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TRIMETHYLAMINE OXIDE, BETAINE AND OTHER OSMOLYTES IN DEEP-SEA ANIMALS: DEPTH TRENDS AND EFFECTS ON ENZYMES UNDER HYDROSTATIC PRESSURE

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Abstract - Most shallow teleosts have low organic osmolyte contents, e.g. 70 mmol/kg or less of trimethylamine oxide (TMAO). Our previous work showed that TMAO contents increase with depth in muscles of several Pacific families of teleost fishes, to about 180 mmol/kg wet wt at 2.9 km depth in grenadiers. We now report that abyssal grenadiers (*Coryphaenoides armatus*, Macrouridae) from the Atlantic at 4.8 km depth contain 261 mmol/kg wet wt in muscle tissue. This precisely fits a linear trend extrapolated from the earlier data. We also found that anemones show a trend of increasing contents of methylamines (TMAO, betaine) and scyllo-inositol with increasing depth. Previously we found that TMAO counteracts the inhibitory effects of hydrostatic pressure on a variety of proteins. We now report that TMAO and, to a lesser extent, betaine, are generally better stabilizers than other common osmolytes (myo-inositol, taurine and glycine), in terms of counteracting the effects of pressure on NADH K_m of grenadier lactate dehydrogenase and ADP K_m of anemone and rabbit pyruvate kinase.

Key words: Trimethylamine oxide, betaine, inositol, taurine, deep-sea, osmolyte, pyruvate kinase, lactate dehydrogenase, *Coryphaenoides*

INTRODUCTION

The deep sea is the largest habitat on Earth, and yet remains one of the least explored. Physiological and biochemical adaptations of organisms to the deep sea are also understudied, including those that allow survival under high hydrostatic pressure. High hydrostatic pressure has broad effects on biochemistry, inhibiting protein folding and assembly and ligand binding when there is a positive volume change involved. This change often results from the release and expansion of bound water molecules that are densely clustered around some protein surfaces and ligands. While some deep-sea proteins have evolved poorly-understood features that reduce pressure effects, some exhibit significant pressure sensitivities and thus seem incompletely adapted (13). In recent years, we have found evidence that certain solutes called organic osmolytes may help offset effects of pressure.

Organic osmolytes are most commonly found in marine osmoconformers, which include most types of marine organisms. Osmoconformers have internal osmotic pressures equal to seawater at approximately 1000 mOsm. However, while their extracellular fluids are typically dominated by NaCl, their cells do not have high salt concentrations; instead cells accumulate organic osmolytes to about 600 mOsm (with about 400 mOsm arising from basic inorganic and organic solutes). Organic solutes fall into a few categories: neutral amino acids (e.g. glycine, taurine); sugars and polyols (e.g. inositols); methylsulfonium compounds and methylamines, especially glycine betaine (hereafter called betaine) and trimethylamine N-oxide (TMAO) and urea (18). Marine invertebrates (at least those living in shallow habitats) use primarily free amino acids and sometimes methylamines

Abbreviations: **bet**: betaine; **glyc**: glycine; **HPLC**: high-performance liquid chromatography; **LDH**: lactate dehydrogenase; **mInos**: myo-inositol; **PK**: pyruvate kinase; **sInos** and **scy-Inos**: scyllo-inositol; **TMAO**: trimethylamine N-oxide

as osmolytes. Among the vertebrates, osmoconformers include elasmobranchs (sharks, skates, rays), which use urea and TMAO as their major organic osmolytes. In contrast, most other marine vertebrates are hypo-osmotic osmoregulators; that is, they maintain internal osmotic pressures at levels considerably less that 1000 mOsm (about 350 mOsm in most shallow marine teleosts fishes) (18).

