling	0.1	0.6							Tokutake et al. (2010)
Epididymal adipose tissue (6)	High carbohydrate diet	Acyl-CoA cycling	0.6	0.3							Tokutake et al. (2012)
Mouse											
Liver (6)		Enzymatic assay	77.3	75.6							McAllister et al. (1988)
Liver (3)		HPLC/MS	65.6	78.8							Gao et al. (2007)
Liver (4)		Enzymatic Assay	75.0								Zhang et al. (2007)
Heart (3)		HPLC/MS	37.0	29.8							Gao et al. (2007)
Heart (4)		Enzymatic Assay	18.0								Zhang et al. (2007)
Muscle (3)		HPLC/MS	8.5	3.3							Gao et al. (2007)
Kidney (4)		Enzymatic Assay	4.0								Zhang et al. (2007)
Brain (4)		Enzymatic Assay	0.5								Zhang et al. (2007)
Human											
Liver (5)		HPLC	154.0	43.0							Corkey et al. (1988)

Tissue/ Cell Type	Additional information	Method of detection	nmol/g wet weight		pmol/mg PCA pellet weight[a]		nmol/g protein[b]		pmol/10^6 cells[c]		Reference
			CoA	Acetyl CoA	CoA	Acetyl CoA	CoA	Acetyl CoA	CoA	Acetyl CoA	
Cultured Cells											
HepG2 (25)		**HPLC**	2.6	3.3	**6.9**	**8.9**	34.7	44.6	23.1	29.7	**This Study**
HepG2 (27)		**Recycling Assay**	1.9	3.9	**5.1**	**10.5**	25.4	52.4	16.9	34.9	**This Study**
HepG2		Fluorometric Assay							59.6		Palekar (2000)
Rat Hepatocytes (2)		HPLC	35.9	34.8							King et al. (1988)
Hek293 (6)		**HPLC**	2.1	3.8	**5.6**	**10.3**	27.8	51.7	18.5	34.5	**This Study**
Hek293 (24)		**Recycling Assay**	1.8	3.5	**4.8**	**9.6**	24.1	47.8	16.1	31.9	**This Study**
MEF (4)		**HPLC**	1.7	3.5	**4.6**	**9.4**					**This Study**
MOLT (3)		**HPLC**	11.8	0.0	**31.8**	**0.0**					**This Study**
K562 (4)		**HPLC**	6.7	0.0	**18.1**	**0.0**					**This Study**
pancreatic β cells (6)		HPLC					106.0	135.0			Corkey et al. (1989)
Hepa 1c1c7 cells		liquid chromatography/ electrospray ionization mass spectrometry	300.0	450.0							Basu et al. (2011)

Table 4.1: Summary of CoA and acetyl CoA levels in tissues and cultured cells. CoA and acetyl CoA levels in various tissues and cultured cells, measured by the method stated have been compared. The number of samples is shown in parentheses. [abc] CoA levels were measured in this chapter in pmol/mg pellet. CoA levels were converted into nmol/mg wet weight[a], nmol/g protein[b], pmol/10^6 cells[c] using linear regression values obtained from samples standardised by all methods.

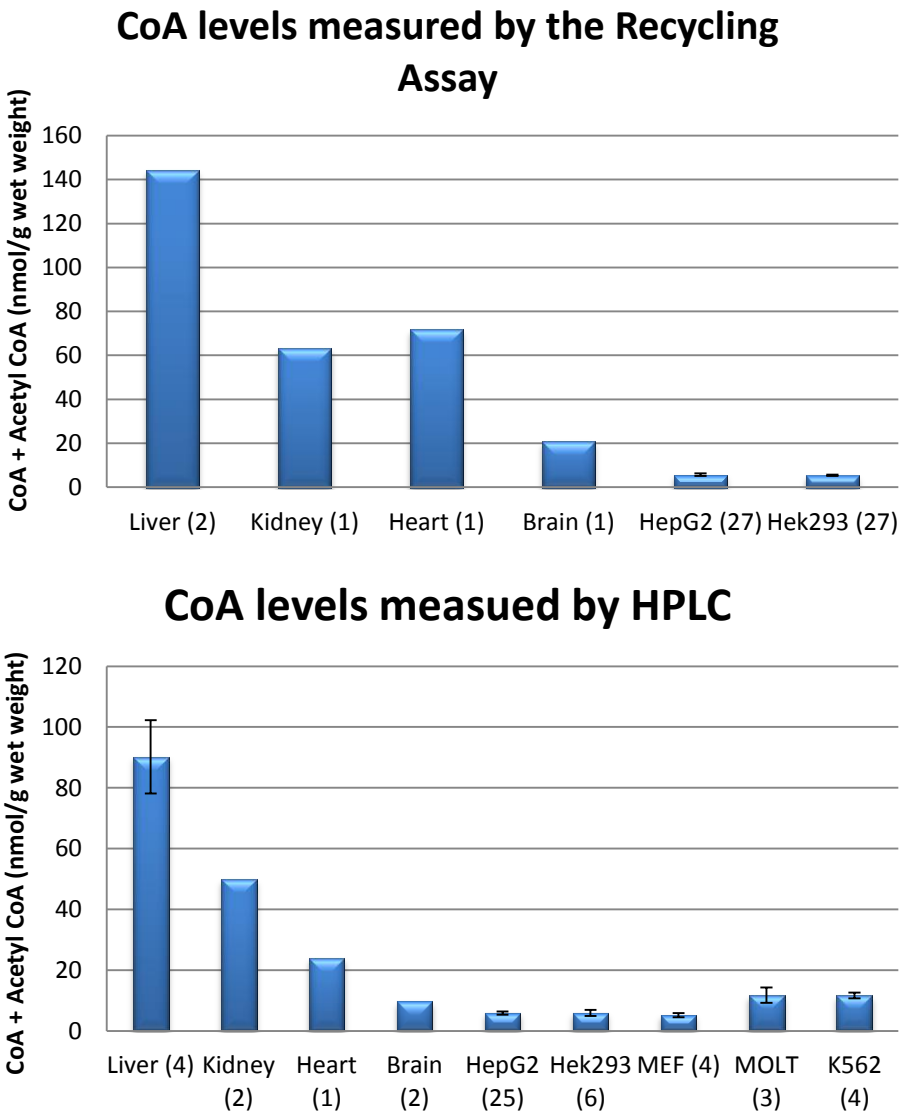


Figure 4.1: Summary of Total CoA levels measured. CoASH + acetyl CoA levels were measured in rat tissue and cell extracts by the enzymatic recycling assay **(A)** or HPLC **(B)**. The number of samples (from independent experiments) is shown in parentheses.

This pellet contains acid insoluble components of the cell including denatured protein and long chain fatty acyl-CoA thioesters. Preliminary experiments using HepG2 and Hek293 cells showed a good linear correlation between cell number, PCA pellet weight, cell wet weight, protein concentration and LDH content. This enables CoA levels in cultured cells to be expressed using different standardisation methods and allows comparison against literature values.

There have been a large number of studies reporting CoA levels in tissues. However there are limited reports in cultured cells (*Table 4.1*). Reported values for CoA vary to quite an extent for a given tissue. For example in the reports mentioned here, free CoASH levels in rat liver range from 22.3 - 135 nmol/g wet weight (acetyl CoA: 5-100.5 nmol/g wet weight). This is likely to be due to differences in the conditions used. CoA levels in adult rat tissues obtained during this study generally fell between the ranges observed previously *(Table 4.1, Figure 4.1)*. When measuring by HPLC, levels of CoASH were highest in liver (CoASH: 62.3 nmol/g wet weight, acetyl CoA: 27.9 nmol/g wet weight), followed by kidney (CoASH: 43.5 nmol/g wet weight, acetyl CoA: 6.3 nmol/g wet weight), heart (CoASH: 13.8 nmol/g wet weight, acetyl CoA: 50.8 nmol/g wet weight) and brain (CoASH: 7.5 nmol/g wet weight, acetyl CoA: 2.2 nmol/g wet weight). Similarly, when combined levels of CoASH + acetyl CoA were measured by the recycling assay the highest CoA levels were seen in liver (144.2 nmol/g wet weight), followed by heart (71.9 nmol/g wet weight), kidney (63.2 nmol/g wet weight), and brain (20.7 nmol/g wet weight). The differences in CoA levels obtained by the two analytical methods were probably due to the fact the tissues used for each method were from different rats and a low sample number was used. It is unlikely that the differences are due to difference in the two analytical methods because when the same sample of powdered tissue was analysed by both methods, very similar results were achieved. In this study, the focus was on the two cell

lines HepG2 and Hek293. Liver tissue is known to have the largest concentration of CoA among tissues due to its high metabolic activity, and its CoA level is the most responsive to changes in metabolic state. HepG2 cells are a human liver carcinoma cell line which retain some of the characteristics of liver *in* vivo (Brandon et al. 2006; Chen et al. 2002; Feng et al. 2001; Murakami 2002). Hek293 is derived from human embryonic kidney cells and is also used here due to its ease of maintenance and genetic manipulation. However, it is important to note that because they are experimentally transformed, they are not representative of kidney cell function (Graham et al. 1977; Shaw et al. 2002). When measuring by HPLC, HepG2 CoASH levels were 6.93 ± 1.0 pmol/mg PCA pellet (2.57 nmol/g wet weight) and acetyl CoA levels were 8.91 ± 0.95 pmol/mg PCA pellet (3.30 nmol/g wet weight) (n=25). Recycling assay values were comparable, with CoASH levels of 5.08 ± 0.61 pmol/mg PCA pellet (1.88 nmol/g wet weight) and acetyl CoA levels of 10.48 ± 1.22 pmol/mg PCA pellet (3.88 nmol/g wet weight) (n=27). Hek293 CoA levels were also in the same range, with CoASH and acetyl CoA levels of 5.55 ± 1.2 pmol/mg PCA pellet (2.06 nmol/g wet weight) and 10.34 ± 2.26 pmol/mg PCA pellet (3.83 nmol/g wet weight), respectively, when measured by HPLC (n=6); and CoASH and acetyl CoA levels of 4.82 ± 0.67 pmol/mg PCA pellet (1.79 nmol/g wet weight) and 9.56 ± 0.77 pmol/mg PCA pellet (3.54 nmol/g wet weight), respectively, when measured by the recycling assay (n=24).

Notably, CoASH and acetyl CoA levels were much lower in both HepG2 and Hek293 cells compared to liver. The possibility was considered that this large difference may be due to the method of standardisation. It was observed protein amount per gram of wet weight of liver is four fold higher than in HepG2 cells. This may suggest that there are more liver cells/g wet weight compared to cultured cells, although the high protein concentration in liver may also be due to blood proteins, since these are not removed during the freeze clamping process used to collect liver samples. Assuming liver contains approximately

$15x10^7$ hepatocytes per g tissue wet weight (Carthew et al. 1997), liver contains approximately 500 pmol CoASH/10^6 cells. This is still over 20-fold higher than the CoASH level in HepG2 cells expressed per 10^6 cells (approximately 20 pmol/10^6 cells) (Palekar 2000). Therefore, the contrast between liver and HepG2 cells does not appear to be due to the method of standardisation. The difference in CoA levels between HepG2 cells and liver tissue is also unlikely to be due to the fact that the tissues are from rat, as opposed to the cells which were derived from humans, since CoA levels reported in human liver tissue is very similar to, if not higher than levels reported in rat liver *(Table 4.1)*. Finally, the variation in CoA levels between liver and cultured cells is not due to sample preparation and analytical methods, since percentage recovery experiments in Chapter 3 determined that CoA was not lost during the extraction procedure. The only other study reporting CoASH levels in HepG2 cells found was by Palekar (2000), who reported CoASH levels of 59.6 pmol/10^6 cells. Although this is 2.5 times higher than what was determined in this study, it is still 10 fold less than in liver. CoASH and acetyl CoA were also measured by HPLC in other cultured cells including mouse embryonic fibroblasts (MEF), and the human leukaemia cell lines MOLT and K562. Similarly to HepG2 and Hek293, the CoASH levels were lower than in liver in these cultured cells (15-30 pmol/mg PCA pellet).

4.2.2 Characterisation of CoA levels under different conditions in cultured cells

CoA levels are known to change in tissues in response to a number of extracellular stimuli. Cultured cells were treated with various stimuli to determine the conditions under which CoA levels change and also how responsive these cells are to the conditions tested.

4.2.2.1 The effect of FBS

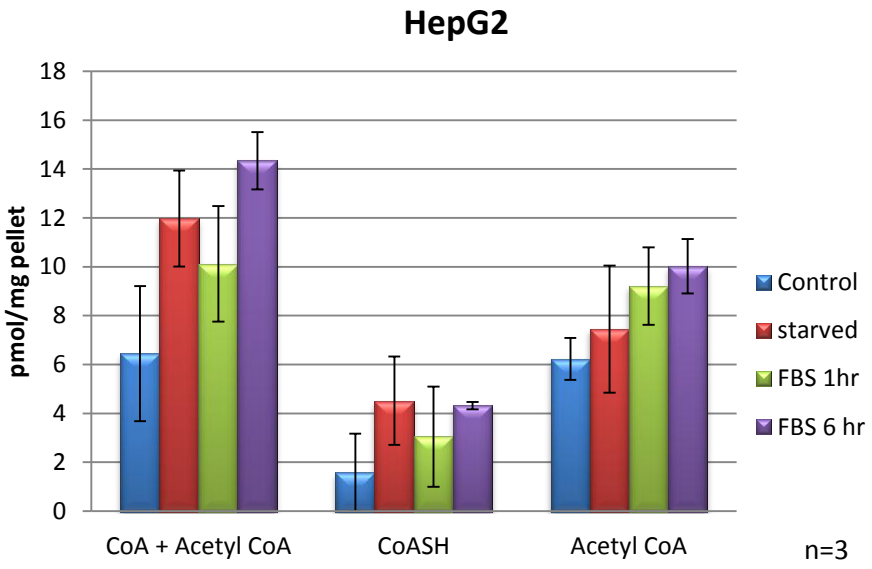
Initially, to characterise how cultured cells behave, HepG2 and Hek293 cells were starved of FBS for 24 hrs followed by 10% FBS stimulation in Dubecco's Modified Eagle

Medium (DMEM) for time points ranging between 1 and 6 hrs *(Figure 4.2)*. Starvation and serum stimulation does not significantly affect CoA levels in HepG2 cells. In Hek293 cells, FBS stimulation for 1 hr increases free CoASH levels, which then return to basal levels after 6 hrs. This is also seen as a significant decrease in combined CoASH + acetyl CoA levels. These results suggest that growth factor signalling pathways do not regulate CoA levels in HepG2 cells. The increase in CoA levels in Hek293 cells might indicate an increase in CoA biosynthesis, or more likely an increase in the conversion of acetyl CoA to CoASH, which can be seen between FBS starved conditions and 1 hr FBS stimulation, although is not statistically significant. FBS contains many components, including nutrients and mitogenic stimuli that may affect CoA levels in different directions, so it is difficult to interpret the exact cause for the fluctuations in CoA levels. Tissue specific differences between Hek293 and HepG2 cells may account for the different responses.

4.2.2.2 The effect of Glucose and Insulin

Studies with radioactive [^{14}C]pantothenate indicate that CoA synthesis in rat liver and perfused heart is inhibited following 1 hr incubation with glucose at the first step of the biosynthesis pathway, PanK, due to an increase in the accumulation of [^{14}C]pantothenate. Incubation for 1 hr with insulin in the presence of glucose further inhibits the rate of pantothenate phosphorylation, whereas insulin on its own does not show any inhibition, indicating that insulin does not inhibit PanK directly (Robishaw et al. 1982; Smith 1978). Furthermore, the levels of CoA are reduced in liver and hepatocytes in the presence of insulin and cytosolic CoA levels are increased 150% in diabetic hearts (mitochondrial 30%) (Neely et al. 1982; Reibel et al. 1981a; Reibel et al. 1981b; Smith and Savage 1980). The mechanism by which insulin regulates CoA levels is still unclear, although it is thought it may be due to its action on carbohydrate metabolism.

A



B

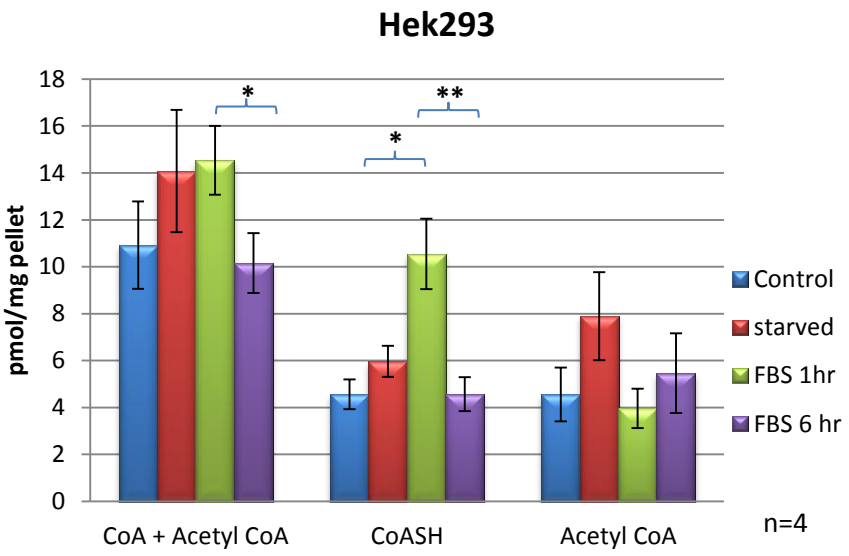


Figure 4.2: The effect of FBS starvation and stimulation on CoA levels in HepG2 and Hek293 cells. HepG2 (**A)** and Hek293 **(B)** cells were incubated in FBS free DMEM for 24 hours at 37˚C, 10% CO_2, then stimulated with 10% FBS for either 1 or 6 hours. Control cells were incubated in DMEM + 10% FBS. CoA levels were measured in cell extracts and quantified by the recycling assay. Free CoA levels were calculated from the difference between total and acetyl CoA. $p<0.05$ (*); $p<0.001$ (**)

To determine whether CoA levels would decrease specifically upon glucose and insulin stimulation, HepG2 cells were incubated in serum and nutrient free medium, and then stimulated with increasing concentrations of glucose (0-25 mM), with or without insulin. The expected decrease in CoA levels in HepG2 cells was not observed upon treatment with different concentrations of glucose for 16 hrs (*Figure 4.3)*. A possible decrease in free CoASH levels occurred with 10 nM insulin treatment in HepG2 cells after 1 hr, however this was not statistically significant and after 6 hrs there was no difference compared to the control (*Figure 4.4A)* 100 nM Insulin had no effect on CoA levels in Hek293 cells despite the activation of the insulin signalling pathway as indicated by the phosphorylation of its downstream target, Protein Kinase B (PKB) (*Figure 4.4B&C*).

4.2.2.3 The effect of Fatty Acids

Recent data in the literature demonstrates significantly lower CoA levels in rat liver as well as cerebellum, medulla oblongata and skeletal muscle in rats fed a high fat diet compared to rats fed a high protein or high carbohydrate diet (Tokutake et al. 2010). CoASH and acetyl CoA levels were evaluated by HPLC after incubating HepG2 cells with the long chain fatty acid, 0.5 mM palmitate (C16) bound to 1% BSA and 1 mM carnitine for 6 or 24 hrs in FBS-free DMEM with 5 mM glucose. Carnitine is required for the transport of fatty acyl CoA across the inner mitochondrial membrane into the mitochondria. It is added to ensure there is sufficient carnitine to transport fatty acids into the mitochondria, because regulation of CoA levels by fatty acids might require β-oxidation in the mitochondrial matrix. There was no effect on CoA levels after 6 hrs incubation with palmitate, although after 24 hrs there was a statistically significant decrease in combined CoASH + acetyl CoA

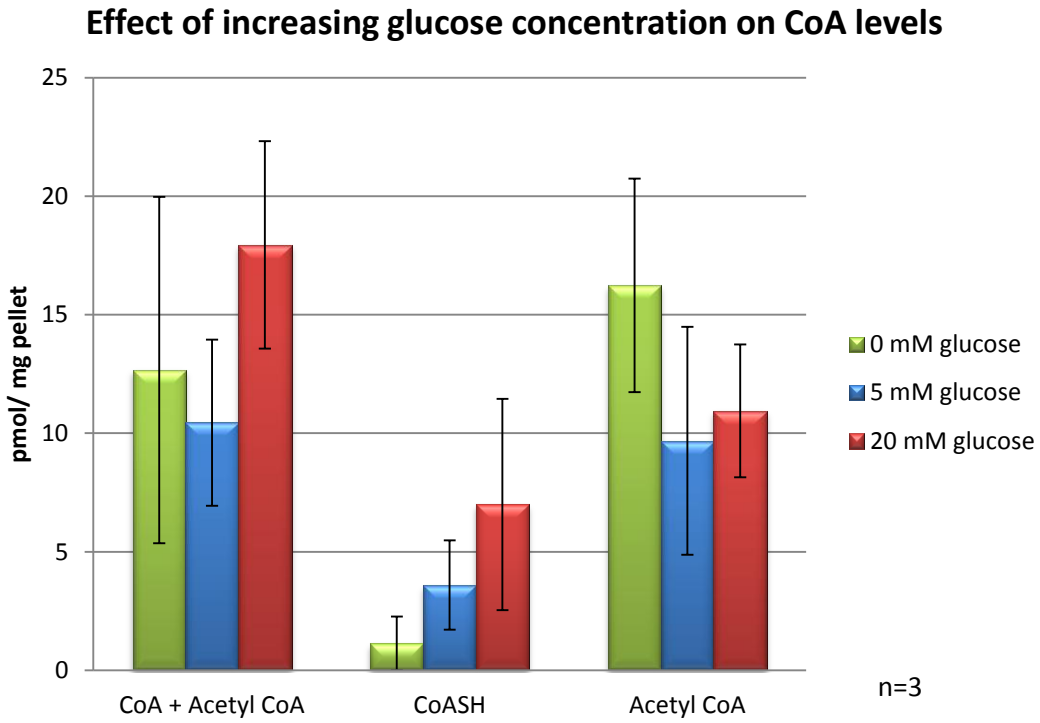


Figure 4.3: The effect of Glucose on CoA levels in HepG2 cells. HepG2 cells were incubated in FBS-free DMEM with low glucose (5 mM) and 1% BSA for 24 hours at 37˚C, 10% CO_2. Cells were then stimulated for 24 hours with concentrations of glucose ranging from 0-20 mM.

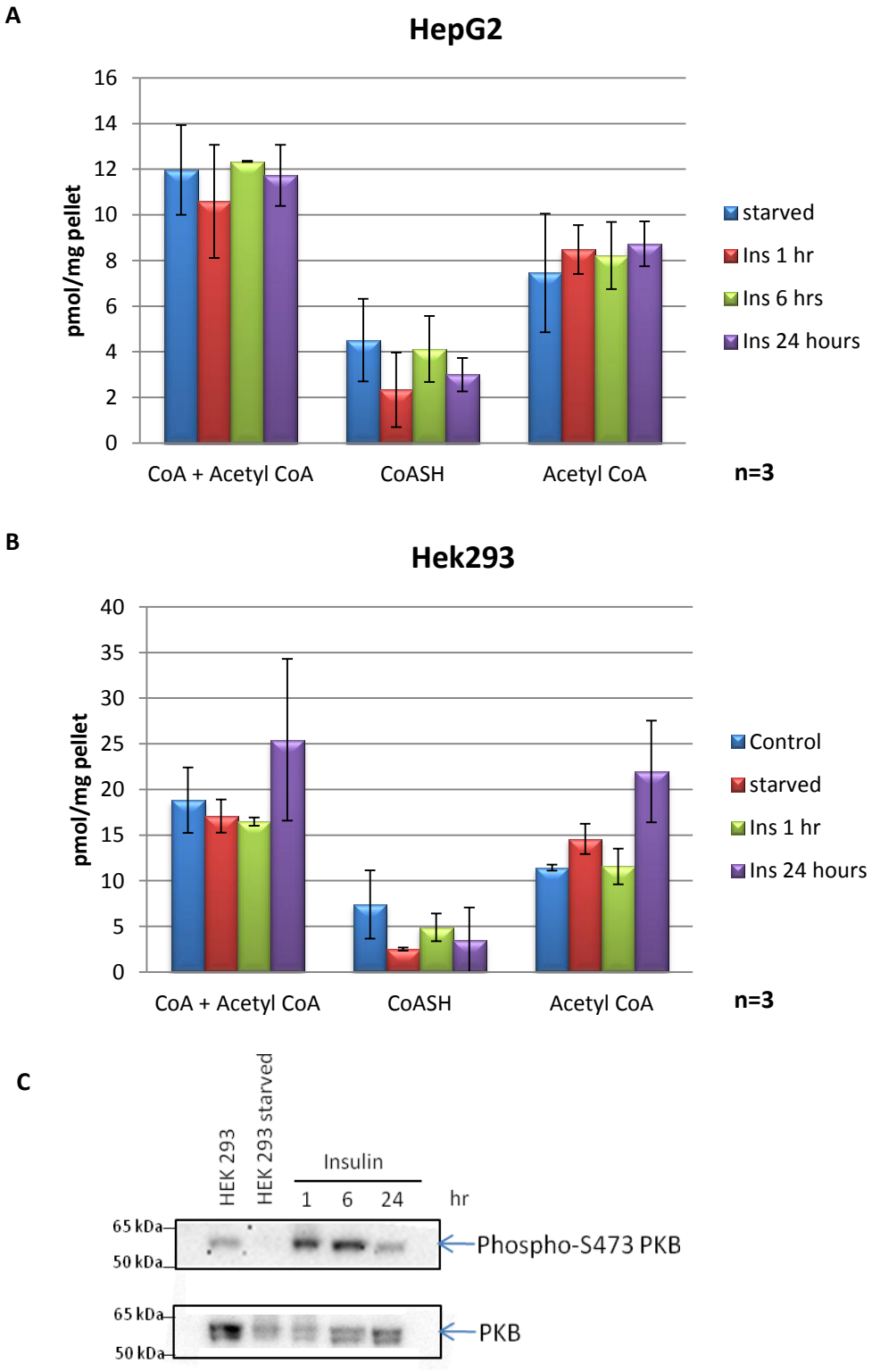


Figure 4.4: The effect of insulin on CoA levels. HepG2 (**A**) and Hek293 (**B**) cells were incubated in FBS free DMEM for 24 hours at 37˚C, 10% CO_2, then stimulated with 10 nM (HepG2)/ 100 nM (Hek293) Insulin for the various time points indicated. CoA levels were measured in cell PCA extracts, which were quantified by the recycling assay. Free CoA levels were calculated from the difference between total and acetyl CoA. C) Western blot analysis of phospho-S473 PKB indicated insulin signalling was activated by 100 nM Insulin stimulation in Hek293 cells.

levels (*Figure 4.5A*). This decrease was likely to be due to a decrease in free CoASH as there were no significant changes in acetyl CoA levels (*Figure 4.5B&C*).

