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REVIEW

Co-evolution of proteins and solutions: protein adaptation versus cytoprotective micromolecules and their roles in marine organisms

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ABSTRACT

Organisms experience a wide range of environmental factors such as temperature, salinity and hydrostatic pressure, which pose challenges to biochemical processes. Studies on adaptations to such factors have largely focused on macromolecules, especially intrinsic adaptations in protein structure and function. However, micromolecular cosolutes can act as cytoprotectants in the cellular milieu to affect biochemical function and they are now recognized as important extrinsic adaptations. These solutes, both inorganic and organic, have been best characterized as osmolytes, which accumulate to reduce osmotic water loss. Singly, and in combination, many cosolutes have properties beyond simple osmotic effects, e.g. altering the stability and function of proteins in the face of numerous stressors. A key example is the marine osmolyte trimethylamine oxide (TMAO), which appears to enhance water structure and is excluded from peptide backbones, favoring protein folding and stability and counteracting destabilizers like urea and temperature. Co-evolution of intrinsic and extrinsic adaptations is illustrated with high hydrostatic pressure in deep-living organisms. Cytosolic and membrane proteins and G-protein-coupled signal transduction in fishes under pressure show inhibited function and stability, while revealing a number of intrinsic adaptations in deep species. Yet, intrinsic adaptations are often incomplete, and those fishes accumulate TMAO linearly with depth, suggesting a role for TMAO as an extrinsic 'piezolyte' or pressure cosolute. Indeed, TMAO is able to counteract the inhibitory effects of pressure on the stability and function of many proteins. Other cosolutes are cytoprotective in other ways, such as via antioxidation. Such observations highlight the importance of considering the cellular milieu in biochemical and cellular adaptation.

KEY WORDS: Osmolytes, Cytoprotection, Hydrostatic pressure, Deep sea, Trimethylamine N-oxide

Introduction: intrinsic and extrinsic biochemical adaptations

The study of biochemical adaptations to the vast diversity of environments on Earth has yielded major insights into fundamental properties of life and its evolution. The majority of this work has focused on intrinsic macromolecular adaptations: changes in protein functional characteristics and amino acid sequences, in the genetic sequences that code for them and in regulatory mechanisms (e.g. for differential gene expression in different environments). Often overlooked is the fact that most proteins operate in a special cellular environment selected to be quite different from the external environment. This milieu, which provides extrinsic components profoundly important for protein structure and function, consists primarily of micromolecules, the dominant one being water. As Gerstein and Levitt (1998) memorably stated: 'When scientists publish models of biological molecules in journals, they usually draw their models in bright colors and place them against a plain, black background. We now know that the background in which these molecules exist – water – is just as important as they are.'

And yet, the importance of other cellular micromolecules is often overlooked, especially those that physical chemists call cosolutes or cosolvents. The former term emphasizes interactions with other dissolved compounds, the latter emphasizes interactions with water. The original work on such micromolecules was with salinity and osmotic stress, i.e. their properties as osmolytes in marine organisms. More recently, the cytoprotective role of organic micromolecules has received attention with respect to countering the effects of other stressors, including high hydrostatic pressure in the deep ocean. As Scherer (2013) puts it: 'Protein interactions in water are also clearly mediated by the other solution components...Cosolutes, including the important biological osmolytes…denaturants such as urea…as well as different salts...are inextricably linked to…the stability and interactions of proteins in solution.'

For membrane proteins, the particular lipids that surround them provide another important micromolecular environment. A high concentration of total cellular proteins, a 'macromolecular milieu', can also enhance individual protein stability (reviewed by Somero, 2003). In this review, we will focus primarily on the small 'cytoprotective' cosolutes/cosolvents that cells accumulate to high levels in conjunction with certain physical and chemical stressors. We will then turn to proteins under hydrostatic pressure in the deep sea to illustrate the interplay of extrinsic and intrinsic adaptations.

Types of cytoprotective micromolecules and their presence in marine organisms

As noted earlier, the first cytoprotective micromolecules to be well documented were osmolytes. These are inorganic ions, e.g. K^+ as the primary cellular cation in most cells, and certain small organic solutes, whose concentrations via colligative effects help to maintain cell volume by balancing intra- and extracellular osmolalities or reducing evaporation. Osmolytes are typically regulated in response to water stress, e.g. a change in external osmolality or desiccation. As extensively reviewed elsewhere (Yancey et al., 1982; Yancey, 2005), organic osmolytes fall into a few broad categories across the spectrum of life: polyols and sugars, free amino acids, methylamines and methylsulfonium solutes, and urea (Fig. 1). Within all but the urea category, numerous different molecules have been found in different organisms, environments and even within different organs in an individual. Some are widespread; e.g. the methylamine betaine (glycine betaine; N-trimethylglycine) has been found in all domains and eukaryotic kingdoms.

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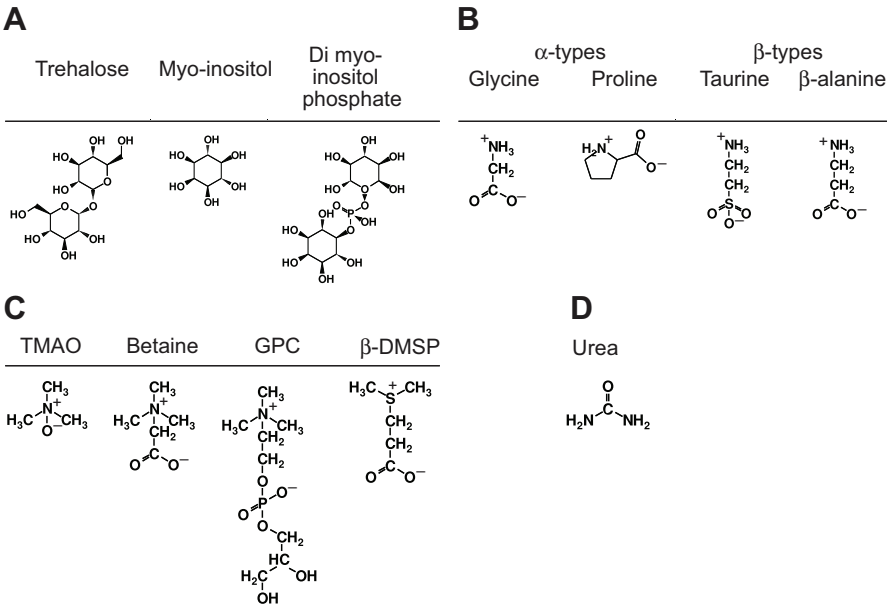