Organic solutes used as osmolytes are thought to be accumulated because, unlike common inorganic ions, most of them (with the exception of urea) do not perturb proteins; i.e. they are "compatible" solutes (3). Importantly, many osmolytes –especially methylamines– are not merely compatible, but actually stabilize the functions and structures of proteins. In doing so, these osmolytes can counteract the effects of perturbants such as urea (a protein destabilizer), temperature and NaCl. For example, shallow-water cartilaginous fishes typically have urea at 300-400 mM as an osmolyte, accompanied by TMAO at 150-200 mM in cells. At this ratio (about 2:1), urea's inhibitory effects are offset by TMAO's stabilizing effects (18).

In our recent work, we have found that osmolyte compositions of deep-sea animals differ from those of shallow-living relatives, which tend to be dominated by taurine, glycine and other neutral amino acids. In contrast, some species of deep-sea polychaetes, echinoderms, gastropods and octopods have substantial levels of scyllo-inositol, a solute not reported at osmotically significant levels in other animals (21). Many deep-sea animals also have high levels of methylamines, including betaine, glycerophosphorylcholine and, most strikingly, TMAO (5,20,21). TMAO is found in cells of some shallow-living osmoconformers and teleost fishes (osmoregulators), but (except in elasmobranchs) usually at modest levels (e.g. 70 mmol/kg fresh weight or less in bony fishes). However, we found an approximately linear increase with depth (to 2.9 km depth) in muscle TMAO contents in several families of bony fishes (macrourids, morids, zoarcids, scorpaenids) from the eastern Pacific Ocean. For example, TMAO was found at about 180 mmol/kg in macrourid (grenadier) fishes from 2.9 km (5,6). A similar pattern was found in several different types of osmoconformers (e.g. decapod crustaceans, skates, carnivorous clams).

In this study, we analyze a macrourid fish from a much greater depth (4.8 km) and a different ocean to test the scope of this TMAO pattern. We also compare shallow and deep Cnidarians (sea anemones) to test whether this phylum also exhibits osmolyte differences in the deep.

We also continue testing our hypothesis that TMAO (and perhaps other organic osmolytes) in deep-sea animals serve to counteract the inhibitory effects of hydrostatic pressure on proteins (5). We have found that TMAO can counteract pressure effects on protein stability and assembly, yeast growth, and enzyme kinetics (5,19,20). Two examples of kinetics involve the ADP K_m of pyruvate kinase (PK) and the NADH K_m of lactate dehydrogenase (LDH). Both K_ms are pressure-sensitive for deep-sea as well as shallow PK and LDH homologues (although deep homologues of LDH are less pressure sensitive than shallow homologues; 12,13). TMAO can offset most or all inhibition by pressure in the physiological range (5,20). Here, we test the effects of other organic osmolytes on PK and LDH to determine whether these solutes can also offset the effects of pressure or whether TMAO is uniquely suited for this role.

MATERIALS AND METHODS

Abyssal grenadiers (*Coryphaenoides armatus*; Family Macrouridae) were collected by net from 4.8 km depth on the Porcupine Abyssal Plain (North Atlantic) and frozen at –80˚C. White muscle tissue was analyzed for TMAO according to Kelly and Yancey (6). The anemone *Actinauge abyssorum* was collected from 1.9 and 2.9 km depth off the Oregon coast as described by Kelly and Yancey (6). A subtidal anemone *Stomphia didemon* was collected at 90 m depth in Monterey Bay, California, by P. Whaling of the Monterey Bay Aquarium Institute, using a remotely operated vehicle. An intertidal anemone, *Telia (Urticina) crassicornis*, was collected on the coast near Bellingham, Washington State. All anemones were frozen on dry ice and then stored at –70˚C until analyzed for organic osmolytes, using high-performance liquid chromatography (HPLC) as described by Yin *et al*. (22).