4.2.3 The effect of signalling pathway inhibitors

Association of CoASy with S6K and p85α subunit of PI3K provides a potential link between CoA biosynthesis and the mTOR and PI3K signalling pathways. No changes in CoASy activity were observed *in vitro* in previous studies from the association of CoASy and S6K, however it is possible that components upstream or downstream of S6K might regulate CoASy *in vivo*. Indeed, mTOR, p85α and p110α subunits of PI3K were also detected in the mitochondrial fraction and p85α associates with CoASy on the outer mitochondrial membrane in Hek293 cells (Breus et al. 2009; Nemazanyy et al. 2004). mTOR is a key regulator of cellular growth, which requires the synthesis of fatty acids for membranes. As CoA is an important factor in fatty acid synthesis, mTOR signalling may be accompanied by changes in CoA levels.

4.2.3.1 Regulation of CoA levels via the mTOR pathway

To establish whether mTOR signalling regulates CoA levels, HepG2 cells were treated with 100 nM rapamycin (mTOR inhibitor) for 24 hrs in DMEM containing FBS. The fluctuations in CoASH and acetyl CoA levels detected by HPLC upon rapamycin treatment were not statistically significant *(Figure 4.6A)*. PP242 is another mTOR inhibitor, which is selective and ATP competitive. Treatment with 2.5 μM PP242 did not appear to cause any changes to CoASH or acetyl CoA levels in either Hek293 or HepG2 cells after analysis by the recycling assay *(Figure 4.6B&C)*. Both these inhibitors effectively decreased mTOR activity, as indicated by the markedly reduced phosphorylation at the mTOR target site (threonine 389) of S6K *(Figure 4.6D)*. These data indicate that mTOR signalling does not affect CoA levels over a 24 hr treatment in cultured HepG2 or Hek293 cells.

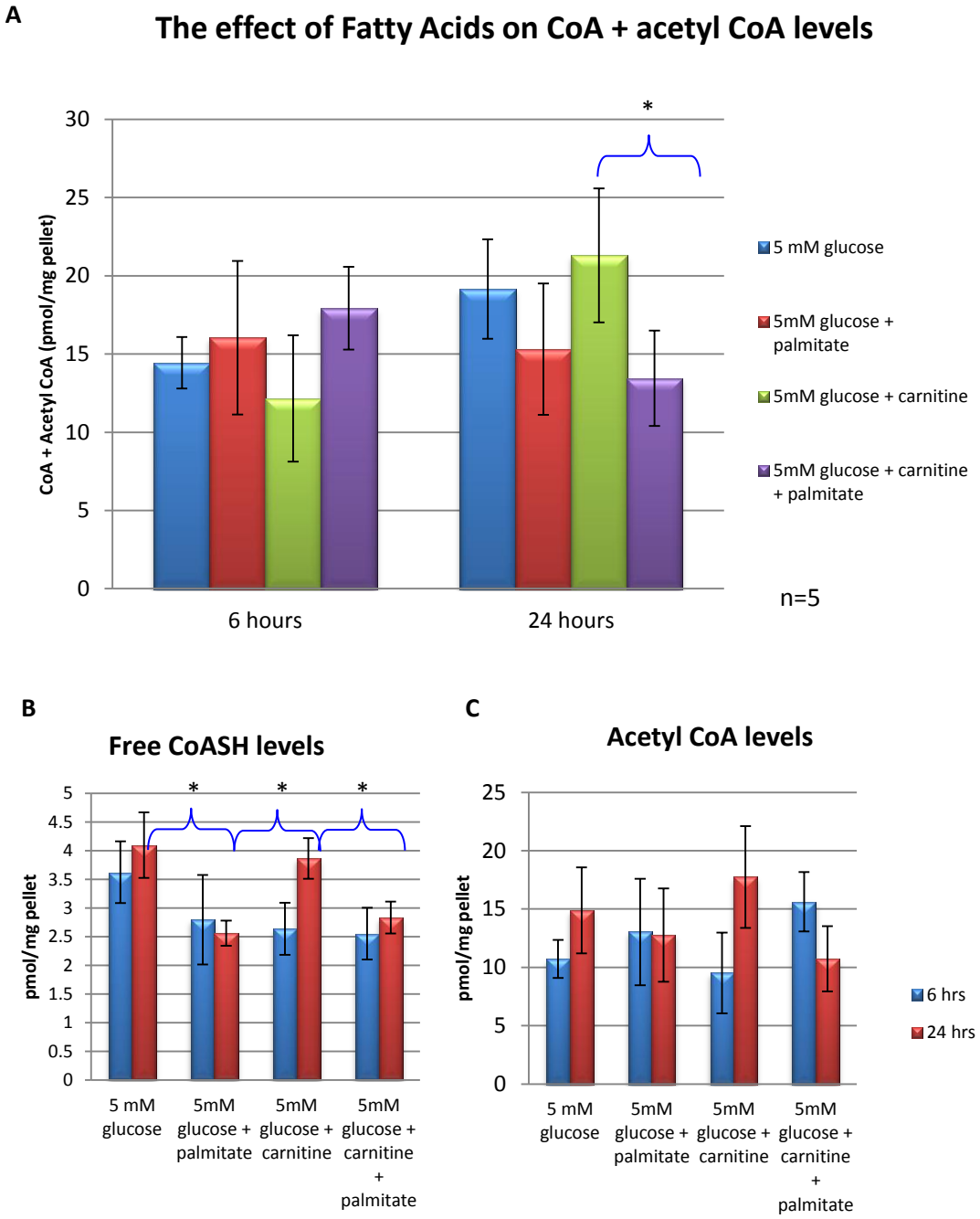


Figure 4.5: The effect of Fatty Acids on CoA levels in HepG2 cells. HepG2 cells were incubated in DMEM + 5 mM glucose, 1% BSA for 16 hours at 37°C, 10% CO_2. Cells were then incubated with 0.5 mM palmitate, 1 mM carnitine or 0.5 mM palmitate + 1 mM carnitine for either 6 or 24 hours at 37°C, 10% CO_2. Combined CoA + acetyl CoA (**A**), free CoASH (**B**) and acetyl CoA (**C**) levels were then measured in PCA extracts by HPLC. $p<0.05$ (*).

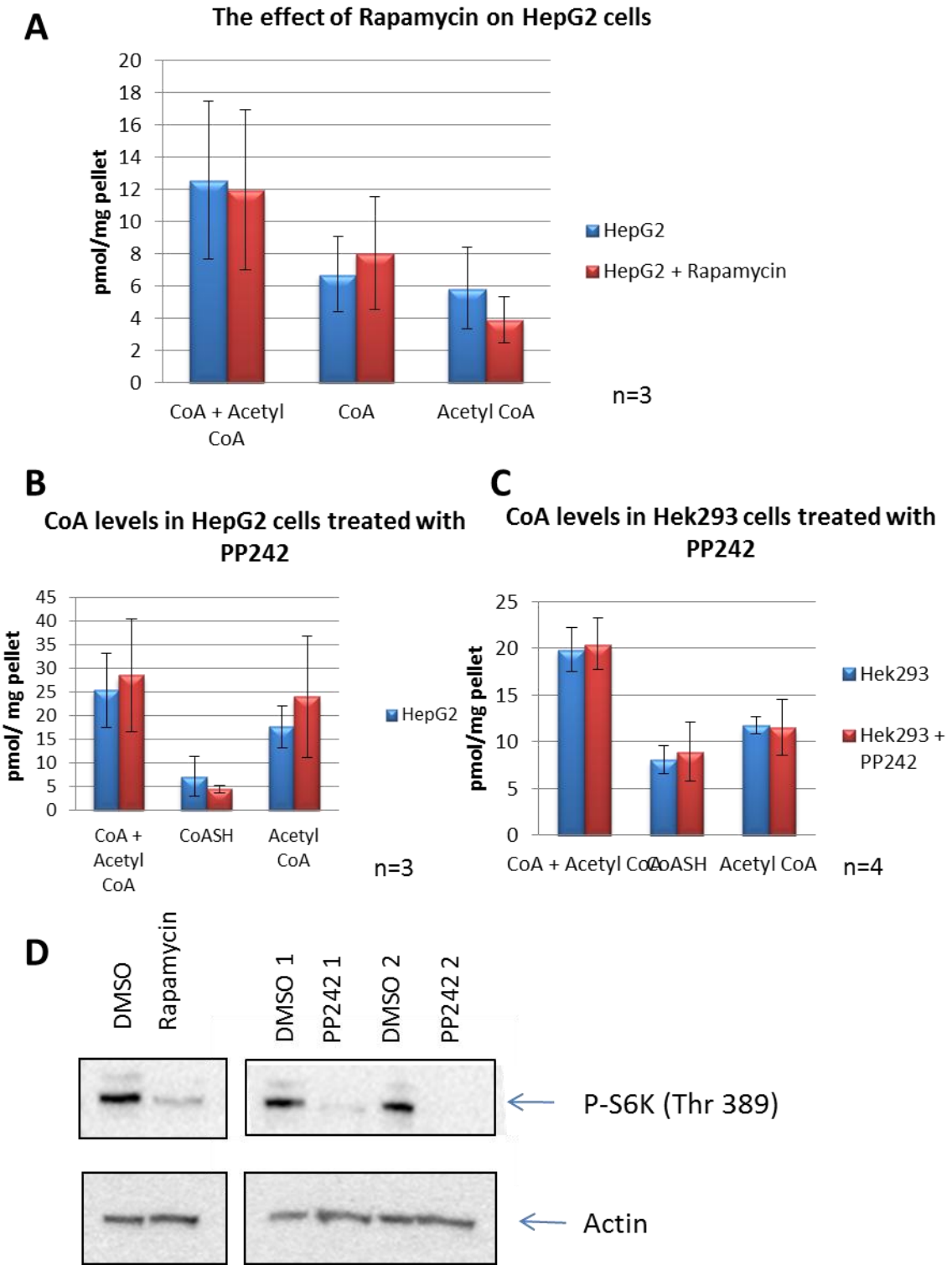


Figure 4.6: The effect of mTOR signalling on CoA levels. HepG2 or Hek293 cells were incubated in DMEM + 10% FBS in the presence 100 nM Rapamycin (mTOR inhibitor) (**A**), or 2.5 µM PP242 (mTOR inhibitor) (**B&C**) for 24 hours at 37˚C, 10% CO_2. CoASH + acetyl CoA as well as free CoA and acetyl CoA were quantified in PCA cell extracts using HPLC (**A**) or the recycling assay (**B&C**). **D)** Western blot analysis of mTOR activity, exemplified by S6K phosphorylation after 100 nM rapamycin or 2.5 µM PP242 treatment in HepG2 cells.

4.2.3.2 Regulation of CoA levels via the PI3K pathway

To ascertain whether PI3K signalling affects CoA levels through its association with CoASy, HepG2 cells were treated with 50 µM LY294002 (generic inhibitor of PI3 kinase) for 24 hrs in the presence of 10% FBS. Even with a high concentration of the inhibitor, there was not a statistically significant change in CoA levels when measured by the recycling assay *(Figure 4.7)*. This implies that CoA levels are not affected by PI3K signalling in HepG2 cells.

4.2.3.3 Regulation of CoA levels via the MAPK pathway

Mitogen-activated protein kinase (MAPK) signalling can also regulate cell growth and proliferation. Cells undergoing these processes require more energy and substrates, so consequently would have an increased metabolism. To find out whether MAPK signalling affects CoA levels, HepG2 cells were treated with 20 µM PD98059 (inhibitor of MEK1 and MEK2). Very little change was observed in CoASH and acetyl CoA levels after analysis by HPLC *(Figure 4.8)*. It is therefore unlikely that CoA levels in HepG2 cells are affected by MAPK signalling.

4.2.4 Manipulation of cellular CoA levels to identify cellular functions affected by changes in CoA

Several different methods were explored to modulate intracellular CoA levels in order to study the role of CoA as a regulator of cellular function.

4.2.4.1 Regulation of CoA levels by Bezafibrate

Fibrate drugs are agonists for the peroxisome proliferator activator (PPAR) receptor, which generally results in expression of genes involved in fatty acid metabolism. CoA levels have been shown to increase upon treatment with fibrates in both tissues (liver, kidney, heart, skeletal muscle) (Halvorsen 1983; Horie et al. 1986; Voltti et al. 1979) and cultured cells (HepG2) (Ramaswamy et al. 2004). It has been previously confirmed that

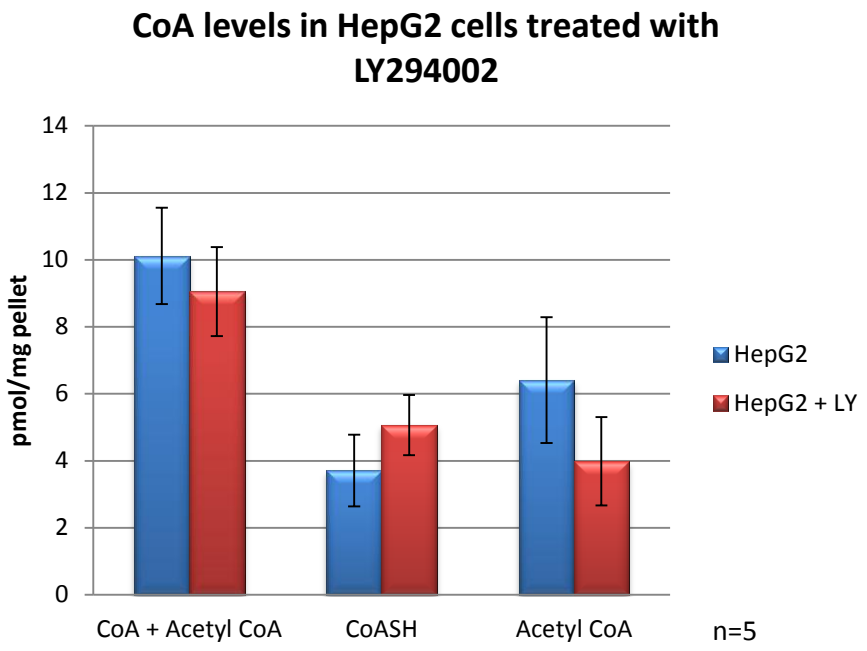


Figure 4.7: The effect of a PI3K signalling on CoA levels. HepG2 cells were incubated in DMEM, 10% FBS in the presence of 50 µM LY294002 (PI3K inhibitor), for 24 hours at 37˚C, 10% CO_2. CoASH + acetyl CoA as well as free CoA and acetyl CoA were quantified in PCA cell extracts using the recycling assay. Free CoA levels were calculated from the difference between total and acetyl CoA.

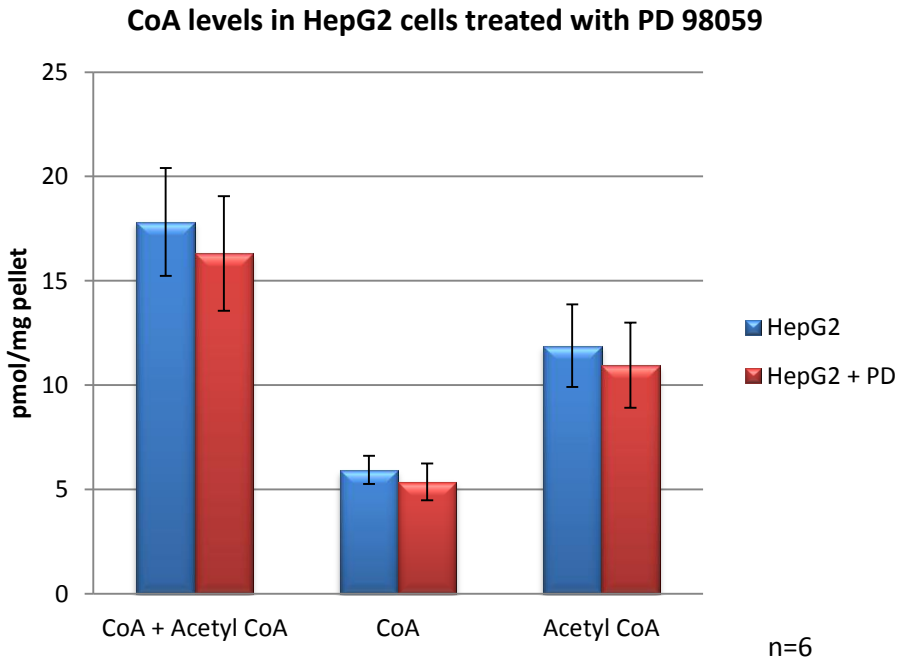


Figure 4.8: The effect of MAPK signalling on CoA levels. HepG2 cells were incubated in DMEM, 10% FBS in the presence of 20 µM PD98059 (MAPK inhibitor), for 24 hours at 37˚C, 10% CO_2. CoASH + acetyl CoA as well as free CoA and acetyl CoA were quantified by HPLC.

bezafibrate specifically increases PanK1α expression through activation of PPARα. The increase in PanK1α expression following bezafibrate treatment resulted in a 2.6 fold increase in CoA levels (Ramaswamy et al. 2004). In order to reproduce this effect, HepG2 cells were incubated with bezafibrate in DMEM containing 10% FBS for 24 hrs. A 2 fold increase was detected in combined CoASH + acetyl CoA levels in HepG2 cells by the recycling assay *(Figure 4.9A)*, following an increase in PanK1β expression *(Figure 4.9B).* Most of this change appeared to be due to an increase in acetyl CoA, rather than free CoASH. It is possible that free CoASH levels are tightly regulated. Alternatively CoA is rate-limiting for reactions that require CoA as a cofactor, and any resulting changes in CoA might be seen via the converted acylated CoA products. A 1.6 fold increase in CoA levels was also detected after bezafibrate treatment in Hek293 cells *(Figure 4.10C)*. These data indicate that PanK regulation of CoA biosynthesis still takes place in these cells. Bezafibrate is known to have effects other than increasing PanK1 expression, therefore is not suitable for the study on the role of CoA as a regulator of cellular processes and a more specific method of manipulating CoA levels is required.

4.2.4.2 Overexpression of CoASy

Bezafibrate effects showed that changing CoA biosynthetic enzymes is a good way to change CoA levels. Initially, tetracycline-inducible stable cell lines of Hek293 over-expressing CoASy wild type (WT) and a dominant negative mutant which completely disrupts PPAT activity, CoASy*203 were tested. Altering CoA levels through CoASy expression is potentially easier than altering CoA levels through PanK expression since PanK has a number of isoforms (1-4) and also these cell lines have previously been established in this laboratory. To determine whether over-expression of this enzyme results in an increase in CoA levels, Hek293 parental (empty vector), and Hek293 over-expressing WT and mutant forms of CoASy were treated with 1 μg/mL tetracycline in DMEM with 10% FBS

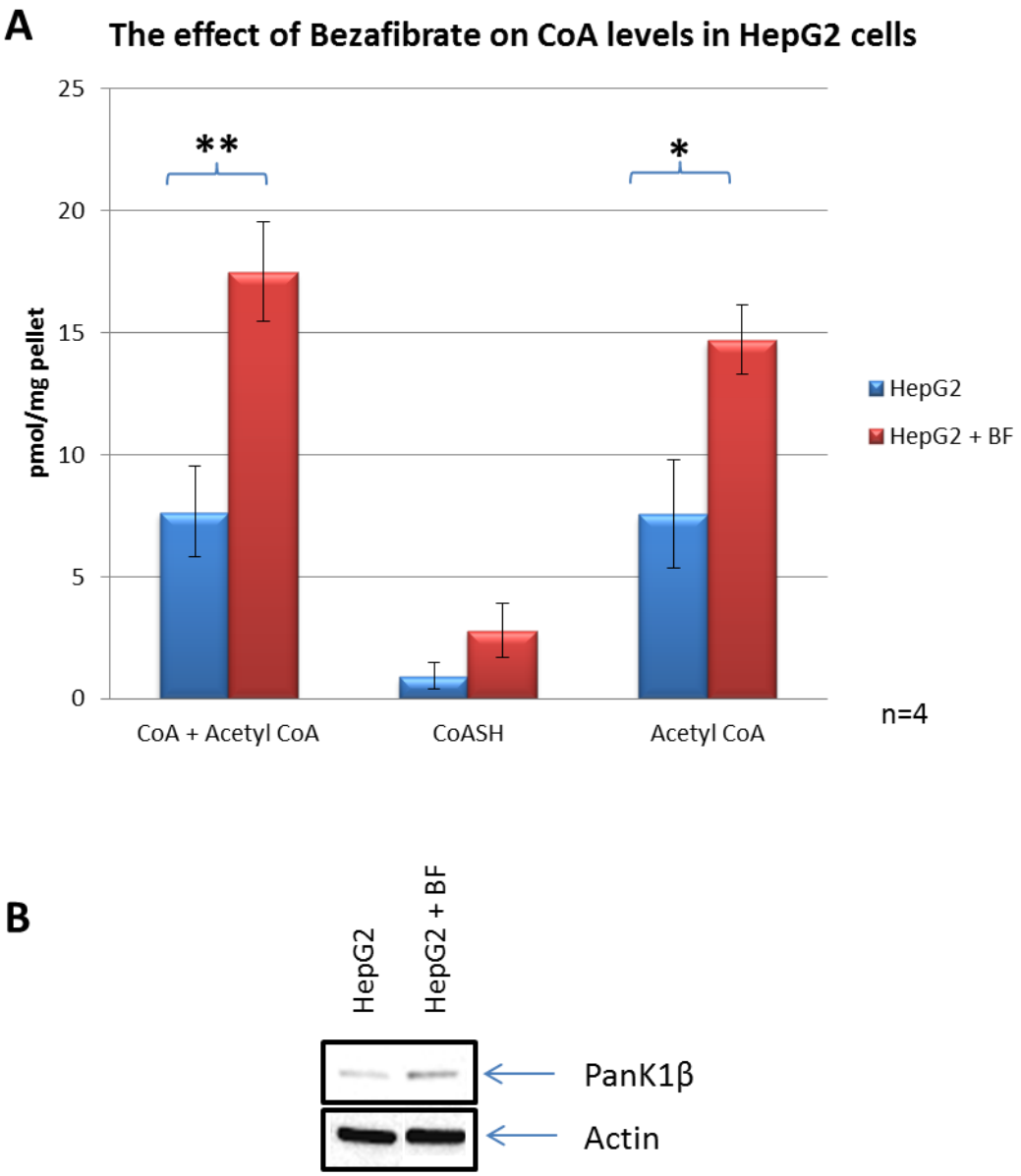


Figure 4.9: Bezafibrate increased CoA levels in HepG2 cells. HepG2 cells were incubated in DMEM, 10% FBS in the presence of DMSO or 0.5 mM BF for 24 hours at 37˚C, 10% CO_2. **A)** Total CoA and acetyl CoA levels were quantified in cell extracts using the recycling assay. Free CoA levels were calculated from the difference between total and acetyl CoA. **B)** Western blot analysis of PanK1β expression in HepG2 lysates. $p<0.05$ (*); $p<0.001$ (**)

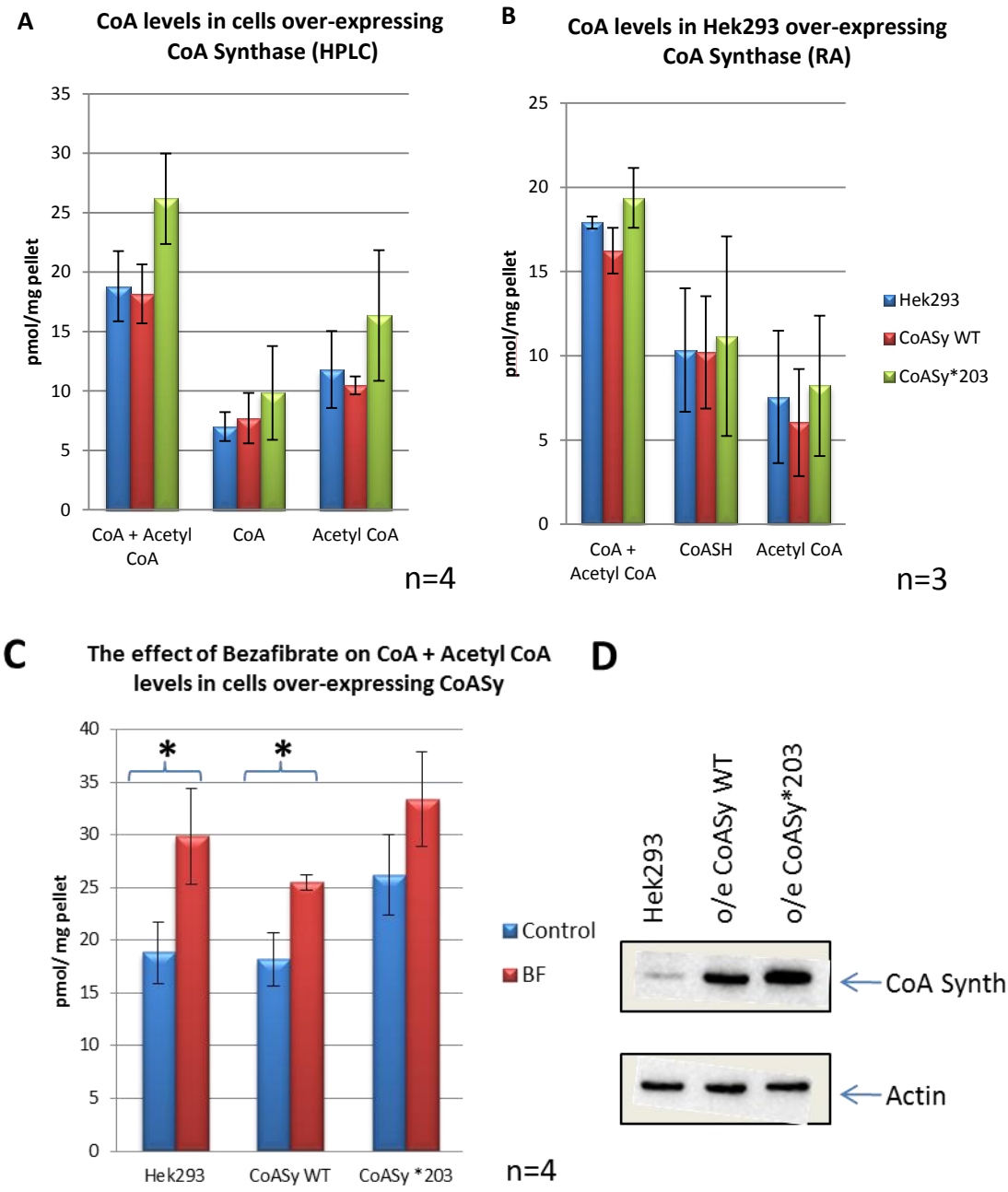


Figure 4.10: CoA levels in cells over-expressing CoA Synthase. Expression of CoASy was induced by adding 1 µg/ml tetracycline to Hek293 parental, Hek293 over-expressing CoASy WT or Hek293 over-expressing CoASy*203 cells in DMEM, 10% FBS at a confluency of 60% and incubating for 24 hours at 37˚C, 10% CO_2. Combined CoA + acetyl CoA, free CoASH and acetyl CoA levels were measured by HPLC (**A**) and the enzymatic recycling assay (**B**). Combined CoA + acetyl CoA levels were measured in all cell lines after a further 24 hour incubation with 0.5 mM BF at 37˚C, 10% CO_2 by HPLC (**C**). CoASy expression was measured in Hek293 parental, Hek293 over-expressing CoASy WT and mutant CoASy*203 cell lysates by Western Blot analysis (**D**) $p<0.05$ (*).

for 24 hrs. CoA levels were measured in all these cell lines by the recycling assay and HPLC. Surprisingly the expected increase in CoA levels with the wild type CoASy over-expressing cells or the decrease with CoASy*203 mutant cells was not seen when measuring by both methods *(Figure 4.10 A&B)*. This may be because under these conditions PanK is the major rate-limiting enzyme and the endogenous level of CoASy is sufficient to maintain normal turnover of CoA, especially when the basal rate of CoA biosynthesis is very low, as appears to be the case with these cells. Even when PanK was hyper activated with the hypolipidemic drug bezafibrate there was still no difference in CoA levels between Hek293 cells over-expressing CoASy, CoASy*203 and Hek293 parental control *(Figure 4.10C).* CoASy WT and CoASy*203 are both over-expressed *(Figure 4.10D)*, so these results imply that CoASy is not the main control point of CoA biosynthesis in Hek293 cells under normal conditions.