Lactate dehydrogenase (LDH, A_4 isozyme) from grenadier white muscle and pyruvate kinase (PK) from the deep anemone's internal septa were extracted as previously described (5, 20). Briefly, LDH was assayed in 4 mM pyruvate, 80 mM Tris-HCl buffer (pH 7.14 at 10˚C) with varying concentrations of NADH; PK was assayed with ADP as varied substrate, in 80 mM Tris-HCl (pH 7.14 at 10˚C), 1.5 mM phosphoenolpyruvate, 0.1 mM fructose diphosphate, 0.15 mM NADH, 100 mM KCl, 10 mM $MgSO_4$, and 6 units/ml of rabbit LDH (Type II, Sigma Chem., St. Louis, MO, USA). PK from rabbit muscle and other chemicals were purchased from Sigma Chem. Kinetics of the enzymes were measured in a temperature-controlled pressure cell as described by Gillett *et al*. (5) and Yancey *et al*. (20). Seven different substrate concentrations were run (in duplicate) for each K_m value calculated. K_m and V_{max} (maximal velocity) values were calculated using the weighted linear regression of Wilkinson (16), and data were statistically analyzed by ANOVA.

RESULTS

Osmolyte analyses

White muscle tissue of Atlantic grenadiers (*C. armatus*) from 4.8 km contained TMAO at 261 ± 12 mmol/kg (n=5). This precisely fits a linear depth trend extrapolated from previous data on Pacific gadiform fish, i.e. grenadiers (including *C. armatus*) and cods from shallow water (Fig. 1).

The osmolyte compositions of the anemones also differed, with some possible depth trends. The intertidal species *T. crassincornica* was dominated by glycine, followed by taurine and betaine. The subtidal anemones (*S. didemon*) had approximately equal contents of glycine and taurine, with low contents of betaine. The intermediate-depth anemones (*A. abyssorum* from 1.9 km) were

Table 1 Contents of major organic osmolytes (in mmol/kg wet wt), with standard deviations, in sea anemones

Depth	Myo-Inositol	Scy-Inositol	Taurine	Glycine	Betaine	TMAO	Other Aas
Intertidal	1.6 ± 0.2	n.d	24.6 ± 9.9	54.6 ± 7.5	3.9 ± 0.7	n.d.	21.7 ± 6.4
90 m	0.9 ± 0.2	n.d.	50.1 ± 4.4	50.4 ± 4.9	4.2 ± 0.5	n.d.	10.3 ± 1.5
1.9 km	2.0 ± 0.3	11.1 ± 1.6	31.7 ± 2.3	17.8 ± 2.0	12.0 ± 0.4	10.6 ± 1.7	32.1 ± 3.4*
2.9 km	4.2 ± 0.5	16.9 ± 2.9	23.1 ± 2.1	12.7 ± 1.5	33.4 ± 1.7	31.6 ± 3.3	3.9 ± 1.4

n.d.: not detected (<0.1 mmol/kg wet wt); AA: amino acids. Three animals per group were analyzed. The 1.9 and 2.9-km anemones were the same species (*A. abyssorum*). Scy-inositol: scyllo-inositol; *primarily β-alanine

dominated by taurine and ß-alanine, followed by glycine, betaine, scyllo-inositol and TMAO. Their deeper (2.9 km) conspecifics were dominated by equal contents of TMAO and betaine, followed by taurine, scyllo-inositol and glycine (Table 1, Fig. 2). TMAO, betaine and scyllo-inositol contents were positively correlated with depth, while glycine contents were negatively correlated. Excluding the intertidal anemones (which live under different stresses than the fully submerged species), taurine contents were negatively correlated with depth (Table 1, Fig. 2). (Note that the total osmolyte contents are considerably less than cellular concentrations, because