4.2.4.3 The effect of Hopantenate on CoA levels

Hopantenate (Hopan) is a structural analogue of pantothenate and is a PanK inhibitor in mice, which has been shown to decrease CoA levels in liver and kidney (Zhang et al. 2005). HepG2 cells were seeded at 30% confluency in DMEM containing 10% FBS for 4 hrs. After removing the media, cells were then incubated in pantothenate free DMEM (no FBS) with various concentrations of pantothenate, ranging from 0-16 mM, added independently, with or without the addition of 200 µM Hopan for 24 hrs. No significant changes in combined CoASH + acetyl CoA levels were detected by HPLC when changing the concentration of pantothenate in the media or when incubating Hopan with HepG2 cells (*Figure 4.11A).* Surprisingly cells are able to grow normally, even without added pantothenate in the medium. Therefore to determine if pantothenate is required for these cells, HepG2 cell growth was measured in the absence of pantothenate using the cell titre blue fluorometric assay. Only after seeding HepG2 cells at a high density (2000 cells/well –

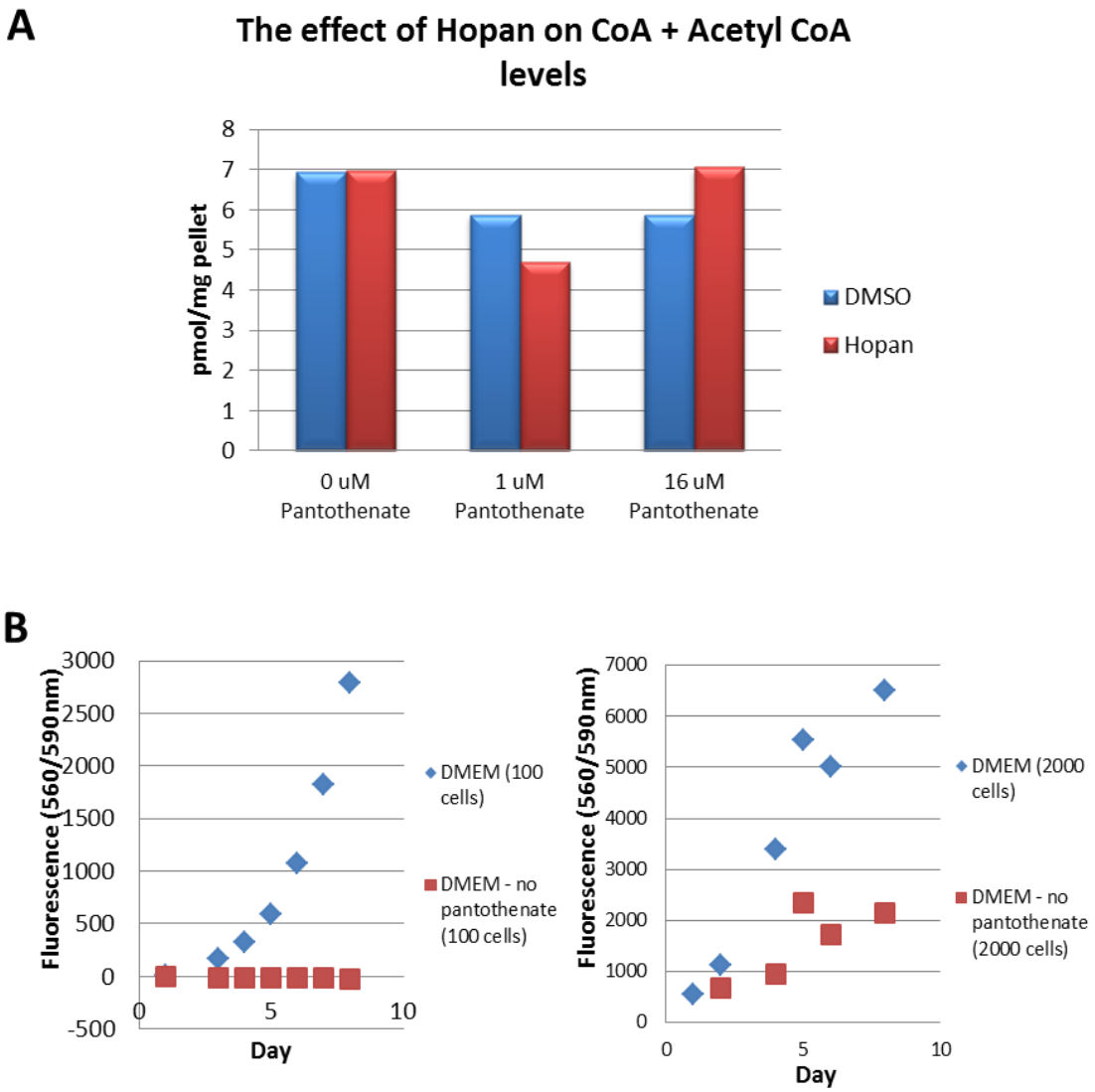


Figure 4.11: The effect of Pantothenate and Hopan on CoA levels in HepG2 cells. A) HepG2 cells were seeded at a confluency of 30% and incubated for 4 hours in DMEM, 10% FBS at 37°C, 10% CO_2. DMEM was removed and cells washed with PBS before incubating cells in pantotheniate free DMEM +/- pantothenate +/- 200 µM Hopan as indicated, for 48 hours. at 37°C, 10% CO_2. Combined CoA + acetyl CoA levels were quantified in cell extracts using HPLC. **B)** Growth analysis of HepG2 cells in pantothenate free media. HepG2 cells were seeded into 96 well plates at either 100 cells/well or 2000 cells/well and cell growth was measured in pantothenate free DMEM and normal DMEM using cell titre blue reagent, measuring 560/590 fluorescence.

96 well plate) was any cell growth seen, however this was greatly impaired compared to when pantothenate was present in the media (*Figure 4.11B)*. Together, these data indicate that cells need pantothenate to grow, however once they accumulate enough CoA, cells are able to survive in the absence of pantothenate. This suggests the extracellular supply of pantothenate is not essential for cell maintenance, possibly due to CoASH having a long half-life and/or a CoASH or pantothenate recycling mechanism is in place.

4.2.4.4 The effect of Pantethine on CoA levels

Pantethine has been shown to restore CoA levels in a PanK2 deficient PKAN (Pantothenate Kinase-associated neurodegeneration) *Drosophila* model, as well as in dPanK/fbl RNAi-treated S2 cells (Rana et al. 2010). Pantethine is a disulphide of pantetheine and both molecules can be phosphorylated by PanK and converted into 4'-phosphopantetheine (an intermediate in the canonical *de novo* CoA biosynthesis pathway) (Abiko 1967). Alternatively, a novel pathway for CoA biosynthesis, independent of PanK, has been proposed (Rana et al. 2010). HepG2 cells were supplemented with 100 µM pantethine in DMEM containing 10% FBS to see whether this might result in an increase in CoA levels. However, treatment up to 6 days did not result in a statistically significant change in combined CoASH + acetyl CoA levels. This may indicate that pantethine only can increase CoA levels when the canonical pathway is impaired.

4.2.4.5 Delivery of CoA using liposomes and cell penetrating peptides

The delivery of a number of biological compounds into mammalian cells has been improved by fusing them to cationic peptides and is a way to specifically increase CoA. These peptides are able to overcome the lipophilic barrier of the cellular membrane and are known as protein transduction domains or cell penetrating peptides. Cell penetrating peptides have been used to deliver a wide variety of cargoes including proteins, DNA, antibodies, toxins and nanoparticular drug carriers such as liposomes inside cells (Guidez

2004; Vives 2003). In these studies, the TAT peptide (GRKKRRQRRRPQ), derived from the transactivator of transcription of the HIV virus, was linked to a terminal cysteine and incubated with CoA dimer to attach CoA through a disulphide bond. The linkage of the TAT peptide with CoA would potentially transport CoA into mammalian cells following incubation, and free CoASH would be released in the reducing conditions inside the cell *(Figure 4.12)*. HepG2 cells were incubated with 10 µM TAT peptide, as well as poly-arginine (Arg9) (another cell penetrating peptide method) and also liposomes loaded with CoA to try and deliver CoA into cells, however none of the methods used could successfully increase CoA levels in cells.

4.2.5 Generation of a stable cell lines over-expressing PanK1β and its characterisation

Zhang *et al.* transiently over-expressed PanK1β and PanK3 in Hek293 T cells and in both cases measured a significant increase in CoA biosynthesis using radioactive pantothenate (Zhang et al. 2005). Since this appears to be a good way to specifically increase CoA, stable cell lines over-expressing PanK1β were generated to study the effect of increasing CoA on cultured cells.

4.2.5.1 Generation of Hek293 stably expressing PanK1β

PanK1β was amplified by PCR from the pET28a vector containing PanK1β obtained from ADDGENE. The forward primer contained an EcoRI restriction site as well as 30 bases from the N-terminal sequence that were missing from the ADDGENE construct. The reverse primer contained a NotI restriction site and the final base of the C-terminal sequence, which was missing from the ADDGENE construct. The PCR product was sub-cloned into pET30a and sequenced. The NotI/EcoRI-digested fragment was then ligated into pcDNA3.1(+). Restriction digestion analysis confirmed that PanK1β (~1000 bp) was

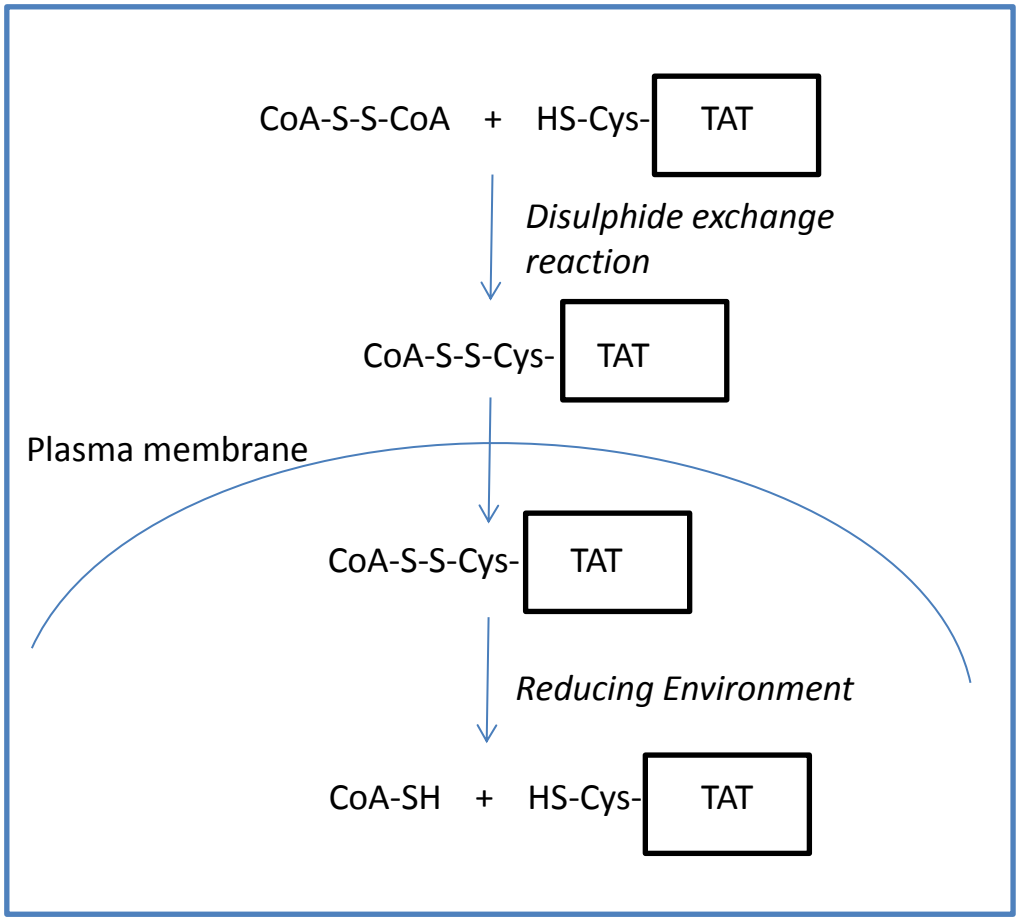


Figure 4.12: Potential method to deliver CoA into HepG2 cells using cell penetrating peptides. Schematic demonstrating how the TAT peptide (GRKKRRQRRRPQ) could be used to transport CoA into cells.

correctly inserted into pcDNA3.1(+) *(Figure 4.13A).* Sequencing analysis with the T7 forward primer and BGH reverse primer confirmed that PanK1β was cloned in the correct orientation and the additional bases were included *(Figure 4.13B&C)*. Purified pcDNA3.1(+)/PanK1β plasmids were transfected into Hek293 and HepG2 cells using ExGen500 reagent. Expression of PanK1β was much more efficient in Hek293 cells compared to HepG2 cells *(Figure 4.14A&B)*, so the generation of stable cell lines was continued in Hek293 cells using Geneticin® selection. After Geneticin® selection, expression of PanK1β was checked again and it was confirmed that stable cell lines over-expressing PanK1β were successfully generated *(Figure 4.14C)*.

4.2.5.2 Analysis of CoA levels in Hek293 over-expressing PanK1β

CoA levels were measured in these Hek293 cells over-expressing PanK1β using HPLC. In cells over-expressing PanK1β there was a 63% increase in free CoASH levels, a 130% increase in acetyl CoA levels, a 240% increase in malonyl CoA levels and a 57% increase in HMG CoA levels, which all contributed to a 2-fold elevation in total acid soluble CoA levels *(Figure 4.15A)*. Interestingly, no increase was observed in long-chain acyl CoA *(Figure 4.15B)*. A two-fold increase in combined CoASH + acetyl CoA levels was observed by the recycling assay *(Figure 4.15C)*. It is important to note that the 3-fold increase in malonyl CoA is a minimum estimate as it is not known whether any other peaks co-elute with malonyl CoA. Alkaline hydrolysis illustrated that no peaks co-eluted with the other CoA esters mentioned, however it was not possible to check this for malonyl CoA as its peak is difficult to identify under normal conditions. An increase in the peak which corresponds with malonyl CoA is clearly visible in Hek293 cells over-expressing PanK1β *(Figure 4.16).*

4.2.5.3 The effect of PanK1β over-expression on cell growth

To investigate whether cell growth was affected by the elevated CoA levels in PanK1β over-expressing cells, Hek293 parental and Hek293 cells over-expressing PanK1β

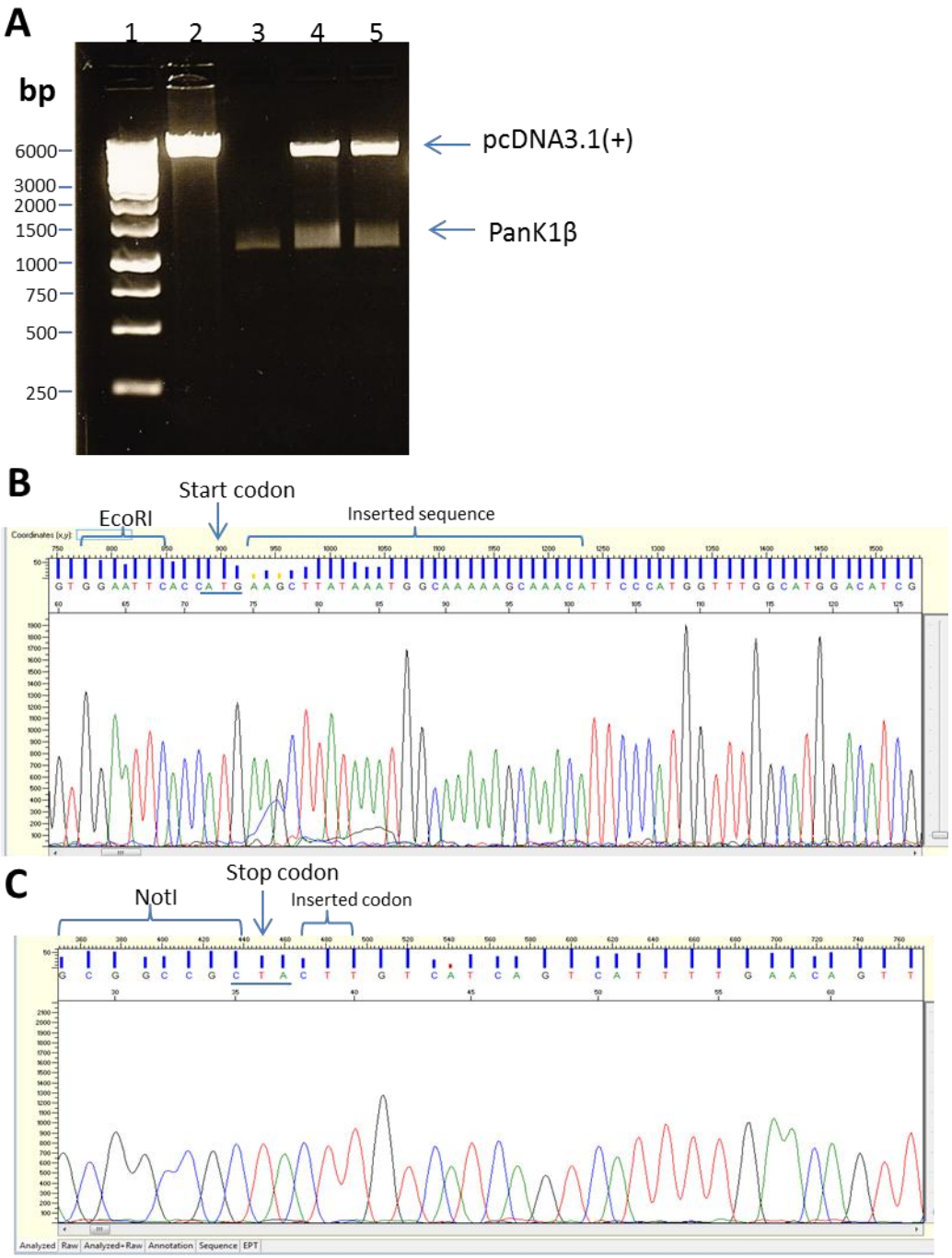


Figure 4.13: Molecular cloning of PanK1β. A) Restriction Digestion analysis shows that PanK1β has been correctly cloned into pcDNA3.1(+). Plasmid pcDNA3.1(+)/PanK1β was restricted with EcoRI and NotI to release the 1 kb PanK1β insert (lanes 4+5). Lane 1 contains the molecular weight marker, Lane 2 contains purified pcDNA3.1(+), Lane 3 contains purified PanK1β DNA (from PCR products). Products were analysed by agarose gel electropheresis. **B)** Sequencing analysis of pcDNA3.1(+)/PanK1β with T7 forward primer shows that the N-terminal region of PanK1β has the EcoRI restriction site and bases missing from the ADDGENE version of PanK1β inserted correctly. **C)** Sequencing analysis of pcDNA3.1(+)/PanK1β with BGH reverse primer shows that the C-terminal region of PanK1β has the NotI restriction site and codon missing from the ADDGENE version of PanK1β inserted correctly.

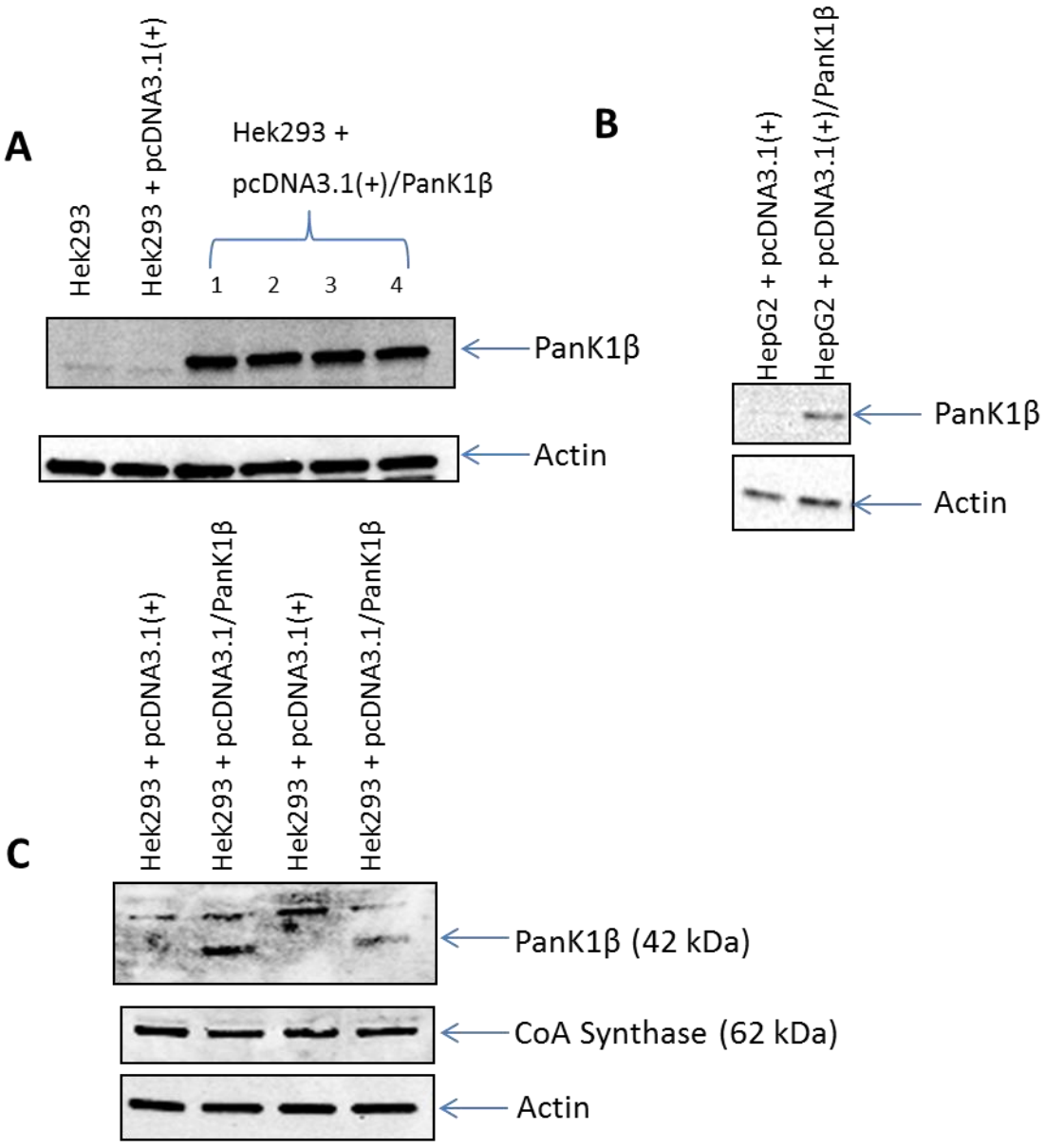


Figure 4.14: Generating stable cell lines overexpressing PanK1β. A) Expression of PanK1β was measured by western blot analysis in Hek293 transiently transfected with PanK1β using Exgen500 transfection reagent. **B)** Expression of PanK1β was measured by western blot analysis in HepG2 transfected with PanK1β using Exgen500 transfection reagent. **C)** Stable cell lines over-expressing PanK1β were generated by treating cells transfected with PanK1β or plasmid alone with 800 µg/mL Geneticin®. PanK1β expression was measured by western blot analysis in parental cells containing the plasmid alone and cells over-expressing PanK1β.

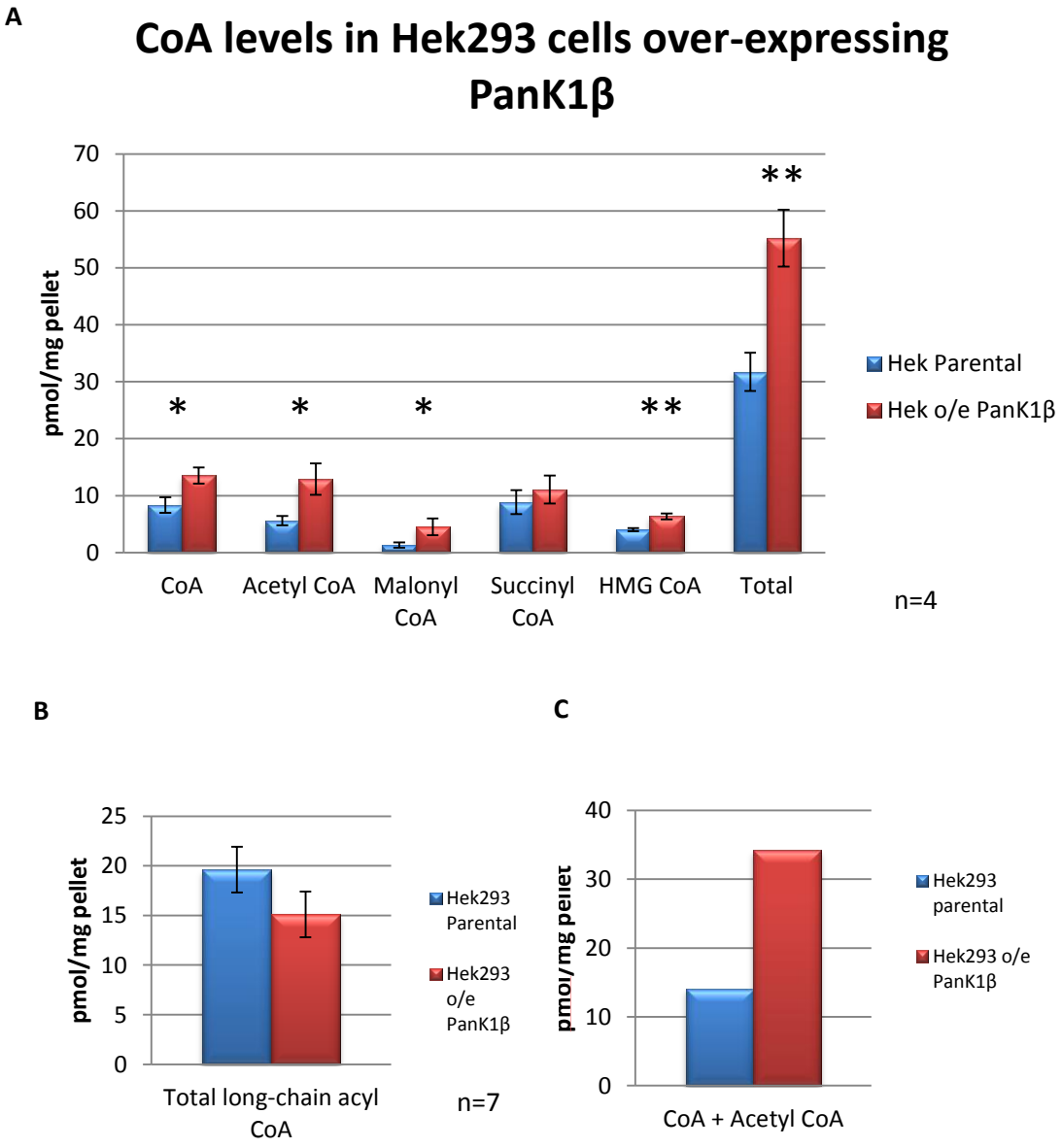


Figure 4.15: The level of CoA increases in cells over-expressing PanK1β. A) CoA and a range of CoA esters were measured in Hek293 parental and Hek293 over-expressing PanK1β cells using HPLC. **B)** Long chain CoA esters were measured by the recycling assay after alkaline hydrolysis of the PCA pellet. **C)** Combined CoA + acetyl CoA levels were measured in cell PCA extracts and quantified by the recycling assay. $p<0.05$ (*); $p<0.001$ (**)

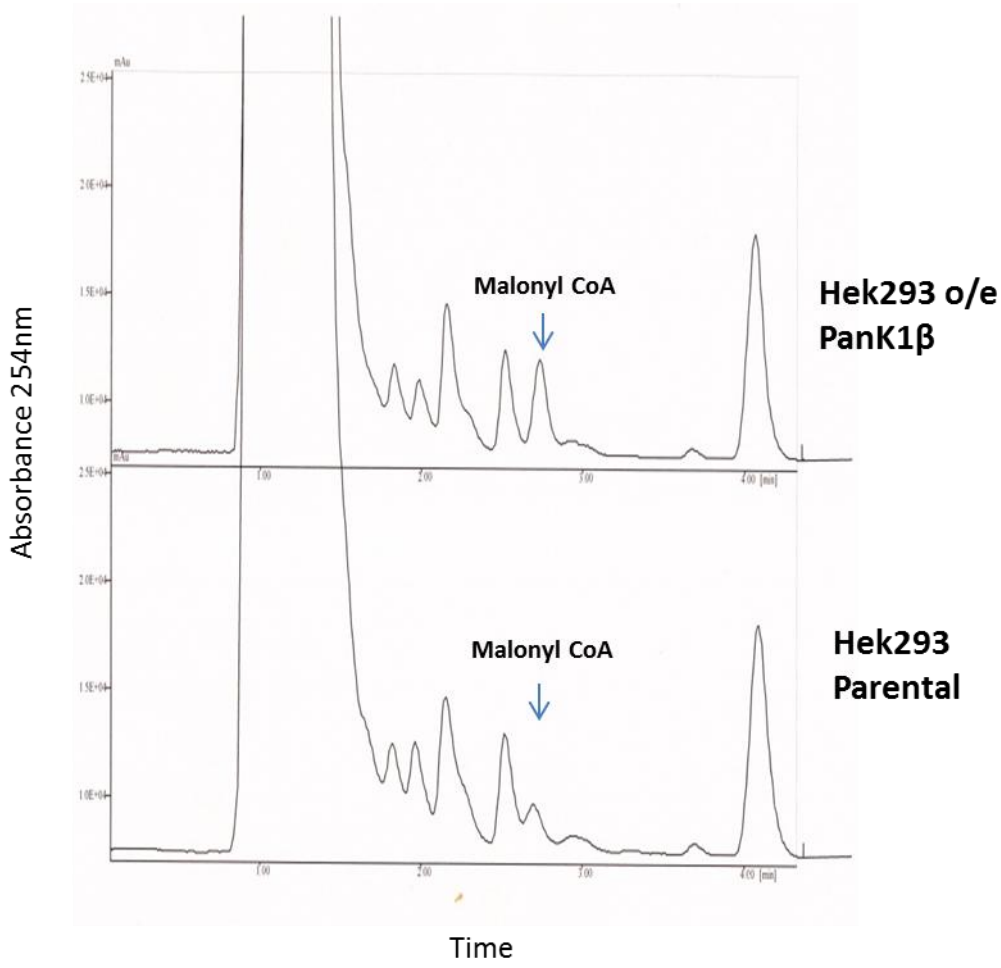


Figure 4.16: Representative HPLC profile showing malonyl CoA peak in control Hek293 cells and cells over-expressing PanK1β. Malonyl CoA values were only quantified from samples showing a distinct peak.

were seeded into 96-well plates (~2000 cells/well) and growth of each cell line was analysed using the cell titre blue assay over a period of up to 10 days. After 5 days, it became clear that the rate at which cells over-expressing PanK1β was slower than that of control cells, with the number of cells about 30-40% lower by the end point *(Figure 4.17A)*. This reduction in cell growth was not seen in cells over-expressing CoASy (WT or *203 mutant) *(Figure 4.17B)*, suggesting specific effects of PanK1β. Consistent with slower growth, protein amount in cell lysates, was also reduced by about 30% in cells over-expressing PanK1β *(Figure 4.17C).*

4.2.5.4 The effect of PanK1β over-expression on signalling pathways involved in cell growth

mTOR and PI3K are central players in the regulation of growth and localise with the site of CoA production (outer mitochondrial membrane). Moreover, unpublished work from this laboratory also provided evidence that CoA interacts with mTOR. To test whether slower growth of cells over-expressing PanK1β is due to changes in mTOR and PI3K signalling, phosphorylation of the downstream targets of mTOR and PI3K was measured using phospho-specific antibodies against S6K and PKB respectively. No change in the phosphorylation of S6K or PKB was observed in PanK1β over-expressing cells *(Figure 4.18A)*, indicating that CoA has no effect on long-term mTOR or PI3K activation. AMPK signalling also was not affected by the increase in CoA levels, suggesting that no changes in AMP: ATP ratio occurred *(Figure 4.18A)*.

CoASy expression did not change in Hek293 cells over-expressing PanK1β *(Figure 4.18B)*, signifying that the increase in CoA levels was solely due to an increase in PanK1β and additional elevation of secondary rate-limiting enzyme in the CoA biosynthetic pathway was not required. This further supports the earlier notion that CoASy is not as important as PanK in the regulation of CoA biosynthesis.

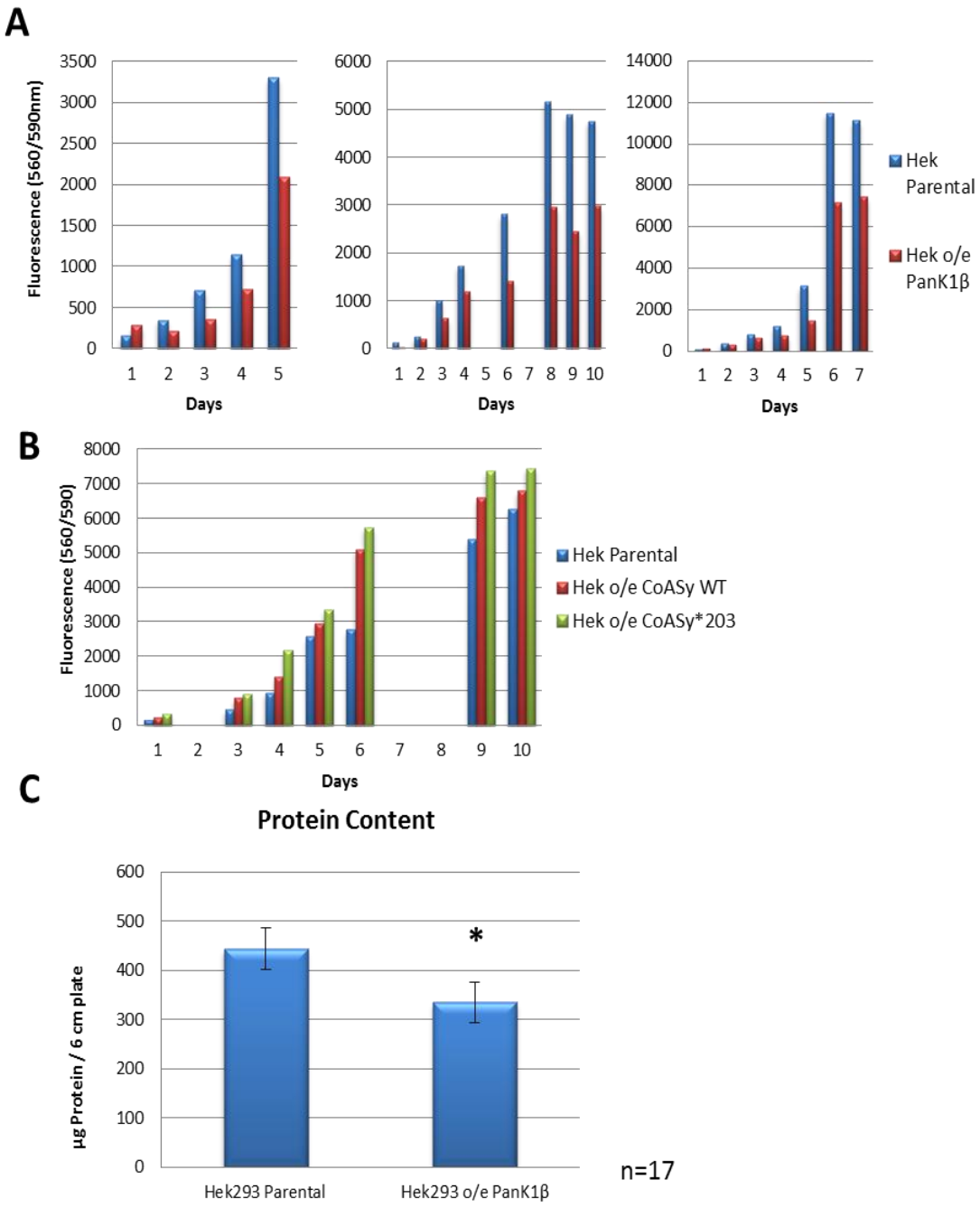


Figure 4.17: The effect of PanK1β over-expression on cell growth. Cell growth was measured in Hek293 parental, Hek293 over-expressing PanK1β in three independent experiments **(A)** and also Hek293 over-expressing CoASy (WT and *203) (one experiment shown out of three) **(B)** as a control using the cell titre blue reagent. Cells were seeded into 96 well plates as approximately 2000 cells/ well. **C)** A similar number of Hek293 parental or cells over-expressing PanK1β were seeded onto 6 cm plates and allowed to grow for the same amount of time until they were 70-80% confluent. Protein concentration was analysed in lysates of these cells using Bradford assay.

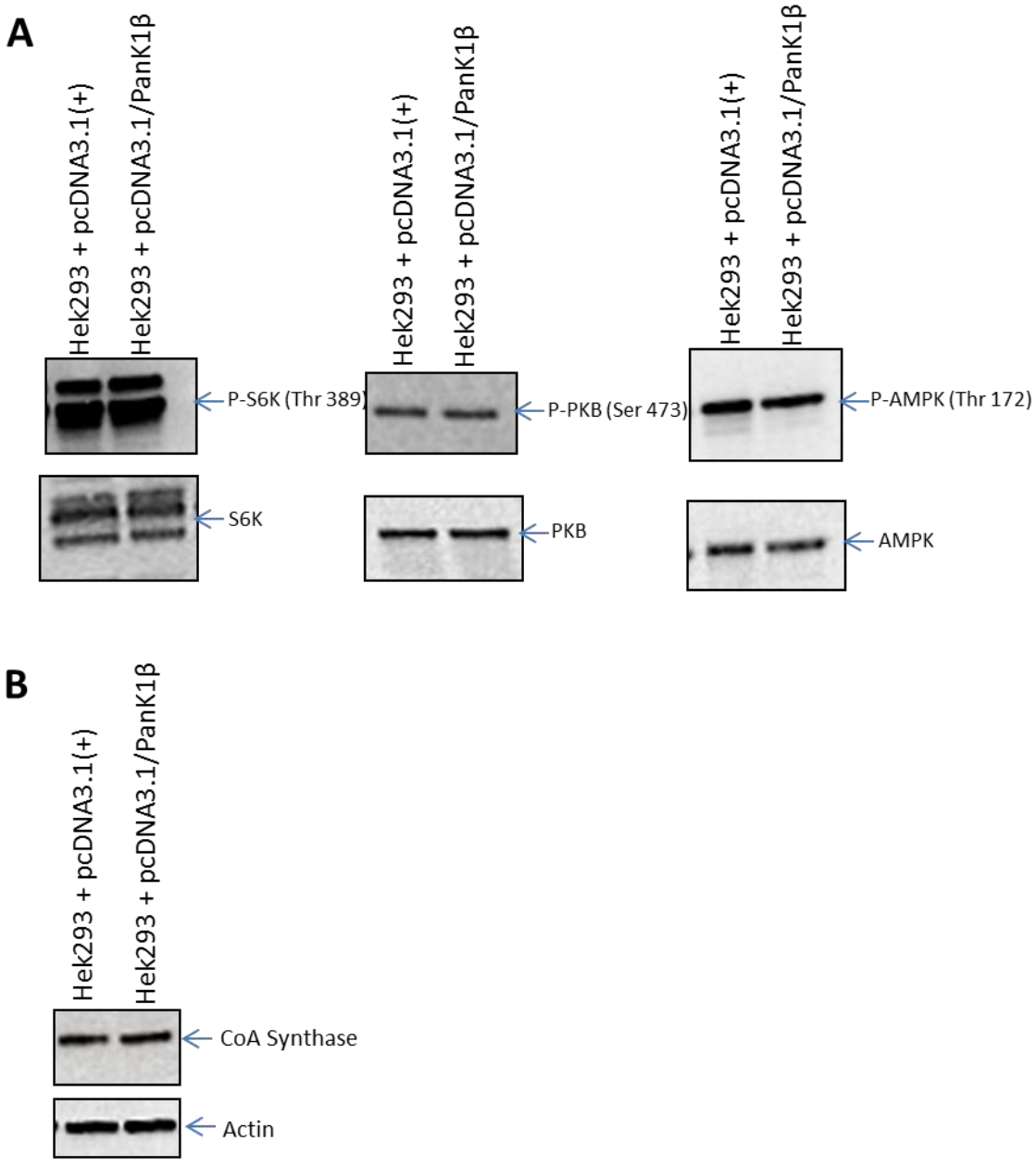


Figure 4.18: The effect of PanK1β over-expression on various signalling proteins. S6K, PKB and AMPK activation **(A)** as well as expression of CoASy **(B)** were measured by western blot analysis in cells over-expressing PanK1β.

4.2.5.5 The effect of PanK1β over-expression on metabolic pathways

Glycolysis is the main metabolic pathway used in fast proliferating cells such as Hek293, as glucose is used to generate biomass as well as ATP (Vander Heiden et al. 2009). It is possible that an increase in CoA might disturb the normal metabolic balance of Hek293 cells. Acetyl CoA levels were also significantly increased, which might suggest an increase in glucose or fatty acid oxidation.

Fatty acid oxidation was compared in Hek293 parental cells and Hek293 over-expressing PanK1β cells by incubating cells with 0.1 mM [9,10 ^{3}H] palmitate (10 mCi/mmol) (bound to 1% BSA). The rate of palmitate oxidation in control cells was 7.5 pmol/min/mg protein whereas the rate in cells over-expressing PanK1β was slightly lower at 6 pmol/min/mg protein (*Figure 4.19)*. The decrease was not statistically significant. To assess whether glucose oxidation differs between Hek293 parental cells and Hek293 over-expressing PanK1β cells, cells were incubated with 5 mM D-[^{14}C(U)] glucose (0.1 µCi/mmol). As with fatty acid oxidation, glucose oxidation also was not significantly affected by increased CoA levels in cells over-expressing PanK1β, with a rate of 119 pmol/min/mg protein for control cells and a rate of 141 pmol/min/mg protein for cells over-expressing PanK1β (*Figure 4.20).*

Lactate production usually correlates with the rate of glycolysis and was also measured in cells over-expressing PanK1β over a time course of 6 hrs. Lactate production was measured spectrophotometrically using LDH, after PCA extraction. Interestingly, lactate production appeared to be about 10-20% higher in cells over-expressing PanK1β and this observation was intensified to about 40% higher when standardising against protein concentration *(Figure 4.21A&B)*. The significance of this effect will be discussed below.

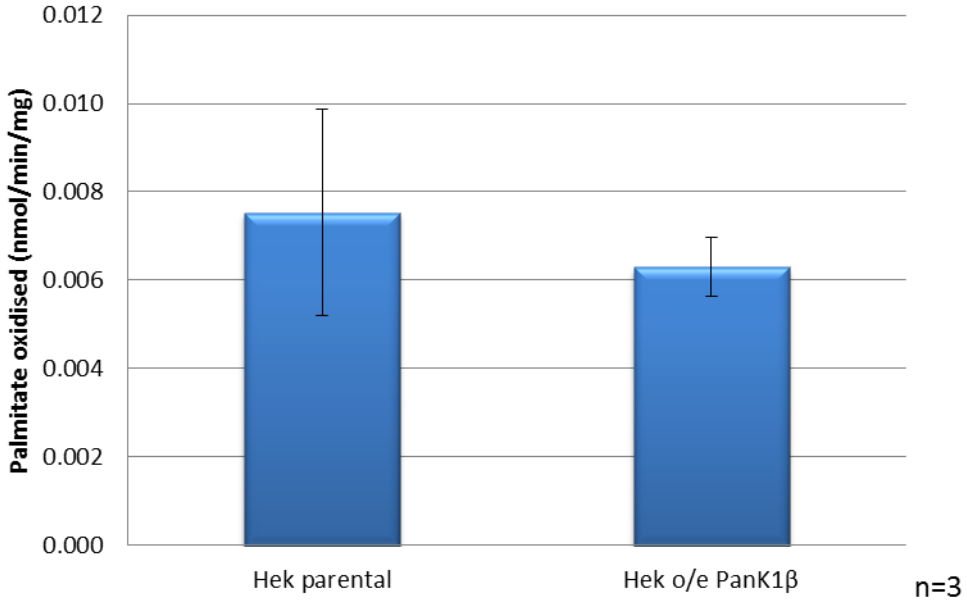


Figure 4.19: Fatty acid oxidation in cells over-expressing PanK1β. Fatty acid oxidation was measured in Hek293 parental cells and Hek293 cells over-expressing PanK1β by incubating cells for 1 hr with 0.1 mM [9,10 ^{3}H] palmitate bound to 10% BSA (10 mCi/mmol). Cells were killed with 5% PCA and palmitate was extracted using a chloroform and methanol extraction procedure. The amount of $^{3}H_2O$ produced from oxidation of [9,10 ^{3}H] palmitate was measured by scintillation counting. The results are shown after standardising by µg protein

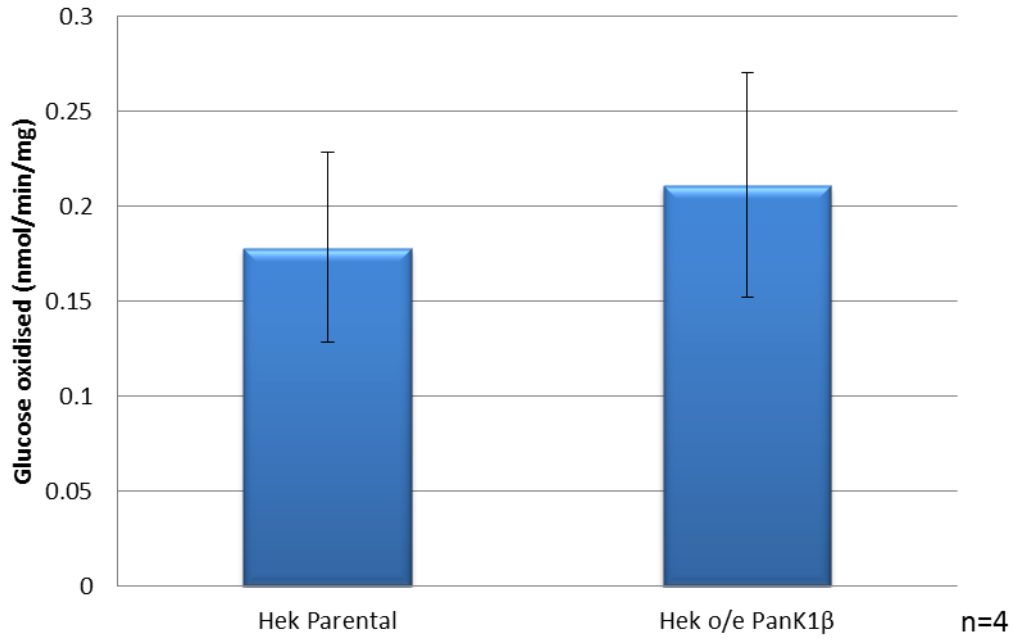
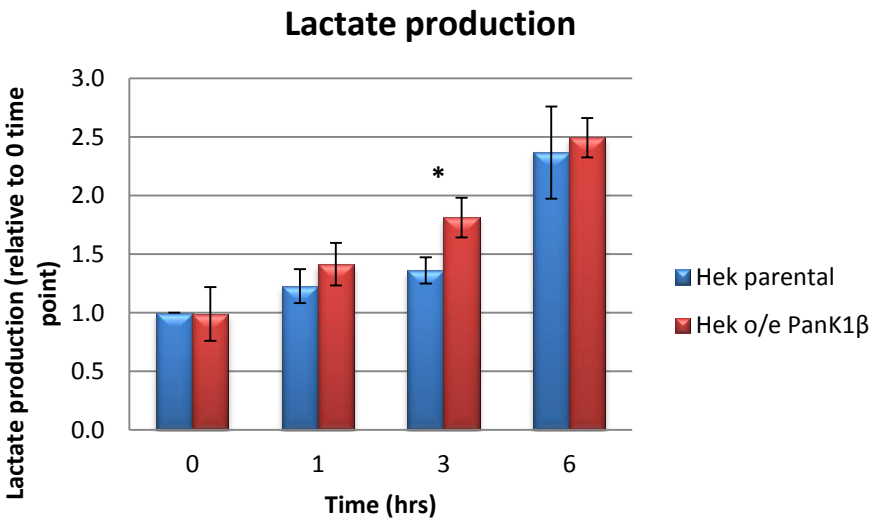


Figure 4.20: Glucose oxidation in cells over-expressing PanK1β. Glucose oxidation was measured in Hek293 parental cells and Hek293 cells over-expressing PanK1β by incubating cells for 90 mins with 5 mM D-[^{14}C(U)] glucose (0.1 µCi/mmol). Cells were killed with 5% PCA and [^{14}C] CO_2 produced was trapped in benzethonium hydroxide for 1 hr after lysing the cells with PCA. [^{14}C] CO_2 was measured by scintillation counting. The results are shown after standardising by µg protein.