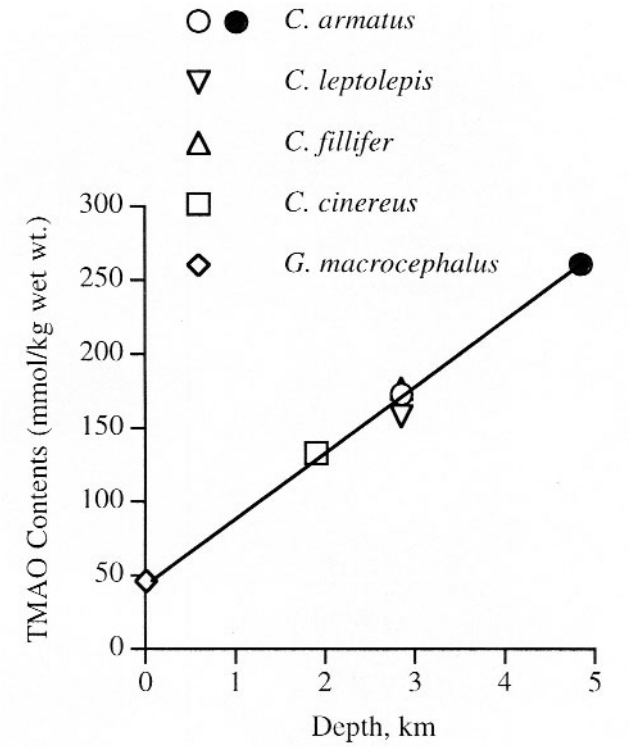

Fig. 1 TMAO contents in muscles of cods (*Gadus macrocephalus*) from shallow water and grenadiers (*Caryphaenoides* spp) from deeper waters. Open symbols are from Gillett *et al*. (5) and Kelly and Yancey (6); the filled circle is new. The regression line yields this equation: TMAO=0.0437 x depth + 44.0 (r^2=0.99).

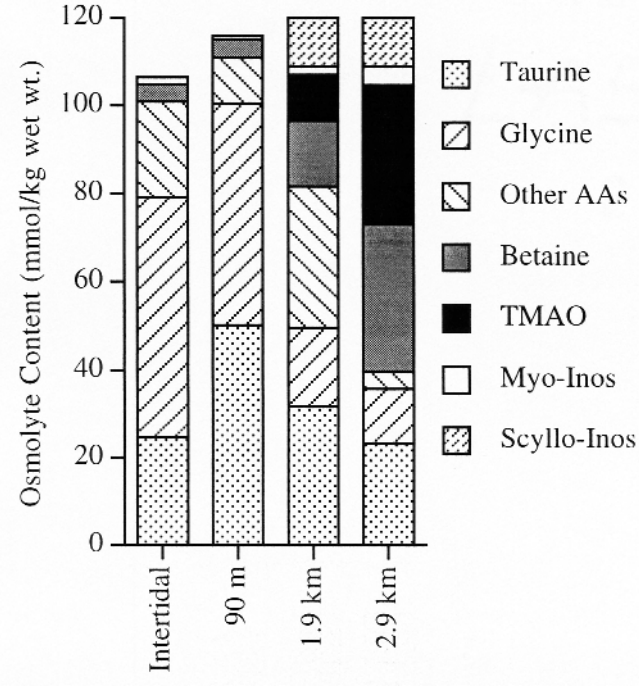

Fig. 2 Contents of organic osmolytes in septal tissues of sea anemones (data from Table 1). Inos: inositol

contents are derived from whole tissue which includes both extracellular fluid, which has no organic osmolytes, and intracellular fluid, which contains the organic osmolytes.)

Enzyme kinetics

Three enzymes were tested at 0.1 MPa (1 atm) and 25 MPa (250 atm), with and without 250 mM of the solutes TMAO, glycine, betaine and myo-inositol. Although scyllo-inositol is the polyol found in some deep-sea invertebrates such as anemones (see Introduction and Table 1), myo-inositol was selected for testing because scyllo-inositol in usable quantities is not available.