A



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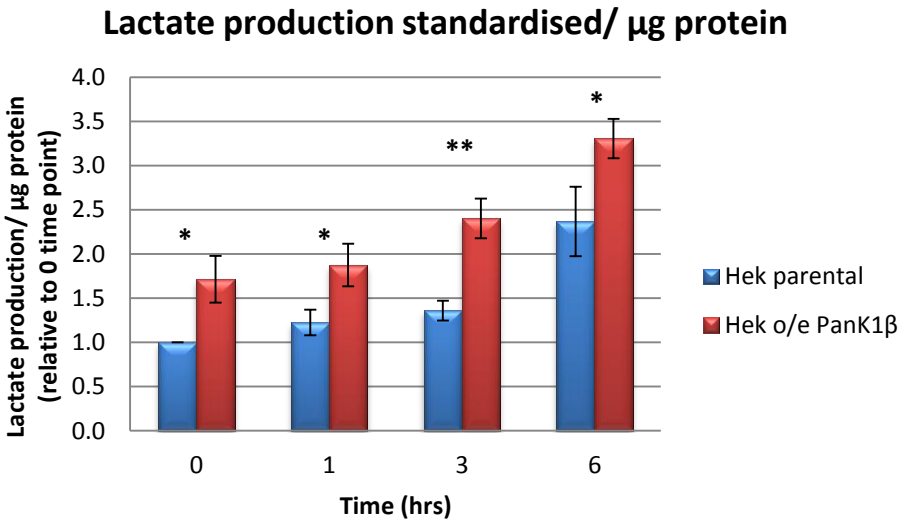

Figure 4.21: Lactate production in cells over-expressing PanK1β. A) Lactate production was analysed after incubating Hek293 parental and over-expressing PanK1β cells with PBS, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 5 mM glucose for the time points up to 6 hours. Cells were killed with 5% PCA and lactate was assayed spectreophotometrically. **B)** Lactate production was recorded relative to the 0 time point and also standardised against µg protein.

4.2.5.6 The effect of PanK1β over-expression on protein acetylation

A study from Sibon's group suggested changing intracellular CoA level alone can influence the acetylation of proteins (Siudeja et al. 2011). Together with the observation here that acetyl CoA levels as well as free CoASH levels were increased *(Figure 4.15A)*, one might expect acetylation of proteins to also increase in cells over-expressing PanK1β. Western blot analysis using antibodies against acetyl lysine residues confirmed that acetylation did increase in cells over-expressing PanK1β on a wide range of proteins *(Figure 4.22A)*. Indeed, histones and tubulin are commonly acetylated proteins and here it is possible to see an increase in acetylation of proteins at molecular weights corresponding to histones (11-21 kDa) and tubulin (50-55 kDa). Acetylation has been shown to regulate a number of processes such as chromatin remodelling, cell cycle, DNA repair, splicing, nuclear transport, and actin nucleation (Choudhary 2009). There is no simple relationship between protein acetylation and cell growth and there are conflicting results in the literature, however increasing acetylation by histone deacetylase (HDAC) inhibitors has an anti-proliferative effect, which is why HDACs are often targets for cancer (Sambucetti 1999). As expected, incubation of Hek293 cells with HDAC inhibitor, TSA, caused inhibition of cell growth *(Figure 4.22B).*

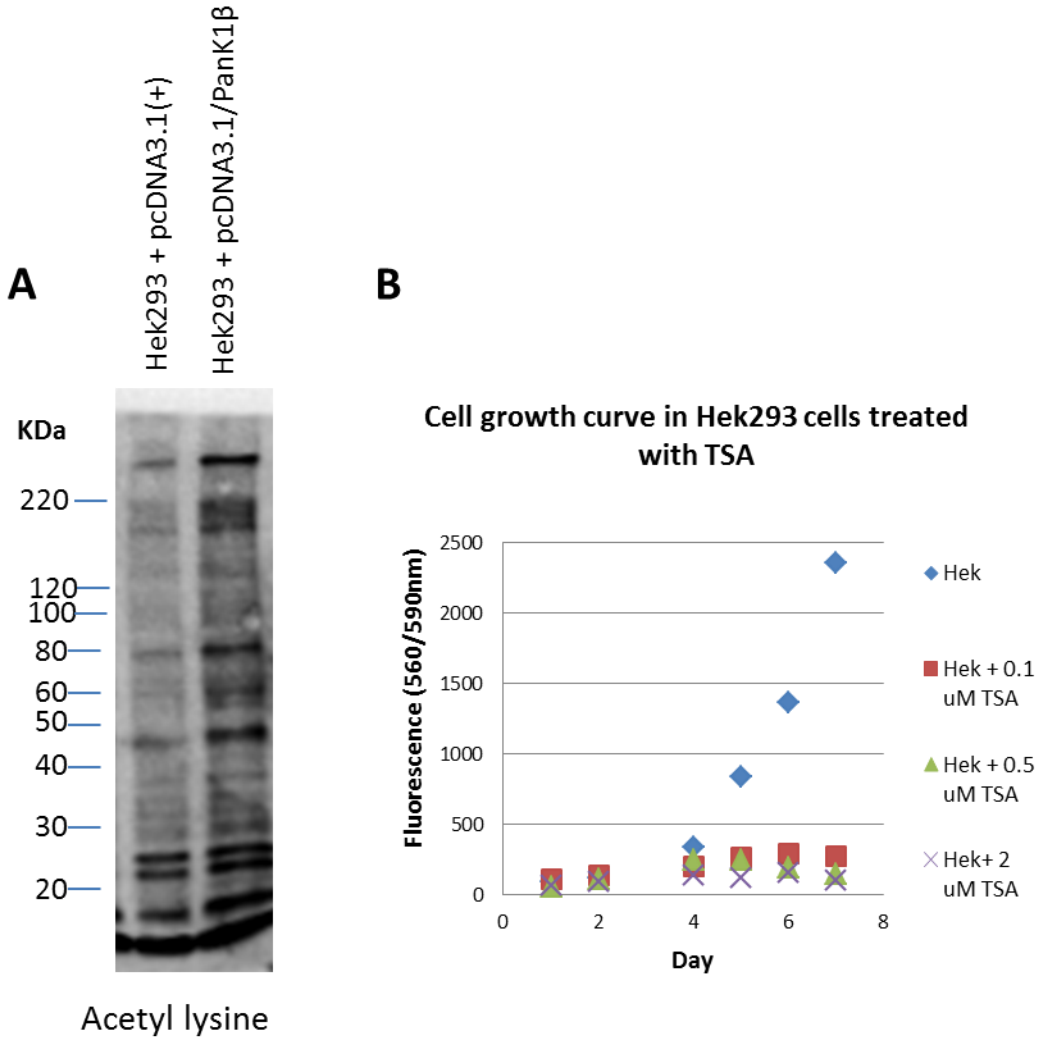

Figure 4.22: The effect of PanK1β over-expression on protein acetylation. A) Proteins acetylated on lysine residues were measured using antibodies against acetyl lysine residues by western blot analysis in cells over-expressing PanK1β. **B)** Hek293 cells were incubated with various concentrations of TSA indicated and growth of cells was monitored over 7 days using the cell titre blue reagent.

4.3 Discussion

The aims of this chapter were to determine the suitability of using cultured cell lines as a model for studying CoA; to identify the role of the interaction between CoASy and mTOR and PI3K signalling proteins; and to investigate the effects that manipulation of CoA levels have on cellular function. HepG2 cells were chosen as a cultured cell model as they were thought to represent liver, which is a highly metabolically active tissue and consequently has high levels of CoA.

One of the most striking observations here was that HepG2 cells contained much lower levels of CoA than liver. There are several reasons why this difference may have occurred. Firstly, unlike liver in adult rats, HepG2 cells are cultured in medium containing high levels of growth factors as well as non-physiologically high levels of glucose (25 mM). Glucose has previously been shown to inhibit CoA synthesis in liver and heart (Smith 1978; Robishaw et al. 1982). Secondly, HepG2 cells are a hepatoma cell line and previous studies have shown that liver tumours contained approximately 5 times less CoA compared to healthy liver. The low levels of CoA may also be related to the ability of cells to oxidise fatty acids. Tissues with high fatty acid oxidation capacity such as liver and kidney tend to have high CoA content. Cancer cells typically rely on glycolysis. The low fatty acid oxidation capacity, combined with high levels of glucose in the culturing medium may force HepG2 cells down the glycolytic route for energy metabolism and this may result in low CoA levels.

HepG2 cells were also not particularly responsive to stimuli previously reported to change CoA levels in liver. For example, both insulin and glucose did not significantly affect CoA levels in the cultured cells tested. Incubation with fatty acids was an exception, in which the decrease in CoA observed here corresponded with the literature, where rats fed a high fat diet had reduced CoA levels in a number of tissues compared to rats fed a high

carbohydrate diet (Tokutake et al. 2010). This may be related to degradation of CoA by fatty acids reported by Lopaschuck (Lopaschuk et al. 1986). However, generally the low levels of CoA and the lack of agreement with liver indicate that HepG2 cells may not be a suitable model for studying the physiological function of CoA in liver *in vivo*.

Another objective of this study was to elucidate the relative importance of CoASy in the regulation of CoA levels. Following the observation that CoASy associates with mTOR, S6Kand p85αPI3K, it was tested whether mTOR and PI3K signalling could affect CoA levels. CoA levels were not changed by inhibition of PI3K and mTOR, therefore mTOR and PI3K signalling do not appear to be a mechanism for regulating CoA biosynthesis in these cells. There is the possibility that inhibiting PI3K and mTOR signalling might result in a range of contrasting metabolic effects that in turn may increase CoA utilisation or degradation yet the overall levels of CoA remain constant. Another possible explanation may be that because the levels of CoA in cultured Hek293 and HepG2 cells are so low, CoASy may not be working as a rate-limiting enzyme. Here, it was confirmed that although CoASy has previously been identified as rate-limiting following the observation of the accumulation of 4' phosphopantetheine (4'PP) (Jackowski and Rock 1981; Jackowski and Rock 1984; Rock et al. 2000), PanK is the major control point for CoA biosynthesis in Hek293 cells. This was demonstrated by the results showing that CoA levels did not change when CoASy was over-expressed, or even when PanK1α was hyper activated with bezafibrate. Furthermore, no change in CoASy expression was observed when CoA levels were elevated upon PanK1β over-expression. These studies suggest that regulation of CoASy does not contribute to overall control of CoA levels in the conditions used here.

When investigating different methods of manipulating cellular CoA level for the final objective, it was interesting to find that CoA levels in HepG2 cells were not affected upon Hopan treatment, or even when pantothenate was completely absent. This suggests

that there is a large buffering capacity to keep CoA levels within a tight range and alternative routes to CoA biosynthesis, such as conversion through its esters, play a large role in the maintenance of CoA levels in HepG2 cells. Indeed, Zhang *et al*. identified an accumulation of acyl carnitines in the liver of mice treated with Hopan, which is consistent with the cycling function of carnitine to accept acyl groups from acylated CoA and release free CoASH during the process (Zhang et al. 2007). Moreover, metabolomic and gene expression analysis identified adjustments that focussed on preserving the CoA concentration. Also, although Zhang *et al.* found a decrease in CoA levels in liver and kidney upon Hopan treatment, no changes were displayed in heart or brain. These tissue specific differences could result from a lower turnover rate of CoA in heart and brain, corresponding to a longer half life and may also be why no changes in CoA are seen in HepG2 cells.

A novel finding here was that increasing CoA appears to have an anti-proliferative effect on cells. Why cell growth was inhibited remains unknown, but it does not appear to involve changes in mTOR or PI3K signalling pathways which play a central role in growth regulation. As activation of AMPK was not detected, it is unlikely that cells over-expressing PanK1β were energetically compromised. Also, since there was no change in growth after CoASy over-expression, the effects of CoA on growth are likely to be specific for PanK1β.

The possibility was considered that an increase in CoA might cause a metabolic shift which is unfavourable for Hek293 growth. No changes in glucose or palmitate oxidation were detected following an increase in CoA, however the possibility that palmitate or glucose oxidation was increased immediately after PanK1β overexpression cannot be ruled out. Even if this was the case, these effects were not sustained and therefore could not account for slower growth.

The increase in lactate production observed in this chapter, may suggest an accumulation of pyruvate. Pyruvate kinase (PK) is the rate-limiting enzyme that mediates the final step of glycolysis. There are several isoforms of PK and the dominant form in Hek293 cells is PKM2, which is also exclusively over-expressed in embryonic and tumour cells (Bluemlein 2011). PKM2 is an inactive form of PK and it has been suggested that its overexpression results in the accumulation of glycolytic intermediates, which can be used for biosynthetic processes and cell growth. It is thought that high expression of PKM2 contributes to cancer cell growth and a study by Christofk *et al.* suggested that an increased flux through the PK reaction slows down cancer cell growth (Christofk et al. 2008). Therefore, although speculative, if PKM2 plays a similar role in Hek293 cells in promoting cell growth, the slower growth of PanK1β over-expressing cells may be related to activation of the PK reaction. It has previously been shown that PanK4 associates with PKM2, however it is not known how this association affects the activity of either enzyme or whether any other PanK isoforms interact with PKM2 (Li et al. 2005). It is possible that an increase in lactate formation may be through direct interaction of PanK1β with PKM2.

Acetylation is another potential factor investigated here that might affect cell growth. Acetylation is widely thought to be regulated through the balance between HAT and HDAC activities. Recent studies provided some evidence that changes in the supply of its substrate acetyl CoA may also be important (Cai et al. 2011). K_m values of HATs, such as HAT1, PCAF, p300, and GCN5, for acetyl CoA are in the low micromolar range (1-10 μM) (Tanner, Langer, and Denu 2000; Tanner, Langer, Kim, et al. 2000; Bordoli et al. 2001; Poux et al. 2002; Wu et al. 2012). The cytosolic concentration of acetyl CoA is also estimated to be in the low μM range and it is thought that acetyl CoA can freely pass through the nuclear pore complex (Takahashi et al. 2006). Therefore, an increase in cytosolic acetyl CoA would alone be expected to drive acetylation of histones, transcription factors, signalling

and metabolic proteins, providing the appropriate HATs are activated. This effect may be more pronounced in cultured cells which contain less CoA, and the assumption is made that cytosolic acetyl CoA is also proportionately lower. Acetylation of some proteins has been implicated in the inhibition of cell growth and HDAC inhibitors have been studied as possible anti-cancer agents. Although precise relationship between acetylation and growth is likely to be complex and depend on the proteins acetylated, the data presented here is consistent with the idea that global increase in acetylation through PanK1β overexpression may have inhibitory effect on cell growth.

Chapter 5: Results

CoA Binding Proteins

Chapter 5: CoA Binding Proteins

5.1 Introduction

Coenzyme A has the potential to regulate proteins through several different mechanisms; as a cofactor; through allosteric interaction; through the formation of di-sulphide bonds via its thiol group. There are many accounts in the literature where CoA acts as a cofactor during a wide range of metabolic reactions. However the reports of CoA interacting through allosteric or di-sulphide modification are limited.

One of the more commonly known regulatory interactions of CoA is with PanK, which regulates the primary step in the CoA biosynthesis pathway. CoA is involved in the feedback regulation of PanK in both prokaryotes and eukaryotes and is competitive with respect to ATP (Kupke et al. 2003; Rock et al. 2000; Song and Jackowski 1994; Vallari et al. 1987; Yun et al. 2000). The different mammalian isoforms of PanK are regulated by free CoASH as well as its thioesters to various degrees (Leonardi et al. 2005).

CoA has also been reported to regulate the enzyme pyruvate dehydrogenase kinase (PDHK) in pig heart muscle (Siess and Wieland 1982). CoA was shown to increase the activity of PDHK, with its thiol group acting as a requirement for the stimulation. Acetyl CoA, benzoyl CoA and CoA dimer were all ineffective at stimulating PDHK and other thiol containing agents such as DTT, β-mercaptoethanol and glutathione were all unable to substitute CoA as an activator of PDHK activity, indicating CoA's specificity for the enzyme. The activation of PDHK stimulates PDH phosphorylation and consequent inactivation, preventing the conversion of pyruvate into acetyl CoA (Siess and Wieland 1982).

Covalent modification of a few proteins by CoA through formation of mixed di-sulphides with cysteine residues or exchange reactions with protein di-sulphides has been identified. These protein modifications were demonstrated for the mutant β-subunit of F_1-ATPase of *E. coli* (Odaka et al. 1993); a flavodoxin of *K. pneumonia* (Thorneley et al. 1992); and for the rat liver mitochondrial matrix enzymes acetyl-CoA acetyltransferase, 3-ketoacyl-CoA thiolase (Huth et al. 1988, 1991) and hydroxymethylglutaryl-CoA synthase (Charlier, Narasimhan, & Miziorko 1997). CoA modification for the liver mitochondrial proteins appeared to protect the proteins from degradation (Huth et al. 2002).

The aim of this study is to gain a greater understanding of the function of CoA as a regulator of cellular processes. In the previous chapter a "top down" approach was used, however in this chapter a "bottom-up" approach was applied with the aim of identifying proteins that directly interact with CoA and establish how they are regulated. Affinity purification with CoA Sepharose is a commonly used approach which has previously been used to purify a number of CoA binding proteins, such as acyl-CoA synthetase and choline acetyltransferase as well as identify novel proteins related to peroxisomal thiolases (Driskell et al. 1978; Kameda et al. 1985; Raeber et al. 1989; Yang 1996). CoA affinity purification was stated to be a specific and rapid procedure, which effectively binds to CoA-dependent enzymes and so CoA Sepharose 4B beads were used in this study to purify CoA-interacting proteins. CoA-interacting proteins were identified by mass spectrometry. The full procedure used to identify CoA-binding proteins is outlined in *Figure 5.1.*

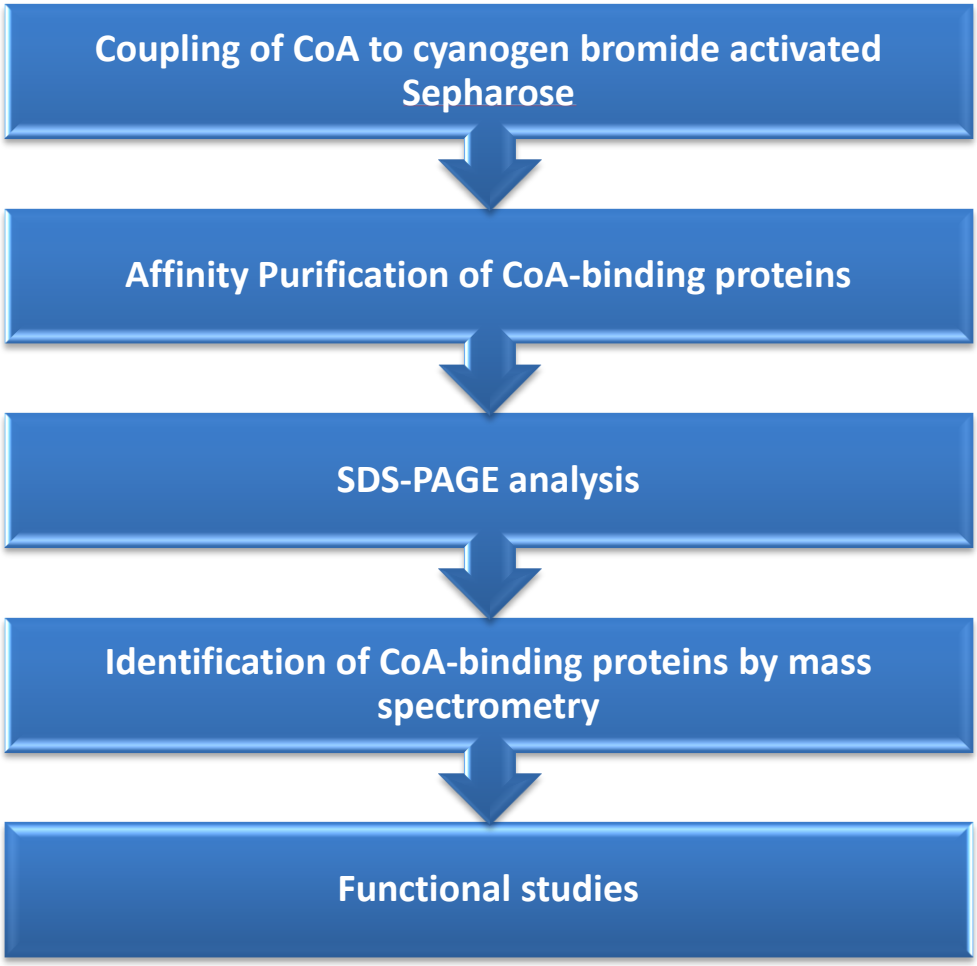

Figure 5.1: Strategy for the Analysis of CoA binding proteins.

5.2 Results

5.2.1 Coupling of CoA to Cyanogen Bromide Activated Sepharose

5.2.1.1 Chemistry of coupling CoA to Sepharose

Activated Sepharose 4B (4% agarose) beads are often used for the preparation of resins which can identify protein interactions through affinity purification. Sepharose beads can be activated with cyanogen bromide (CNBr), which reacts with the hydroxyl groups on Sepharose and results in two types of products: an isourea derivative and a substituted imidocarbonate *(Figure 5.2)*. The isourea is positively charged, so these beads can also act as an anion exchanger. These beads react with nucleophiles, such as primary amines. CoA has two reactive groups: its thiol group and the amine on the adenine ring which both can potentially react with CN group, resulting in the formation of CoA Sepharose beads (*Figure 5.2).*

5.2.1.2 Binding and orientation to CoA Sepharose beads

To identify proteins that are potentially regulated by CoA, CoA was coupled to CNBr-activated Sepharose 4B to generate CoA Sepharose beads. CNBr activated Sepharose was incubated with 6 mM CoA in 100 mM phosphate buffer, pH 6 overnight at 4°C. Samples at the beginning and end of the coupling reaction were analysed by Ellman's test and the coupling efficiency was determined to be approximately 60% (*Figure 5.3A)*.

Since CoA has two potential reactive groups, it was necessary to determine the orientation which CoA binds to activated Sepharose beads. Therefore, CoA Sepharose was prepared under various conditions that blocked the thiol group of CoA and binding patterns of liver lysate proteins were compared (*Figure 5.3B)*. These conditions included using de-sulpho CoA or CoA pre-treated with the alkylating agents, NEM and maleimide. The results indicate that proteins do not really bind when the thiol group is blocked before coupling

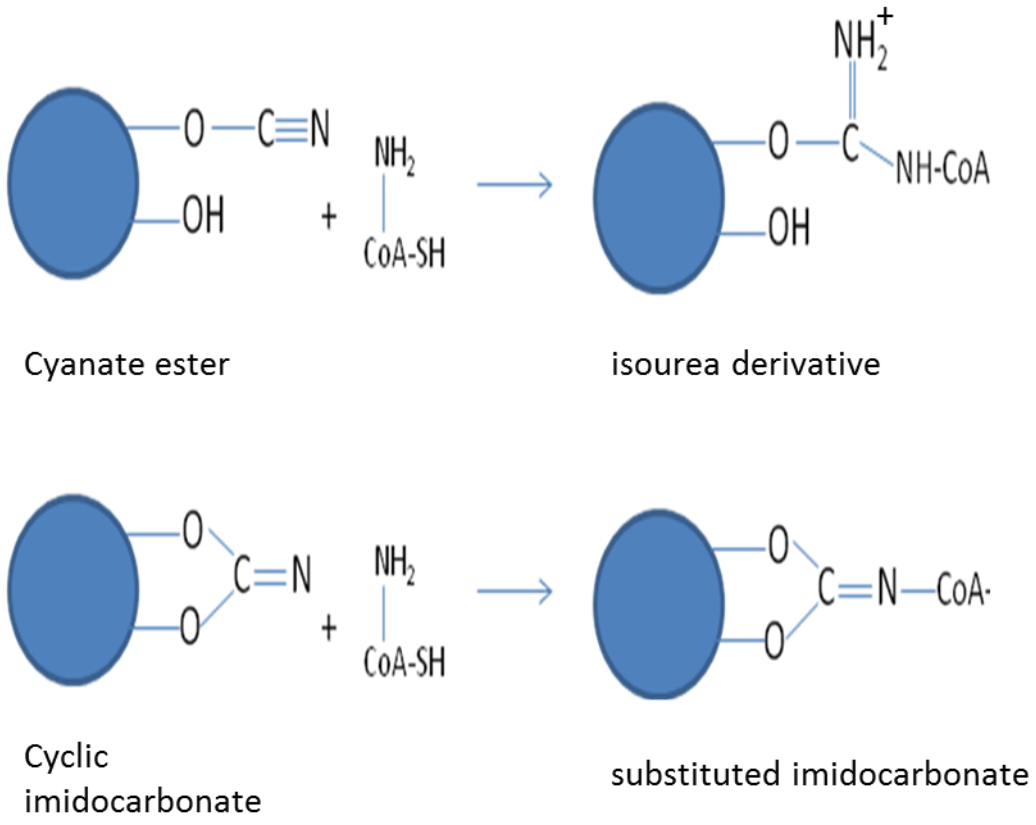

Figure 5.2: Schematic to display how CoA can be coupled to cyanogen coated beads of cyanate ester or cyclic imidocarbonate form. The lone pair of electrons on the adenine NH_2 group of CoA interact with the delta positive carbon of the cyanate/ imidocarbonate group of CoA, resulting in the coupling of CoA to the beads.