The NADH K_m of LDH from *C. armatus* was elevated 25% at high pressure (at 10˚C). TMAO tended to reduce the K_m at 0.1 MPa (though not significantly), but it was able to counteract fully the inhibition at 25 MPa (Fig. 3), as

previously described for *C. leptolepis* LDH (5). Betaine was able to counteract pressure inhibition, but not completely. However, glycine and myo-inositol did not exhibit significant counteracting abilities (Fig. 3). Maximal velocity was not altered by any solutes or by pressure (not shown).

The ADP K_m of rabbit PK increased by 22% at 25 MPa (at 37˚C). Both TMAO and betaine reduced the K_m at 0.1 MPa and completely counteracted the effect of 25 MPa pressure. However, K_m was not altered nor was pressure counteracted by glycine or myo-inositol (Fig. 4). Maximal velocity was not altered by any solutes or by pressure (not shown).

The ADP K_m of deep-sea anemone PK increased by 47% at 25 MPa (at 10˚C). This inhibitory effect was completely counteracted by TMAO, partly counteracted by betaine, and not altered by glycine. Taurine acted as a strong competitive inhibitor (Fig. 5). Maximal velocity was not altered by these solutes or by pressure (not shown). Myo-inositol had complex, unexpected effects. It reduced the ADP K_m by 55% (Fig. 5), more than offsetting the effect of pressure. However, it also reduced the maximal velocity, by 39.9% (± 1.3% standard deviation) at low pressure and by 46.1 % (± 0.5% standard deviation) at high pressure. In other words, binding (as judged as the inverse of K_m) was increased while catalytic rate was decreased by myo-inositol.

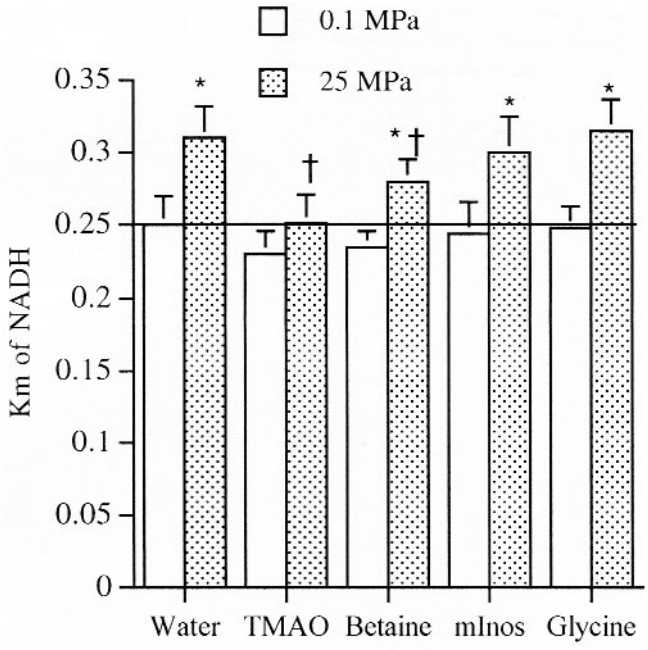

Fig. 3 Effects of various osmolytes (250 mM) on K_m of NADH (in µM with standard deviations) for grenadier lactate dehydrogenase, at low and high pressures. *Significant increase compared to 0.1 MPa water control; †significant decrease compared to 25 MPa water control; mInos: myo-inositol

DISCUSSION

The linear depth correlation in TMAO that we previously reported in various animals (5,6) has now been extended in macrourid fishes to a much greater depth (Fig. 1). It is particularly noteworthy that two of the data points, one at 2.9 km in the Pacific Ocean, and the new data point at 4.8 km in the Atlantic Ocean, are from the same species, *C. armatus*. This pattern lends further support to the hypothesis that TMAO accumulation is related to hydrostatic pressure, the only environmental factor that is also linear with depth. Other factors –e.g. diet, buoyancy, temperature (6) and acylglycerol metabolism (10)– that might explain the occurrence of higher TMAO in deep-sea animals appear to be less able to explain a highly linear pattern.