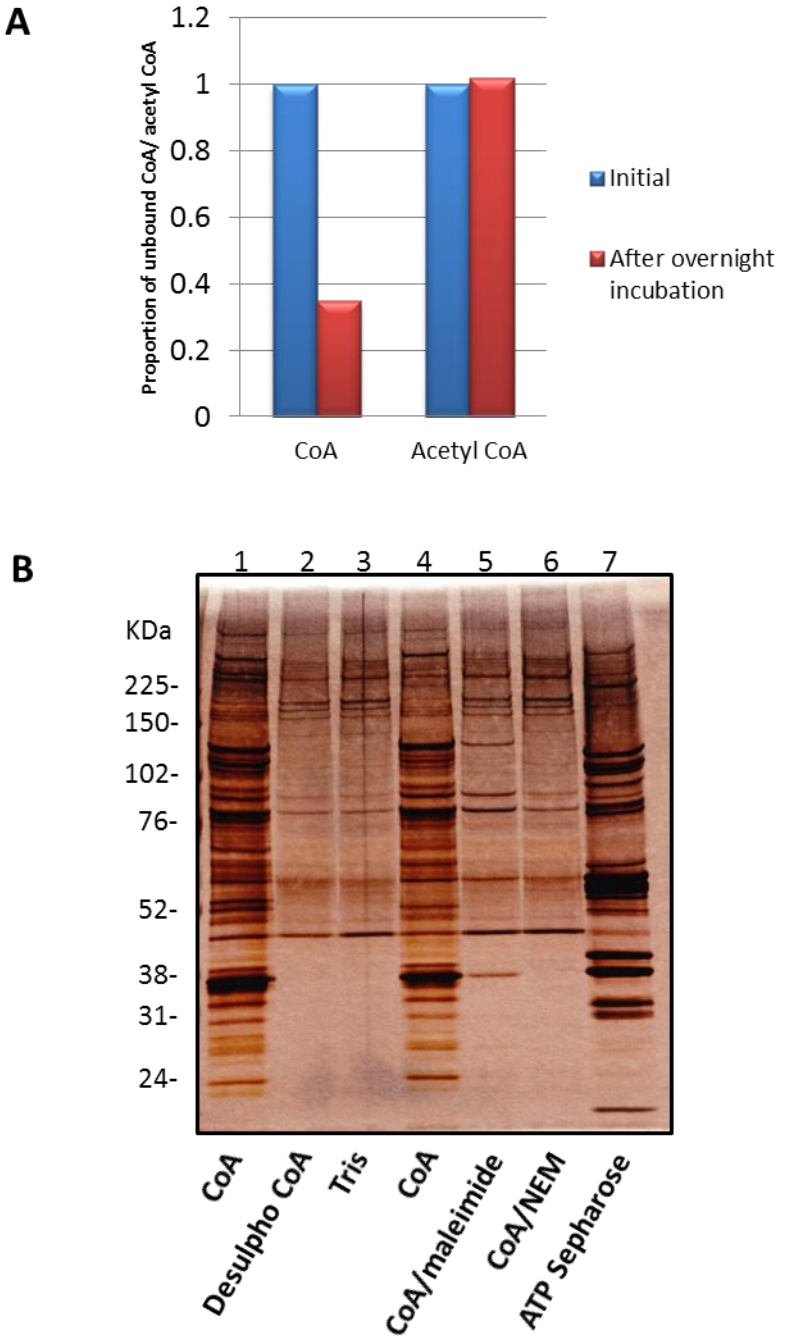

Figure 5.3: The binding and orientation for CoA binding to CNBr activated Sepharose beads. A) 2 µL samples from the supernatant of 100 mM phosphate buffer + 6 mM CoA were taken at the start and end of the coupling reaction. Coupling efficiency of CoA to cyanogen bromide activated Sepharose was measured using Elmans test, where the absorbance of samples was measured at 412 nm after incubating with 0.1 mM DTNB, 100 mM Tris/ HCL pH 8.0 and 2.5 mM sodium acetate for 30 mins at room temperature. Acetyl CoA coupling was measured by HPLC. **B)** Rat liver lysates were incubated with cyanogen bromide-activated Sepharose 4B that had been incubated either with CoA (lanes 1 and 4), de-sulpho CoA (lane 2), Tris (lane 3), or CoA pre-treated with maleimides (lanes 5 and 6). Lysates were also incubated with ATP-Sepharose (lane 7). Proteins that bound to beads were released through boiling in SDS loading buffer, then resolved by SDS-PAGE analysis and silver staining.

CoA to CNBr Sepharose. Therefore, CoA is most likely coupled to Sepharose beads through its thiol group. Although these CoA derivatives do contain adenine NH_2 groups, they seem unable to react with the Sepharose beads, possibly due to the delocalisation of the lone pair electrons to the adenine ring structure.

Additional tests were carried out to eliminate the possibility of proteins interacting with CoA Sepharose through di-sulphide linkage. Treating CoA-coupled beads with 100 mM maleimide, prior to incubation with lysates did not affect the binding pattern of proteins compared to untreated CoA Sepharose. Moreover, incubation of lysates with CoA sepharose in the presence of the reducing agent 10 mM DTT did not affect protein binding patterns *(Figure 5.4)*. These results further support the conclusion that beads are coupled to CoA through the thiol group as proteins do not appear to interact with CoA through di-sulphide bridges.

As CoA appears to bind through the sulfhydryl group, this leaves the adenine nucleotide group exposed for protein binding. Affinity binding experiments were carried out using ATP immobilised on Sepharose through its phosphate group to determine whether the pattern of proteins binding to CoA Sepharose solely represent nucleotide binding proteins. The protein binding pattern of ATP Sepharose is different from CoA Sepharose, indicating that there are a number of proteins that require the pantetheine moiety of CoA, in addition to the adenine group for binding to occur, suggesting that these proteins specifically bind to CoA. In addition, non-specific protein binding through ionic interactions was detected using CNBr Sepharose coupled to Tris (primary amine), which carries the same positive charge as CoA Sepharose.

5.2.1.3 Do proteins that bind to CoA Sepharose bind specifically to CoA?

It is important to check whether the proteins purified by this method are specific to CoA. Rat liver lysates were incubated with Sepharose beads coupled to CoA or Tris for 2

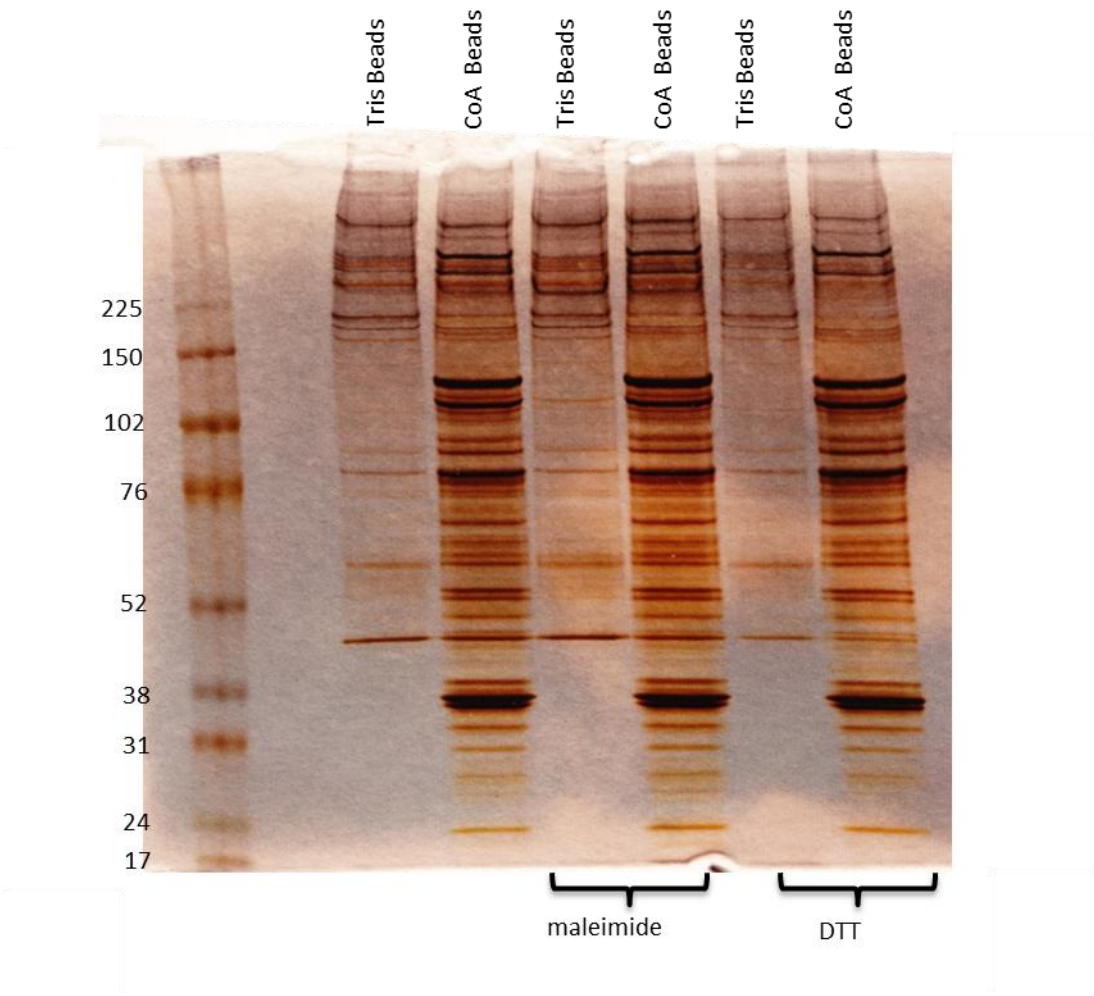

Figure 5.4: Proteins do not bind to CoA through its thiol group. The lysate from rat liver was incubated with Sepharose beads, coupled to CoA or Tris (control beads). Liver lysates were also incubated with beads that had been pre-treated with 100 mM maleimide. Proteins were released through boiling in SDS loading buffer, then resolved by SDS-PAGE analysis and silver staining.

hrs at 4°C, with shaking. Proteins bound to CoA Sepharose were then eluted with 100 mM CoA, ADP or pantothenate *(Figure 5.5)*. Very few proteins were eluted with ADP and none at all were eluted with pantothenate. These data indicate that the majority of proteins purified by this method specifically bind to CoA.

5.2.2 Affinity Purification and SDS-PAGE Analysis

The CoA molecule can interact with proteins in a number of ways *(Figure 5.6)*. Non-specific ionic interactions represent one option; however these can be identified, and consequently eliminated, through binding patterns with Tris beads. Specific interactions include proteins binding through the pantothenate moiety, through the adenosine moiety as well as both the pantothenate and adenosine moieties. It is important to be aware that this method also may pull down proteins which bind to proteins specifically interacting with CoA but do not directly interact with CoA themselves.

Having established a method that has the potential to effectively identify specific CoA interacting proteins, lysates with a normalised protein concentration, from HepG2 cells, Hek293 cells and various rat tissues (liver, kidney, heart, brain) were incubated with the CoA beads and bound proteins were analysed by SDS-PAGE and silver staining *(Figure 5.7A&B)*. There were a number of proteins that did bind to the control beads, through either non-specific binding to the beads, or ionic interactions, however there were also a large number of proteins that appeared to specifically interact with CoA in each of the different lysates. Several CoA binding proteins were observed in all of the lysates (eg. Proteins of about 76 kDa), yet there were also tissue-specific proteins. For example, small molecular weight proteins between 31 and 38 kDa appeared to be prominent in liver and kidney tissue, lower in heart and HepG2 and almost non-detectable in brain and Hek293 cells. Hek293 cells actually have been shown to have more similarities with neuronal cells, rather than kidney cells (Shaw et al. 2002) and this is apparent from the similarities in

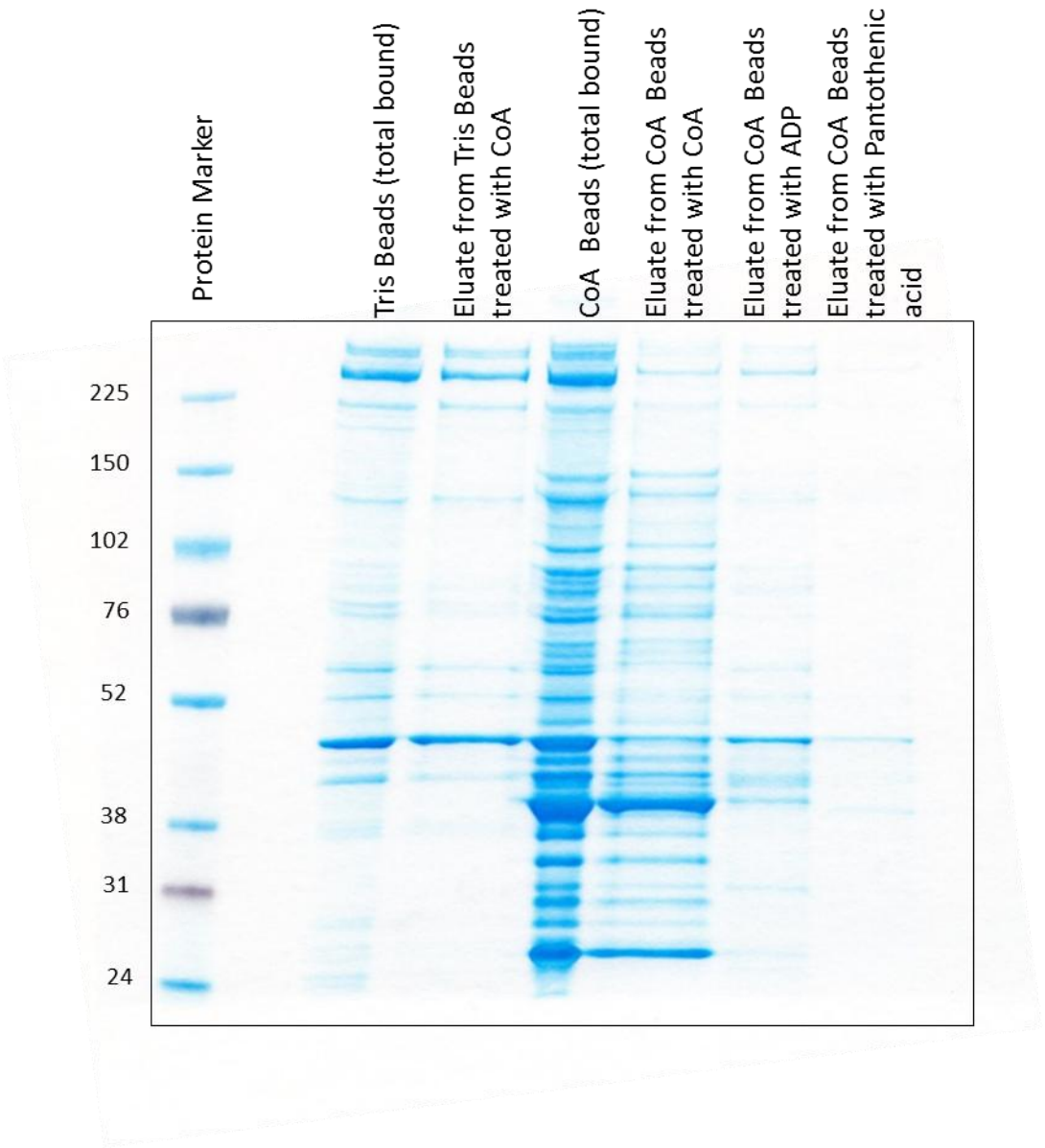

Figure 5.5: Proteins are specifically eluted from CoA beads by CoA. The lysate from rat liver was incubated with Sepharose beads, coupled to CoA or Tris (control beads) for 2 hours, 4°C with continuous mixing. After washing beads with lysis buffer, followed by Hepes buffer + 1 mM DTT, proteins were eluted with either 100 mM CoA, ADP or pantothenic acid. Total proteins that bound to beads were released by boiling in SDS loading buffer. Eluted and total bound proteins were resolved by SDS-PAGE and visualised by Coomassie staining.

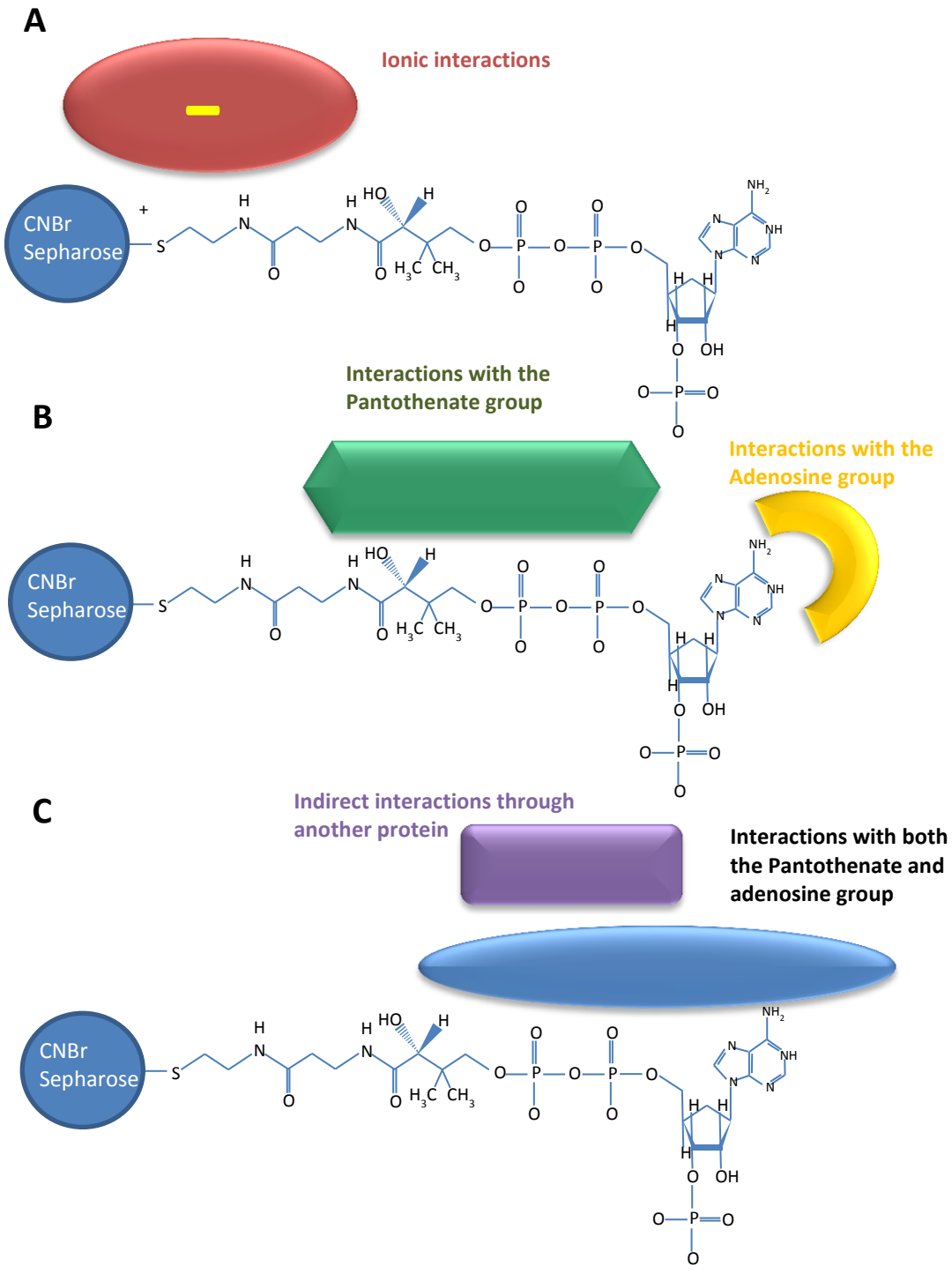

Figure 5.6: Schematic showing the potential interactions that proteins in cell/tissue lysates could use to specifically bind to CoA. These include: **A)** Ionic interactions **B)** Interactions with the pantothenate or adenosine group **C)** Interactions with both the pantothenate and adenosine group or indirect interactions to CoA through binding to a protein which interacts with CoA.

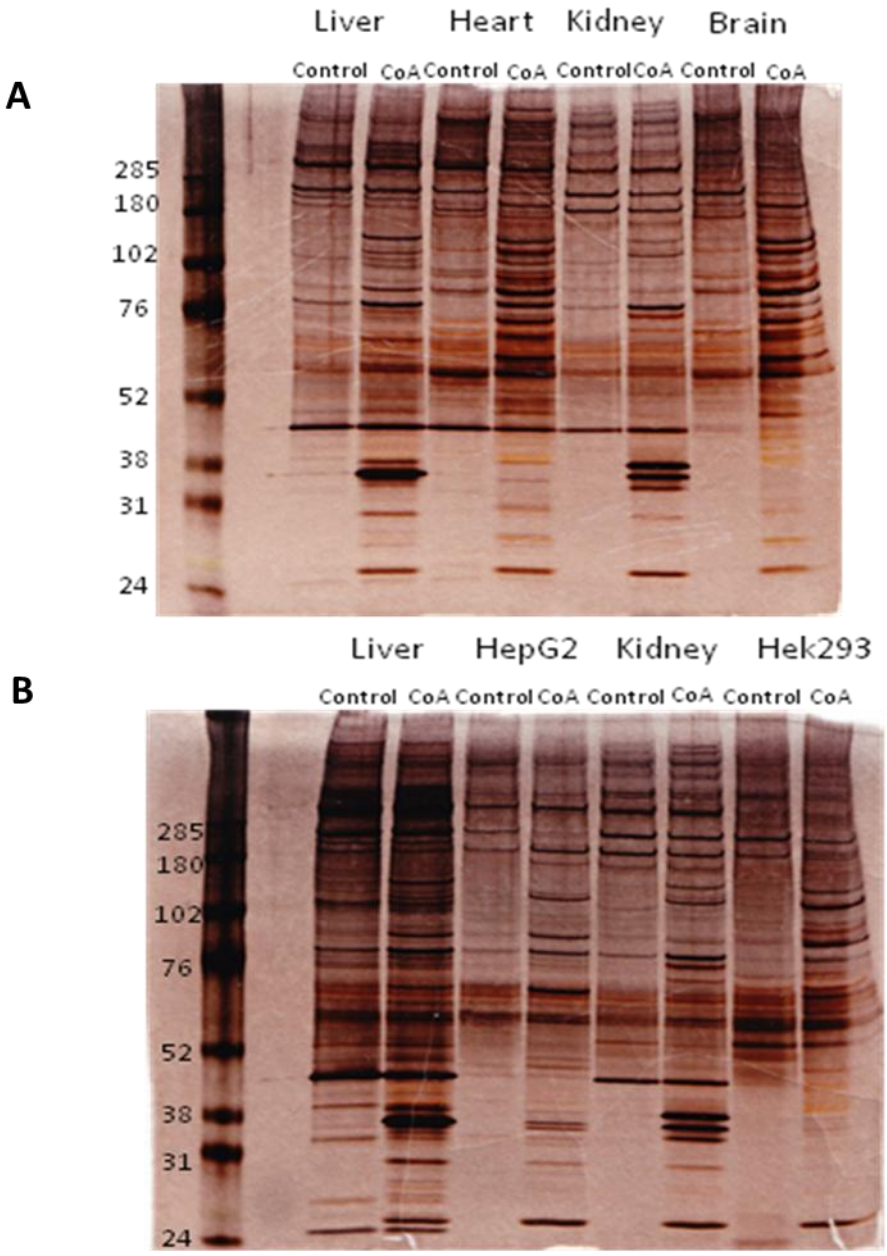

Figure 5.7: Affinity purification of CoA binding partners in various rat tissues and cultured cells. The lysates from rat liver, heart and kidney **(A)** and HepG2 and Hek293 in addition to rat liver and kidney **(B)** were incubated with Sepharose beads, coupled to CoA or Tris (control beads). Beads were washed with lysis buffer, followed by Hepes buffer + 1 mM DTT, before proteins were released through boiling in SDS loading buffer, then resolved and visualised by SDS-PAGE and silver staining.

protein binding patterns between Hek293 and brain tissue in the lower molecular weight region.

Liver was selected for further analysis as it appeared to contain the greatest number of CoA interacting proteins out of all the lysates tested. Further elution of liver proteins bound to CoA beads with increasing concentrations of CoA, confirmed that a large number of proteins specifically bind to CoA *(Figure 5.8)*.

5.2.3 Identification of CoA binding proteins

Specific CoA binding proteins present in rat liver lysate were eluted from Tris and CoA Sepharose by 100 mM CoA and resolved through SDS-PAGE analysis and Coomassie-staining *(Figure 5.9A)*. Each lane of the gel was sliced into 30 pieces, digested with trypsin, and protein identity was analysed by mass-spectrometry.