The results for the anemones (Table 1, Fig. 2) also suggest an increase in methylamines (TMAO plus betaine) with depth, concomitant with a reduction in other common osmolytes (taurine, glycine). We previously found a similar pattern in caridean shrimp, in which TMAO increased and glycine decreased with depth (6).

As noted in the Introduction, previous work in our laboratory found that TMAO can counteract pressure effects on several proteins, while glycine does not (19,20). The results of the current study with LDH and PK suggest

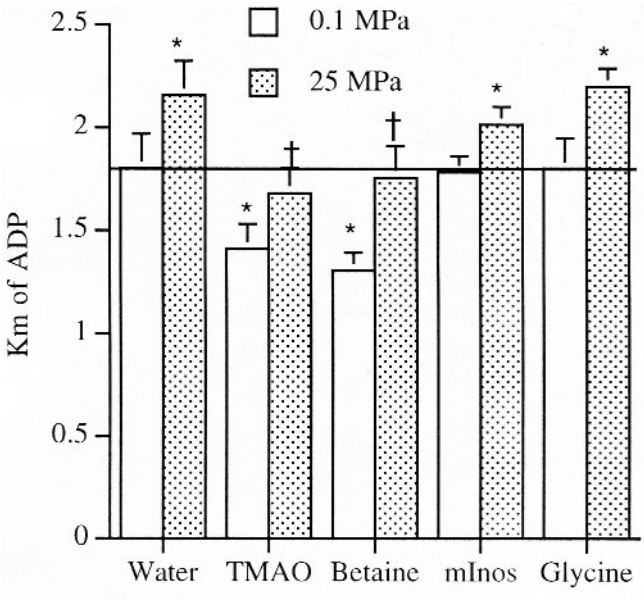

Fig. 4 Effects of various osmolytes (250 mM) on K_m of ADP (in mM with standard deviations) for rabbit pyruvate kinase, at low and high pressures. *Significant increase or decrease compared to 0.1 MPa water control; †significant decrease compared to 25 MPa water control; mInos: myo-inositol

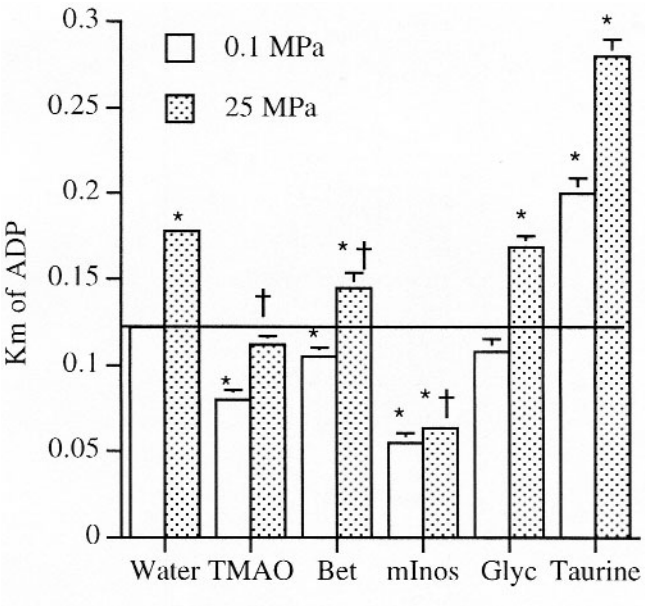

Fig. 5 Effects of various osmolytes (250 mM) on K_m of ADP (in mM with standard deviations) for deep-sea anemone pyruvate kinase, at low and high pressures. *Significant increase or decrease compared to 0.1 MPa water control; †significant decrease compared to 25 MPa water control. Myo-inositol (mInos) also greatly reduced the enzyme maximal velocity, which was unaffected by the other solutes and pressure. Glyc: glycine; Bet: betaine

that TMAO is overall a better stabilizer for counteracting hydrostatic pressure than other osmolytes. The other methylamine tested, betaine, also counteracted pressure effects, though not consistently as well as TMAO. Glycine, myo-inositol and taurine showed no ability (Fig. 3, 4, 5), perhaps explaining in part why two of these osmolytes (glycine, taurine) tend to decrease with depth.