143 CoA binding proteins were identified through mass spectrometry *(Figure 5.9B, Table 5.1)*. These proteins were divided into several groups: CoASH/CoA ester related proteins (17); dehydrogenases (20); other metabolic enzymes (6); kinases (3); acetyl transferases (2); ADP ribosyltransferases (2); Ubiquitin ligase (1); disulphide isomerases (2); G protein signalling proteins (1); scaffold proteins (5); proteins involved in translation/transcription (29); ribosomal proteins (13); cytoskeletal proteins (24); and other proteins (18) including blood and extracellular matirix proteins as well as putative and previously uncharacterised proteins. A number of metabolic enzymes that were expected to bind to CoASH or its thioesters were pulled down, such as 3-ketoacyl-CoA thiolase (*Figure 5.10),* however there were also proteins identified as potentially novel CoA interacting proteins, for example fructose bisphosphate aldolase B *(Figure 5.10)*. Earlier experiments indicated that CoA binds to the Sepharose beads through its thiol group, leaving the nucleotide moiety exposed for protein interaction. This therefore resulted in a number of proteins that were known to bind to ATP (e.g. nucleoside diphosphate kinase,

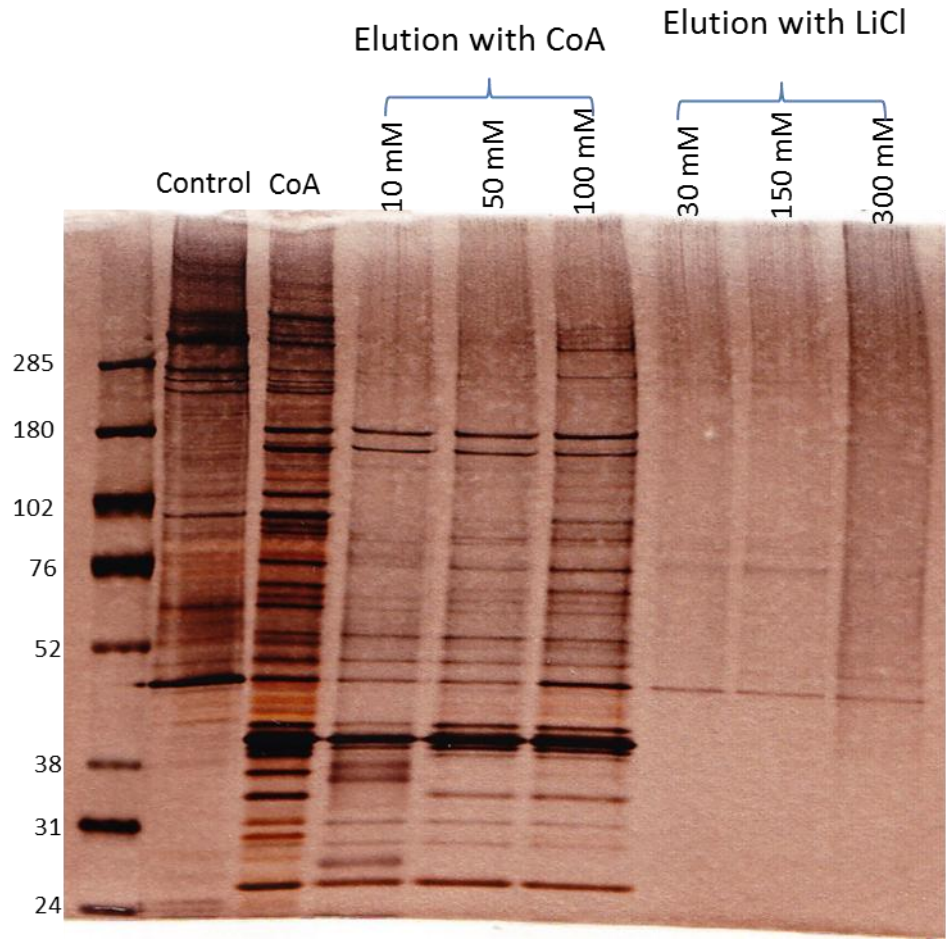

Figure 5.8: Affinity purification of CoA binding partners in rat liver. The lysate from rat liver was incubated with Sepharose beads, coupled to CoA or Tris (control beads). Beads were washed with lysis buffer, followed by Hepes buffer + 1mM DTT, before eluting proteins with increasing concentrations of CoA (10, 50 and 100 mM). The commercially supplied standard used here contains 3 moles of Li^+/ mole CoA, so proteins were also eluted with LiCl as a control. Proteins were boiled in SDS loading buffer, then resolved and visualised by SDS-PAGE and silver staining.

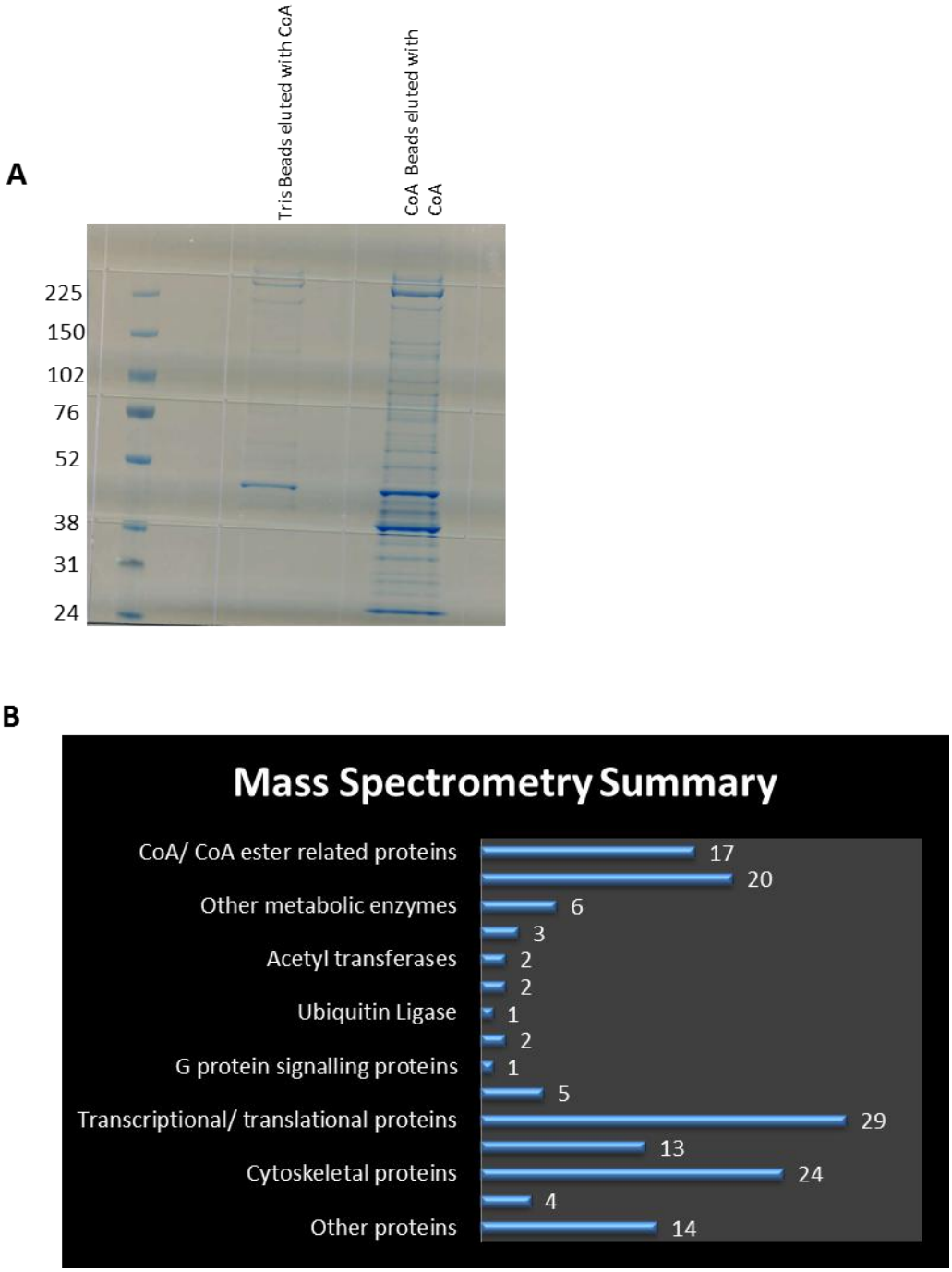

Figure 5.9: A) Image of the Coomassie stained gel used for identification of CoA-binding proteins purified by CoA-Sepharose. Liver lysate proteins eluted from Tris and CoA-Sepharose by CoA were boiled in SDS loading buffer and resolved by SDS-PAGE and Coomassie-stained. Each-lane was sliced into 30 pieces, digested with trypsin, and protein identity analysed by mass-spectrometry. **B)** Summary table of CoA-binding proteins.

Name	Mr (KDa)	Accession No.
2,4-dienoyl-CoA reductase, mitochondrial Rattus norvegicus	36	DECR_RAT
3-ketoacyl-CoA thiolase, mitochondrial Rattus norvegicus	42	THIM_RAT
Acyl-coenzyme A amino acid N-acyltransferase 2 Rattus norvegicus	46	ACNT2_RAT
Acyl-Coenzyme A dehydrogenase family, member 11 Rattus norvegicus	87	B3DMA2_RAT
ATP-citrate synthase Rattus norvegicus	121	ACLY_RAT (+1)
Hydroxyacyl-Coenzyme A dehydrogenase type II Rattus norvegicus	27	B0BMW2_RAT (+1)
Hydroxymethylglutaryl-CoA synthase, mitochondrial Rattus norvegicus	57	HMCS2_RAT (+1)
Peroxisomal 2,4-dienoyl-CoA reductase Rattus norvegicus	31	DECR2_RAT
Peroxisomal acyl-coenzyme A oxidase 1 Rattus norvegicus	75	ACOX1_RAT
Peroxisomal acyl-coenzyme A oxidase 2 Rattus norvegicus	77	ACOX2_RAT
Peroxisomal bifunctional enzyme Rattus norvegicus	79	ECHP_RAT
Peroxisomal D3,D2-enoyl-CoA isomerase Rattus norvegicus	43	Q5XIC0_RAT
Peroxisomal multifunctional enzyme type 2 Rattus norvegicus	79	DHB4_RAT (+1)
Peroxisomal trans-2-enoyl-CoA reductase Rattus norvegicus	32	PECR_RAT
Phytanoyl-CoA dioxygenase, peroxisomal Rattus norvegicus	39	PAHX_RAT
Trifunctional enzyme subunit alpha, mitochondrial Rattus norvegicus	83	ECHA_RAT
Putative uncharacterized protein Zea mays	35	B4FU60_MAIZE (+2)
6-phosphogluconate dehydrogenase, decarboxylating Rattus norvegicus	53	6PGD_RAT (+1)
NADPH:adrenodoxin oxidoreductase, mitochondrial Rattus norvegicus	54	ADRO_RAT
Grhpr protein Rattus norvegicus	36	B0BN46_RAT
Sepiapterin reductase (7,8-dihydrobiopterin:NADP+ oxidoreductase) Rattus norvegicus	28	B2RYK3_RAT (+1)
L-lactate dehydrogenase Rattus norvegicus	36	B5DEN4_RAT (+2)
Biliverdin reductase B (Flavin reductase (NADPH)) (Biliverdin reductase B (Flavin reductase (NADPH)) (Predicted), isoform CRA_b) Rattus norvegicus	22	B5DF65_RAT
Carbonyl reductase [NADPH] 1 Rattus norvegicus	31	CBR1_RAT
Carbonyl reductase 4 Rattus norvegicus	25	CBR4_RAT
Estradiol 17-beta-dehydrogenase 8 Rattus norvegicus	27	DHB8_RAT
Dehydrogenase/reductase SDR family member 4 Rattus norvegicus	28	DHRS4_RAT (+1)
10-formyltetrahydrofolate dehydrogenase Rattus norvegicus	99	FTHFD_RAT (+1)
Glucose-6-phosphate 1-dehydrogenase X Mus musculus	59	G6PD1_MOUSE (+4)
Hydroxysteroid dehydrogenase-like protein 2 Rattus norvegicus	58	HSDL2_RAT
Isocitrate dehydrogenase [NADP] cytoplasmic Microtus ochrogaster	47	IDHC_MICOH (+8)
Oxidoreductase NAD^+-binding domain-containing protein 1 Mus musculus	35	OXND1_MOUSE
Prostaglandin reductase 1 Rattus norvegicus	36	PTGR1_RAT
Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthetase Rattus norvegicus	101	Q5EBC3_RAT
Ab1-219 Rattus norvegicus	52	Q7TP88_RAT (+1)
Quinone oxidoreductase Rattus norvegicus	35	QOR_RAT
Quinone oxidoreductase-like protein 2 Rattus norvegicus	38	QORL2_RAT
Fructose-bisphosphate aldolase B Rattus norvegicus	40	ALDOB_RAT (+1)
ATP synthase subunit alpha, mitochondrial Rattus norvegicus	60	ATPA_RAT
ATP synthase subunit beta Homo sapiens	57	A8K4X0_HUMAN (+10)
5,10-methenyltetrahydrofolate synthetase (5-formyltetrahydrofolate cyclo-ligase) Rattus norvegicus	23	Q5M9F6_RAT
Cytidine monophosphate N-acetylneuraminic acid synthetase Rattus norvegicus	48	Q5M963_RAT
tRNA-dihydrouridine synthase 3-like Rattus norvegicus	72	DUS3L_RAT
Nucleoside diphosphate kinase B Rattus norvegicus	17	NDKB_RAT
Nucleoside diphosphate kinase Rattus norvegicus	19	Q99NI1_RAT
Nucleoside diphosphate kinase A Rattus norvegicus	17	NDKA_RAT
Glycine N-acyltransferase Rattus norvegicus	34	A4PB92_RAT (+1)
Gnpnat1 protein Rattus norvegicus	21	B1H249_RAT (+1)
Parp9 protein Rattus norvegicus	92	A1A5Q1_RAT
Poly [ADP-ribose] polymerase 12 Mus musculus	80	PAR12_MOUSE (+2)
TRIP12 protein (Fragment) Bos taurus	126	Q0P5M6_BOVIN (+2)

Name	Mr (KDa)	Accession No.
Putative disulfide-isomerase Taeniopygia guttata	27	B5FXN8_TAEGU (+7)
Protein disulfide-isomerase A6 Rattus norvegicus	48	PDIA6_RAT
Guanine nucleotide binding protein alpha inhibiting 2 (Fragment) Capra hircus	35	A4F2F7_CAPHI (+16)
AP-2 complex subunit alpha-1 Mus musculus	108	AP2A1_MOUSE
AP-2 complex subunit alpha-2 Mus musculus	104	AP2A2_MOUSE (+6)
AP-2 complex subunit mu-1 Bos Taurus	50	AP2M1_BOVIN (+15)
cDNA FLJ78481, highly similar to Homo sapiens adaptor-related protein complex 2, beta 1 subunit, mRNA Homo sapiens	106	A8K916_HUMAN (+9)
Clathrin light chain B Bos Taurus	25	CLCB_BOVIN (+6)
Constitutive coactivator of peroxisome proliferator-activated receptor gamma Mus musculus	89	F120B_MOUSE
Novel protein, possible orthologue of human peroxisomal proliferator-activated receptor A interacting complex 285 PRIC285 Mus musculus	334	A2AS03_MOUSE (+1)
Staphylococcal nuclease domain-containing protein 1 Rattus norvegicus	102	SND1_RAT
ATP-dependent RNA helicase DDX1 Rattus norvegicus	82	DDX1_RAT
DEAD (Asp-Glu-Ala-Asp) box polypeptide 5 (Fragment) Rattus norvegicus	69	B6DTP5_RAT (+1)
DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 Xenopus tropicalis	54	B1WAU0_XENTR (+10)
DEAD box polypeptide 17 isoform p82 variant (Fragment) Homo sapiens	81	Q59F66_HUMAN
RNA helicase Mesocricetus auratus	73	Q8K5D5_MESAU
Ras GTPase-activating protein-binding protein 1 Mus musculus	52	G3BP1_MOUSE (+1)
Ras GTPase-activating protein-binding protein 2 Mus musculus	54	G3BP2_MOUSE (+1)
Hnrnpl protein (Fragment) Rattus norvegicus	62	B5DFG2_RAT (+1)
Splicing factor proline/glutamine rich (Polypyrimidine tract binding protein associated) Mus musculus	75	A2A7U6_MOUSE (+2)
cDNA FLJ75550, highly similar to Homo sapiens heterogeneous nuclear ribonucleoprotein A1 (HNRPA1), transcript variant 1, mRNA Homo sapiens	34	A8K4Z8_HUMAN (+14)
cDNA FLJ75459 Homo sapiens	43	A8K4L9_HUMAN (+8)
cDNA FLJ77927 Homo sapiens	60	A8K894_HUMAN (+7)
Ewing sarcoma breakpoint region 1 Homo sapiens	65	B0QYK0_HUMAN (+16)
Heterogeneous nuclear ribonucleoprotein D0 Rattus norvegicus	38	HNRPD_RAT
Non-POU domain-containing octamer-binding protein Homo sapiens	54	NONO_HUMAN (+4)
PABPN1 protein Bos Taurus	29	A2VE34_BOVIN (+4)
Predicted protein Nematostella vectensis	26	A7TAM8_NEMVE
Putative uncharacterized protein Mus musculus	42	Q91VM5_MOUSE
Putative uncharacterized protein Mus musculus	88	Q3TVV6_MOUSE (+5)
Putative uncharacterized protein Zea mays	36	C0P9K6_MAIZE (+10)
RNA granule protein 105 Rattus norvegicus	78	A9ZSZ9_RAT (+1)
cDNA FLJ36192 fis, clone TESTI2027450, highly similar to Eukaryotic translation initiation factor 3 subunit 5 Homo sapiens	39	B3KSH1_HUMAN (+11)
Eukaryotic translation initiation factor 3, subunit 1 Mus musculus	36	A2AE04_MOUSE (+9)
Eukaryotic translation initiation factor 3, subunit E interacting protein Homo sapiens	71	B0QY89_HUMAN (+7)
Eukaryotic translation initiation factor 4A isoform 1 Sus scrofa	46	A6M928_PIG (+15)
Eukaryotic translation initiation factor 4A isoform 3 Sus scrofa	47	A6M931_PIG (+2)
40S ribosomal protein S19 Homo sapiens	16	B0ZBD0_HUMAN (+9)
cDNA FLJ40595 fis, clone THYMU2010705, highly similar to 40S RIBOSOMAL PROTEIN S4, X ISOFORM Homo sapiens	30	B2R491_HUMAN (+13)
LOC100037080 protein Xenopus laevis	33	A2BDA2_XENLA (+16)
LOC100037089 protein (Fragment) Xenopus laevis	31	A2BDB1_XENLA (+10)

Name	Mr (KDa)	Accession No.
LOC100125111 protein Xenopus tropicalis	15	A4IH66_XENTR (+9)
LOC100145026 protein (Fragment) Xenopus tropicalis	15	B0BME0_XENTR (+19)
Putative uncharacterized protein Mus musculus	27	Q5YLW3_MOUSE (+3)
Ribosomal protein L11 Mus musculus	20	A2BH07_MOUSE (+7)
Ribosomal protein L23 Mus musculus	15	A2A6F9_MOUSE (+19)
Ribosomal protein S10 Capra hircus	19	A5JST4_CAPHI (+7)
Ribosomal protein S11 Homo sapiens	18	B2R4F5_HUMAN (+7)
Ribosomal protein S5, isoform CRA_b Rattus norvegicus	23	B0BN81_RAT
Rps16 protein Mus musculus	16	A4FUS1_MOUSE (+22)
Actin (Fragment) Euagrus chisoseus	22	B2XY21_9ARAC
Actin (Fragment) Labyrinthula terrestris	34	A4ZA16_9STRA
Actin related protein 2/3 complex, subunit 4, 20kDa, isoform 3 (Predicted) Papio Anubis	20	A9L8Z8_PAPAN (+7)
Actin-related protein 2/3 complex subunit 1B Rattus norvegicus	41	ARC1B_RAT (+4)
Actin-related protein 2/3 complex subunit 2 Bos Taurus	34	ARPC2_BOVIN (+7)
Actin-related protein 3 Mus musculus	47	ARP3_MOUSE (+4)
cDNA FLJ78587 Homo sapiens	50	A8JZY9_HUMAN (+20)
Desmin (Predicted) Callicebus moloch	53	B1MTK2_CALMO (+5)
Filamin, alpha Mus musculus	280	B9EKP5_MOUSE (+2)
Filamin-B Mus musculus	278	FLNB_MOUSE
Gelsolin Bos Taurus	81	GELS_BOVIN (+2)
LIM domain and actin-binding protein 1 Mus musculus	84	LIMA1_MOUSE (+4)
Myo18a protein Mus musculus	232	B2RRE2_MOUSE (+1)
MYO1B protein Bos Taurus	132	A6QLD6_BOVIN (+5)
Myosin, heavy polypeptide 8, skeletal muscle, perinatal Mus musculus	223	B2RWW8_MOUSE (+14)
Myosin-10 Bos Taurus	229	MYH10_BOVIN
Myosin-9 Mus musculus	226	MYH9_MOUSE
Myosin-Ic Rattus norvegicus	120	MYO1C_RAT
Plectin-1 Rattus norvegicus	534	PLEC1_RAT (+9)
Predicted protein Nematostella vectensis	50	A7S027_NEMVE (+53)
Radixin Homo sapiens	69	B0YJ88_HUMAN (+10)
Taf15 protein Rattus norvegicus	60	B2RYG5_RAT
Tropomodulin 3 Rattus norvegicus	39	Q6AXW2_RAT
Tropomyosin 1, alpha Rattus norvegicus	29	Q6AZ25_RAT
Fibronectin Rattus norvegicus	273	FINC_RAT
Kallistatin Rattus norvegicus	48	P97569_RAT (+1)
Plasminogen Rattus norvegicus	91	PLMN_RAT
Heat shock protein 90kDa alpha (Cytosolic), class A member 1, gene 2 Xenopus tropicalis	82	A0JMA1_XENTR (+50)
Trim14 protein Rattus norvegicus	50	B2RYH2_RAT
Protein FAM98A Rattus norvegicus	55	FA98A_RAT
Uncharacterized protein KIAA0564 homolog Mus musculus	213	K0564_MOUSE
Novel protein (2610510H03Rik) Mus musculus	45	A2ATZ4_MOUSE (+1)
UPF0027 protein C22orf28 homolog Mus musculus	55	CV028_MOUSE (+2)
LRRGT00192 Rattus norvegicus	34	Q6QI16_RAT

Name	Mr (KDa)	Accession No.
LOC100145742 protein (Fragment) Xenopus tropicalis	98	B1WB27_XENTR (+7)
Putative uncharacterized protein RPL30 (Fragment) Homo sapiens	13	A8MTA6_HUMAN (+20)
Putative uncharacterized protein ENSP00000352132 Homo sapiens	22	A6NE65_HUMAN (+8)
Erythroid spectrin alpha Rattus norvegicus	277	Q6XDA1_RAT
Erythroid spectrin beta Rattus norvegicus	246	Q6XDA0_RAT
Complement C1q subcomponent subunit C Rattus norvegicus	26	C1QC_RAT
Hemoglobin alpha, adult chain 2 Rattus norvegicus	15	B1H216_RAT (+1)

Table 5.1: Full list of CoA binding proteins purified using CoA Sepharose from rat liver lysate, analysed by mass spectrometry.

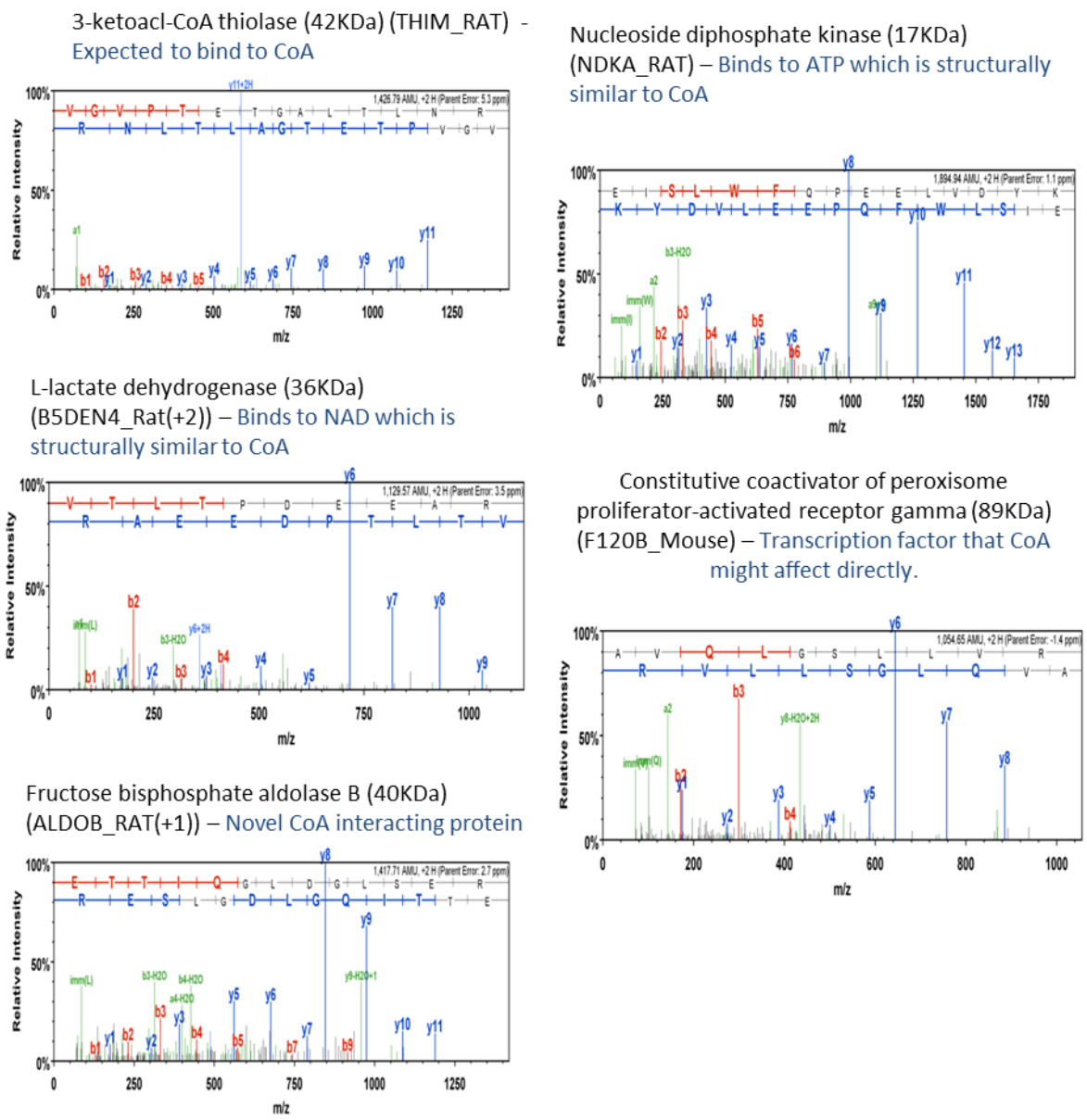

Figure 5.10: Example MS-MS spectra of selcted CoA binding proteins.

Figure 5.10) or NAD^+ (e.g. lactate dehydrogenase, *Figure 5.10)*, which are both similar in structure to CoA due to the presence of the adenine group. Moreover, transcription factors, like PPARγ (*Figure 5.10)* were pulled down through this analysis, indicating that CoA might directly affect transcription.

5.2.4 Functional Studies

The enzymes glucose-6-phosphate Dehydrogenase (G6PDH), lactate dehydrogenase (LDH) and fructose-bisphosphate aldolase (aldolase) are among the proteins that have been identified as potentially novel CoA binding proteins. These enzymes were chosen to carry out more specific functional studies, since they are important metabolic enzymes that were readily available and could be assayed easily. G6PDH and LDH are dehydrogenases and consequently are known to interact with NADP/NADPH and NAD^+/NADH respectively, so it is not unexpected that they would accommodate CoA due to the similarities in structure between these molecules. Interestingly, aldolase is not known to interact with any nucleotide.

G6PDH is the rate-limiting enzyme in the pentose phosphate pathway and converts NADP to NADPH during the reaction involving the transformation of glucose-6-phosphate to phosphoglucono-δ-lactone. G6PDH maintains NADPH levels for the biosynthesis of fatty acids and isoprenoids as well as for maintaining glutathione levels in cells helping to prevent oxidative damage. LDH catalyses the interconversion of pyruvate and lactate, with the simultaneous interconversion of NADH and NAD^+. Pyruvate is the final product of glycolysis and the forward LDH reaction, resulting in lactate production, occurs when oxygen concentrations are low. Aldolase is also an enzyme involved in glycolysis, catalysing the breakdown of fructose 1,6-bisphosphate into glyceraldehyde-3-phospate and dihydroxyacetone phosphate.

To examine whether these enzymes bind directly to CoA, a CoA binding assay was carried out. 50 µg of G6PDH, LDH or aldolase was incubated with Tris or CoA beads for 2 hrs, 4°C, with mixing. Proteins that interacted with the beads were released through boiling with SDS loading buffer, and resolved through SDS-PAGE analysis and Coomassie staining. There appears to be little difference in the binding pattern between Tris and CoA beads for both LDH and aldolase, suggesting that these enzymes do not directly bind to CoA (*Figure 5.11A)*. They were possibly pulled down during affinity purification through binding to another protein that interacts directly with CoA. On the other hand, a much greater proportion of G6PDH binds to CoA beads, compared to Tris beads, signifying that G6PDH does interact directly with CoA (*Figure 5.11A)*. It is interesting that only G6PDH binds directly to CoA, despite the fact that LDH can also hold the adenine nucleotide in its active site. An explanation for this might be because the NADP active site of G6PDH can accommodate the 3'phosphate the ribose sugar of CoA due to the presence of the 2' phosphate on the ribose sugar of NADP/NADPH, however NAD^+/NADH active site of LDH probably does not contain the space for this phosphate group *(Figure 5.11B).*

The intracellular concentration of NADP is estimated to be about 170-220 µM (Olsen et al. 2003), with the cytosolic concentration of NADPH estimated at 10-20 µM (Gupte et al. 2005). Since the cytosolic CoA concentration is also around 20 µM (Robishaw & Neely 1985), it is feasible that CoA could compete with the NADP binding site of G6PDH.

An enzymatic assay was therefore carried out to investigate whether CoA could act as a competitive inhibitor of G6PDH activity. 2 mM glucose-6-phosphate was incubated with 0.5 mM NADP in Tris HCl (pH 7.5) and 10 mM $MgCl_2$ in the presence of increasing concentrations of CoA (5 µM-5 mM). Increasing CoA concentrations did not appear to have any effect on G6PDH activity, measured by changes in NADPH absorption, indicating that CoA does not inhibit G6PDH *(Figure 5.12A)*. Furthermore, CoA does not regulate LDH activity, as expected, at physiological CoA concentrations *(Figure 5.12B)*. The physiological relevance of CoA binding to G6PDH still remains to be elucidated.

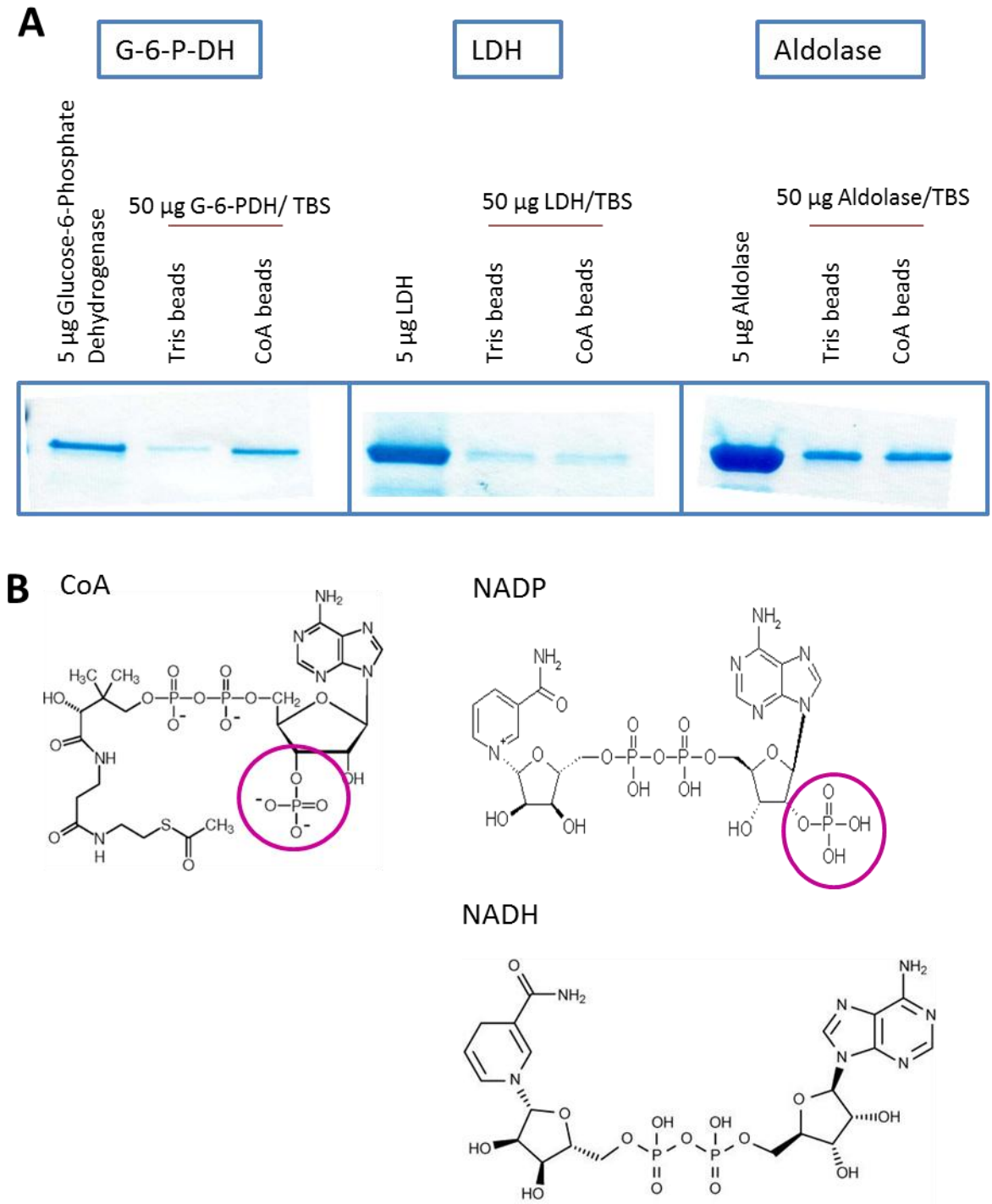

Figure 5.11: CoA binding assays for specific enzymes. A) 50 µg Glucose-6-phosphate dehydrogenase, LDH or Aldolase in Tris buffer (pH 7.5) was incubated with Sepharose beads bound to CoA or Tris (control) for 2 hours, 4°C, with mixing. After washing in TBS, the proteins were released from the beads by boiling in SDS loading buffer. Proteins were resolved and visualised by SDS-PAGE and Coomassie staining. These experiments were repeated three times. **B)** Diagram of the structures of CoA, NADP and NADH, which highlights the phosphate group present on the ribose sugar of CoA and NADP but not NADH.

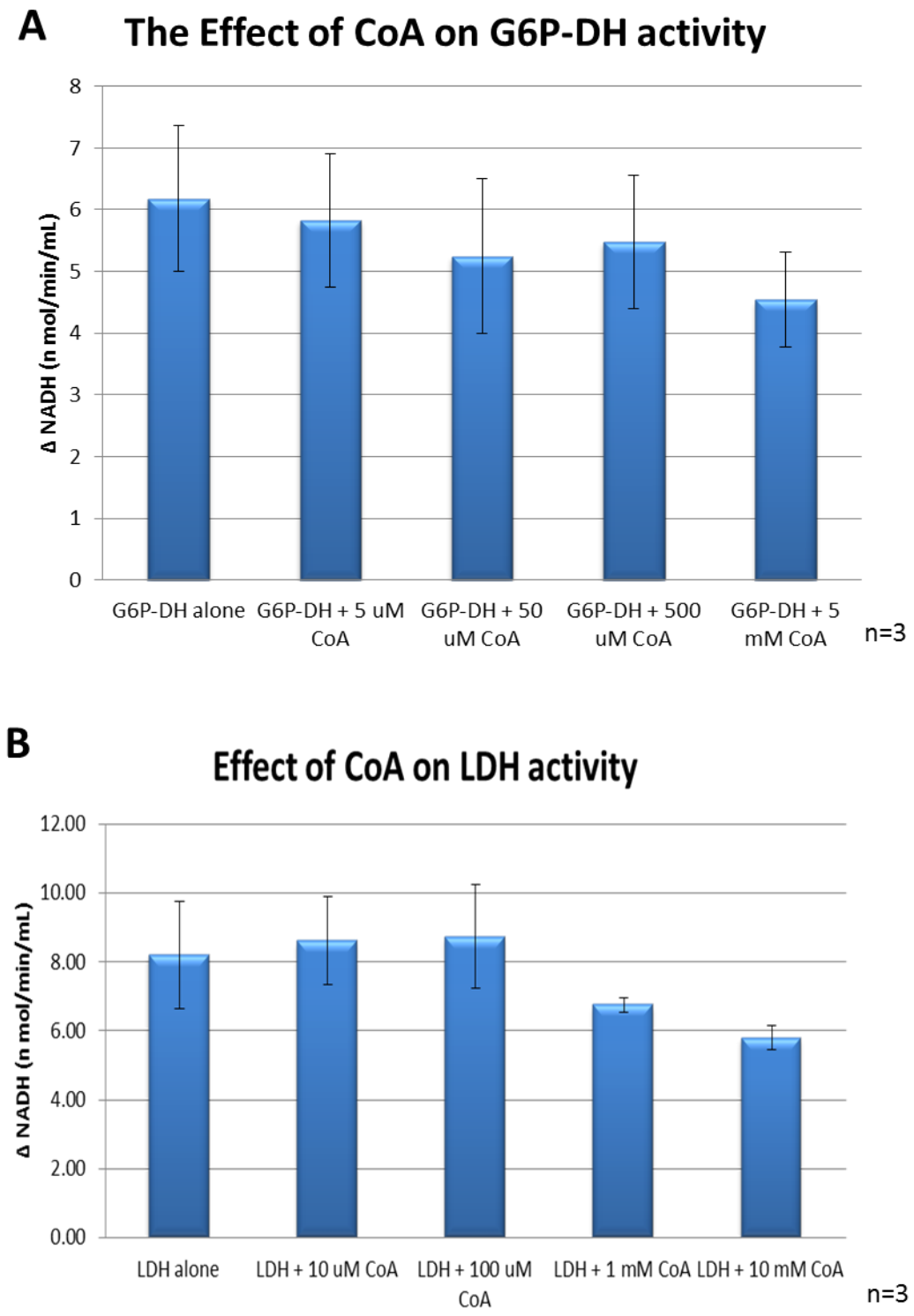

Figure 5.12: CoA has no effect on glucose-6 phosphate dehydrogenase or lactate dehydrogenase activity. A) Glucose-6-phosphate dehydrogenase activity was measured in the presence of 2 mM glucose-6 phosphate, 0.5 mM NADP and 10 mM $MgCl_2$ in Tris HCl (pH 7.5) alone or with concentrations of CoA ranging from 5 µM to 5 mM for 10 mins, 30°C. ΔNADPH was analysed by measuring ΔA_{340} nm. **B)** Lactate dehydrogenase activity was measured in the presence of 1.3 mM pyruvate and 0.3 mM NADH in 65 mM Tris HCl, pH 7.5 alone or with concentrations of CoA ranging from 10 µM to 10 mM for 10 mins, 30°C. ΔNADH was analysed by measuring ΔA_{340} nm.

5.3 Discussion

Affinity purification on CoA Sepharose has been used previously to isolate specific CoA binding proteins, however it has not been mentioned in the literature as a method to identify novel CoA binding proteins. There have been a number of limitations with this method that need to be taken into account. Firstly, not all proteins identified here as CoA binding proteins represent proteins that interact with CoA directly, as exemplified by LDH and aldolase. It is possible these proteins were bound to other proteins that interacted with CoA. For example, aldolase has been implicated in the interaction with several proteins unrelated to glycolytic enzymes such as actin and tubulin, both of which were pulled down in this screen (Volker and Knull 1997; Wang et al. 1996).

It was determined in this chapter that CoA was bound to Sepharose *via* its SH group, leaving the adenine nucleotide exposed. Another limitation with this approach was therefore that CoA beads were also able to bind to adenine nucleotide binding proteins, potentially pulling down proteins that interact with ATP/ADP, NAD^+, NADP and FAD, rather than CoA. Judging by the names of many of these proteins/enzymes, many do appear to bind with these nucleotides, such as the dehydrogenases, helicases and kinases. To eliminate nucleotide binding proteins in future studies, samples could be run through an ATP or NAD^+(H) affinity matrix first, before using CoA Sepharose. However this may also eliminate some genuine CoA-interacting proteins.

CoA is held in a specific orientation with the beads, therefore CoA may not be as flexible when interacting with proteins and possibly may prevent binding. Structural studies have shown that the conformation of CoA when bound to a number of CoA binding proteins can alter between an extended and bent conformation (Engel 1996). It is possible that the steric effect of the Sepharose prevents CoA from forming the bent conformation at its pyrophosphate linkage and consequently interacting with proteins requiring that form.

Furthermore, protein/CoA interactions that also require salt bridges are prevented as the SH group is inaccessible. It is also possible that the beads themselves might hinder protein binding. The addition of a linker between CoA and Sepharose might improve accessibility of proteins interacting with the pantetheine moiety.

Kinases that interacted with CoA were of particular interest in this study in order to identify any signalling pathways CoA might regulate. Only three kinases were detected through this method, all of which were variations on the same enzyme (nucleoside diphosphate kinase). The reason why more kinases were not pulled down by the CoA beads could possibly be because kinases generally are present in very low abundance and the mass spectrometry analysis was not sensitive enough to detect them. It needs to be taken into account that proteins that were extremely abundant (e.g. ribosomal proteins) may have bound non-specifically to CoA.

Despite the limitations, a number of potentially novel CoA-interacting proteins have been identified, and their specific interaction with CoA should be validated in future studies. G6PDH is the main regulatory enzyme in the pentose phosphate pathway, which is essential for the formation of NADPH (required for fatty acid synthesis and prevention of oxidative stress), nucleic acids and nucleotides and aromatic amino acids. Although CoA did bind to G6PDH, physiological concentrations of CoA did not appear to show any regulatory effects on its enzymatic activity. The activity of yeast G6PDH has been previously reported to be down-regulated by palmitoyl CoA (Kawaguchi and Bloch 1974), and this might explain the interaction seen between G6PDH and CoA. The absence of a regulatory effect may be because CoA does not bind strongly enough to G6PDH. Palmitoyl CoA may be more effective due to its long fatty acyl CoA chain.

Furthermore, proteins that interact with the SH group of CoA remain to be investigated, having ensured linkage to CNBr coupled beads through the adenine group.

This may be achieved by the method of Rieke et *al,* where an analogue of CoA, N^6-{N(6-aminohexyl)carbamoylmethyl}-CoA is used to immobilise CoA through its terminal amino group. Initially, bis(CoA) is alkylated with iodoacetic acid, followed by rearrangement to bis(N^6-carboxymethyl-CoA). Next, condensation with 1,6-diaminohexane yields bis{N^6-[N-(6-aminohexyl)-carbamoylmethyl]-CoA}. The bis(CoA analogue) is then split by reducing with DTT. A further reaction with Nbs_2 (5,5'-dithio-bis(2-nitrobenzoic acid), prevents the thiol group of CoA binding to Sepharose and also enables the coupling efficiency of the ligand, based on the amount of 5-thio-2-nitrobenzoic acid liberated after treatment with DTT (Rieke et al. 1979). By leaving the SH group available and carrying out affinity binding experiments under non-reducing conditions, proteins with cysteine residues could form di-sulphide bridges with CoA. Indeed, regulation of proteins through sulfhydryl modification is commonly carried out by glutathione (Bellomo et al. 1987), and a number of proteins such as PTEN and receptor tyrosine phosphatases enzymatic activities depend on their catalytic cysteine residues in the active site (Leslie 2003; Winterbourn 2008; Yu 2005).

Chapter 6: Discussion

Chapter 6: Discussion

CoA has been shown to regulate many important metabolic pathways through its action as a cofactor, however it also has the potential to regulate proteins through allosteric and covalent interactions. Most of the literature focusses on the role of CoA in intermediary metabolism and very little is known about the part it plays as a regulator of cellular function. Furthermore, the levels of CoA are altered in several diseases, including cancer and diabetes, yet the mechanisms behind these changes and the consequences of altered CoA levels are still unclear. The aim of this thesis was to identify novel cellular processes regulated by changes in CoA levels and also to understand in more detail how CoA levels are regulated.

Most studies on CoA in the literature have used whole animals or isolated tissues. In this thesis, cultured cells were used as an experimental model, so it was necessary to ensure that CoA could accurately be measured in this system. Development and optimisation of sample extraction procedure and detection methods (an enzymatic recycling assay and HPLC) resulted in reliable and reproducible techniques that could accurately measure CoA levels within the picomole (or µM concentration) range observed in cultured cell extracts.

Two approaches were used to investigate the role of CoA as a regulator. The "top down" approach was applied in Chapter 4 to identify cellular processes and pathways which are influenced by changes in CoA levels. In Chapter 5 the "bottom up" approach was employed, where a CoA affinity matrix was created to pull down individual proteins that interacted with CoA and establish how they are regulated by CoA. Mass spectrometry analysis of proteins that were affinity purified with CoA identified 143 proteins, many of

which were not previously known to bind to CoA. For example, a number of transcription factors are potential novel binding partners for CoA and require further investigation. Of the three proteins selected for further investigation into CoA regulation, only G6PDH was found to directly interact with CoA. This identified a limitation, where some proteins that potentially bind to CoA binding proteins could also be pulled down with this approach. It may therefore be necessary to investigate more stringent purification conditions in future studies.

In Chapter 4, in addition to the investigation of processes potentially regulated by changes in CoA, several conditions were tested in cultured cells to see whether their effects mediated similar changes in CoA levels to what was observed in the literature. The main limitation encountered here was that CoA levels are much lower in cultured HepG2 cells compared to liver *(Figure 4.1)*. This is an issue because if CoA acts as a regulator, the effects of changes in CoA levels in these cells may not represent the effects in liver *in vivo*.

The low levels of CoA also seen in Hek293 cells might account for the fact that CoASy was not rate-limiting and CoA levels were not regulated through mTOR and PI3K signalling, despite the association of mTOR, S6K and p85αPI3K with CoASy. The effects of the interactions of CoASy with S6K and PI3K still remain to be elucidated. Evidence that CoASy is rate-limiting was previously reported in *E. coli* as well as mammalian COS-7 cells through accumulation of 4'PP (Jackowski and Rock 1981; Rock et al. 2000), however radioactive studies in rat heart did not observe this accumulation unless CoA synthesis was stimulated (Robishaw et al. 1982). This suggests CoASy is not rate-limiting in all systems. There is still the possibility that CoASy over-expression affects the rate of CoA biosynthesis, however overall CoA levels do not change due to rapid recycling (Smith 1978).

Another limitation with this study was that cultured cells did not respond well to conditions previously reported to affect CoA levels in tissues, including glucose and insulin.

It is important to note that a number of reports in the literature did not measure a change in absolute CoA levels despite an increase in the rate of CoA biosynthesis as measured by incorporation of radioactive pantothenate into CoA (Reibel et al. 1981a; Robishaw et al. 1982; Smith 1978). This, together with the observation that a lot of processes are altered to maintain the CoA level, may be partly why the effects with nutrients and hormones did not correlate with the previous data in the literature. Alternatively, the half-life of CoA in the cultured cells studied might be longer than the time-points measured upon treatment with various stimuli, and consequently may be a reason why few changes in CoA levels were seen.

Despite the limitations with this study, several potential processes that may be regulated by CoA were identified. Over-expression of PanK1β resulted in an increase in not just free CoASH levels, but also acetyl CoA, malonyl CoA and HMG CoA. The rise in individual CoA esters not only indicates that CoA is the limiting factor for production of these esters, but also gives an insight into processes this increase in CoA might affect. For example, acetyl CoA can be synthesised through the action of PDH in the mitochondria, ACL in the cytosol, or β-ketothiolase during fatty acid oxidation, so CoA is limiting for at least one of these enzymes. Depending on whether acetyl CoA is synthesised in the cytosol or mitochondria, an increase in acetyl CoA could potentially result in increased glucose and fatty acid oxidation, increased acetylation which was observed in this study, and amino acid, fatty acid and nucleotide synthesis. Malonyl CoA is formed in the cytosol from acetyl CoA by ACC, which is usually tightly regulated by hormones, citrate and AMP. However in these conditions it appears to be dependent on the amount of CoA available. This increase in malonyl CoA levels could promote fatty acid biosynthesis and polyketide biosynthesis as well as inhibit fatty acid oxidation. HMG CoA can be formed in the cytosol and mitochondria by HMG synthase and could activate the mevalonate and ketogenesis

pathways, respectively. Interestingly, no change in long-chain acyl CoA occurred. It has been previously suggested that acyl CoA synthetase is dependent on cytosolic CoASH concentration as its K_m for CoASH is in the low micromolar range, close to the estimated cytosolic concentration of CoASH (Bakken et al. 1991). The lack of an increase in long chain acyl CoA may suggest that Pank1β overexpression mainly increases free CoASH in the mitochondria and causes little change in the cytosol, or some other factors such as supply of fatty acids are limiting for the acyl CoA synthetase reaction. In future studies it would be important to determine the subcellular distribution of CoASH/CoA esters and whether over-expression of PanK1β increases CoASH and CoA esters in mitochondria, cytosol or both compartments. No change in long-chain acyl CoA may also suggest that inhibition of growth by PanK1β over-expression observed here is not mediated by the metabolic enzymes or ion channels that long-chain acyl CoA regulates.

Although these changes in CoA esters, as well as the effect on acetylation and growth observed in Hek293 cells following an increase in CoA biosynthesis may not reflect the effect of increasing CoA in tissues such as liver (in which basal CoA levels are approximately 10-fold higher), these findings may be relevant to some pathologies in which CoA levels are reported to be low. It is interesting to note that CoA levels in cultured cells are reminiscent of disease states such as cancer. It is also important to be aware that the levels of CoA in cultured cells are not only much lower than in tissues such as liver, but the ratio of CoASH to acetyl CoA is reversed. This effect was also seen in liver biopsy samples taken from patients with Reye Syndrome (Corkey et al. 1988). Disease states with low levels of CoA might have similar profiles as the cells studied here, therefore it might be possible to apply the regulatory mechanisms discovered to these pathological conditions. This might include the non-rate-limiting observations seen with CoASy in Hek293 cells.

The reasoning behind the novel finding that increasing CoA appears to have an anti-proliferative effect on cells still remains unknown. One of the possible suggestions, following the observed increase in lactate production, was that an increase in flux through the rate-limiting PK reaction was taking place. Future studies should address this possibility by measuring cellular content of pyruvate and glycolytic intermediates in Pank1β over-expressing cells. Pank1β itself might interact and modulate the activity of PK, however immunoprecipitation studies need to be carried out to determine whether this interaction actually takes place. The effect of over-expressing catalytically inactive Pank1β should also be tested.

The anti-proliferative effect observed following increased CoA levels might also be due to acetylation of proteins regulating cell growth, since increased acetylation was observed when PanK1β was over-expressed in Hek293 cells. Mass spectrometry analysis should be carried out in the future to identify which proteins are acetylated, and specific functional assays for individual proteins could determine whether acetylation of these proteins contributes to decreased cell growth.

It is tempting to speculate a reciprocal relationship between CoA levels and cell growth. It appears cancer cells (e.g. HepG2) and transformed fast growing cells (e.g. Hek293) contain low levels of CoA, and the data here indicate that increasing CoA slows down cell growth in Hek293 cells. It may be the low CoA levels that contribute to the fast growing phenotype of cancer cells. The results involving pantothenate withdrawal indicated that cells are able to grow in the absence of pantothenate, once cells have initially accumulated enough CoA. This supports the concept that cells require relatively little CoA in order to grow. This observation that increasing CoA slows down cell growth still has to be confirmed in HepG2 cells and other cancer cell lines as it may not be true for all cell types and conditions. There is an argument that low CoA levels in these cells are simply due to

the culturing conditions (e.g. high glucose), however cell culturing media are optimised to promote cell growth and maintenance. In other words, if culturing media is responsible for low CoA levels in these cells, one cannot rule out the possibility that maintenance of low CoA by these media may be a necessary factor for promoting cell growth. In future studies, it would be interesting to compare CoA levels in primary hepatocytes, isolated from rat liver, with the CoA levels measured in rat liver tissue and HepG2 cells.

In conclusion, this thesis has provided a greater insight into how CoA is regulated and also the wider role of CoA as a regulator of cellular processes. It was initially shown that mTOR and PI3K signalling do not regulate CoA levels in cultured cells, despite the interaction between CoASy and S6K and p85αPI3K. Increased CoA also does not appear to affect mTOR or PI3K signalling. CoASy was not rate-limiting in Hek293 cells, however, so it cannot be ruled out that CoASy regulates CoA levels *in vivo*. When investigating the role of CoA as a regulator of cellular function, CoA binding studies identified a large number of proteins that were not previously known to interact with CoA. Further functional studies should determine the impact of these interactions. The main finding was that increasing CoA levels results in mechanisms that inhibit cell proliferation. This effect might be mediated through an increase in acetylation or another mechanism associated with high CoA levels. This finding is important because increasing CoA could potentially be used as a method to target cancerous cells by slowing down the fast growing phenotype of these cells.

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