TMAO's effects in counteracting pressure appear to be universal, working on proteins from deep-sea and terrestrial animals (Fig. 4) (19) and on yeast growth (21). This is similar to the well-documented protein-stabilizing capabilities of TMAO that are often universal for other denaturants, e.g. in counteracting the destabilizing effects of urea (18). That TMAO generally works better than other osmolytes in counteracting pressure is similar to findings with urea: TMAO is generally a better counteractant with urea than betaine, sarcosine, proline and glycerol (9,18).

We also found a possible trend of increasing scyllo-inositol with depth in the anemones (Table 1, Fig. 2), as found in some other invertebrate taxa (21). Other researchers have shown that some polyols (e.g. sorbitol) can stabilize microbial proteins against pressure effects (1), suggesting to us that scyllo-inositol might be accumulated for this purpose in deep-sea animals. However, our results with myo-inositol provide mixed evidence: It was unable to counteract pressure increases in K_ms for grenadier LDH and rabbit LDH, and while it did greatly reduce ADP K_m for anemone PK, it also greatly lowered the catalytic rate. It is not clear what the overall effect of this would be if it occurs *in vivo*. Whether scyllo-inositol is a better counteractant than myo-inositol, or its accumulation helps these animals in other non-osmotic ways, or is a byproduct of deep-sea diets, is unknown.

Other organic osmolytes have also been found to have possible relationships to hydrostatic pressure. We found that vesicomyid clams from 2 to 6.5 km depths contain an unidentified serine-phosphate solute which increases linearly with depth, forming over 65% of the osmolyte pool of the deepest species (4). Deep-sea bacteria have been found to accumulate the osmolyte β-hydroxybutyrate in correlation with exposure to pressure as well as to osmotic strength (7). This finding led the authors to propose the term "piezolyte" for osmolytes that are accumulated at high pressure. However, whether the serine-phosphate or β-hydroxybutyrate can offset the effects of pressure is unknown.

It is not known how TMAO and perhaps other osmolyte-like solutes can counteract the effects of pressure, but there are some clues. Timasheff (14) and colleagues have shown that stabilizing osmolytes are preferentially excluded from the hydration layer of proteins, which creates two regions of high order: the protein's hydration layer with relatively low concentration of osmolyte, and distant water relatively enriched in osmolyte. Wang and Bolen (15) have demonstrated that unfavorable interactions between TMAO and peptide backbones explain this strong exclusion. The result of exclusion is that any tendency of a protein to expose more hydrated surface area (e.g. by peptide unfolding or ligand release) to an osmolyte solution will be entropically unfavorable, because more of these ordered regions would result. The mechanism causing the exclusion effect is unclear. One posssibility lies in TMAO's ability to enhance hydrogen bonding among water molecules (2), which may reduce water-protein interactions and thus favor folded, more compact proteins with less surface area exposed to water. This effect may also favor substrate binding to enzymes by reducing water-substrate binding.

In sum, accumulation of TMAO (and perhaps betaine) in some deep-living animals appears to play an important role in adaptation to pressure. However, it should be noted that such osmolytes do not solve all problems associated with pressure. The evolution of pressure-resistant proteins is clearly another common mode of adaptation (8,11,13). Studies on structural adaptations in proteins to pressure show there may be a trade-off in terms of catalytic efficiencies, with deep-sea homologues of enzymes having lower catalytic rates than do counterparts from shallow-living species (13). The presence of methylamines may help ameliorate this "cost" of deep-sea adaptation by

increasing enzyme rates at subsaturating substrate concentrations.

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