Fig. 1. Structures of typical organic osmolytes found in marine organisms. (A) Carbohydrates. (B) Amino acids. (C) Methylamine and methylsulfonium solutes. (D) Urea. TMAO, *N*-trimethylamine oxide; GPC, glycerophosphorylcholine; DMSP, dimethylsulphoniopropionate.

Organic osmolytes were first described in organisms in high-salinity environments. For seawater, salts (mainly NaCl) yield an average osmolality of ∼1000 milliosmoles per kg (1000 mosmol kg^{-1}) whereas the basic solutes in most cells (K^+, metabolites, proteins, etc.) yield only ∼300–400 mosmol kg^{-1}. With respect to this potentially dehydrating situation, marine organisms are classified into two categories. First, osmoconformers prevent osmotic shrinkage by accumulating osmolytes to equalize internal and habitat osmolalities. These include most marine taxa other than most vertebrates and some arthropods. However, although extracellular fluids (ECFs) in multicellular organisms are dominated by NaCl, the major osmolytes inside cells are organic (Fig. 2). These are up- or downregulated in euryhaline species to prevent osmotic shrinkage or swelling as habitat osmolality changes. Key examples include free amino acids and some methylamines in most invertebrate taxa, and trimethylamine N-oxide (TMAO) and other methylamines with urea in chondrichthyans (elasmobranchs – sharks and rays – plus holocephalans). Importantly, urea and methylamines in chondrichthyan cells are typically found at about a 2:1 ratio, at least in organisms inhabiting shallow waters (Fig. 2).

Second, osmoregulators homeostatically regulate internal osmolality. In the oceans, these include vertebrates other than hagfish, chondrichthyans, coelacanths and marine frogs. Osmoregulators typically have special organs (e.g. gills) that work to keep internal body fluids consistent, e.g. at ∼300–400 mosmol kg^{-1} in teleost fishes, obviating the need for organic osmolytes. This level was inherited by terrestrial vertebrates, which are typically at ∼300 mosmol kg^{-1}. However, there are major exceptions to this pattern; for example, in mammals (considered to be exemplary osmoregulators), kidney medulla cells osmoconform to the high osmotic concentrations in that organ's ECF. And, as will be discussed, teleosts in the deepest ocean may achieve osmoconformation, with high levels of TMAO.

Osmolyte properties: inorganic ions versus compatible solutes

A primary question that arose after the discovery of organic osmolytes is: why do osmoconforming cells use these 'expensive' organic solutes instead of inorganic ions to raise cellular osmolality? In particular, NaCl is essentially 'free' in some habitats. However, exposing living cells to external NaCl concentrations higher than

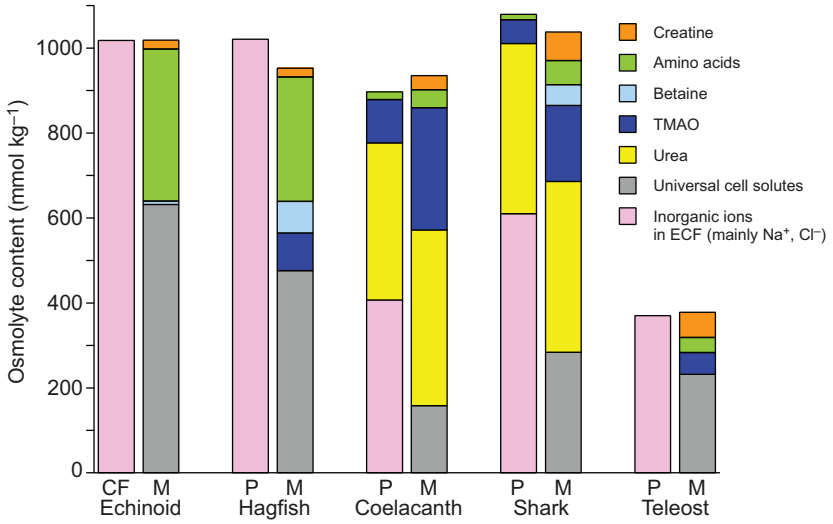

Fig. 2. Osmolyte content of plasma and muscles of marine organisms. Osmolyte concentration of body fluids in mmol l^{-1} and in muscles estimated as mmol kg^{-1} cell water for echinoid echinoderm *Echinus esculentus* (from Robertson, 1980), hagfish *Myxine glutinosa* and *Chimaera monstrosa* (from Robertson, 1976); coelacanth (from Lutz and Robertson, 1971); shark *Scyliorhinus canicula* (from Robertson, 1989); and teleost *Pleuronectes flesus* (from Lange and Fugelli, 1965). CF, coelomic fluid; P, plasma; M, muscle. Because these are whole-muscle extracts including ECF plus intracellular water, watery muscles will have more inorganic ions (universal cell solutes) than cells themselves (e.g. the echinoid).

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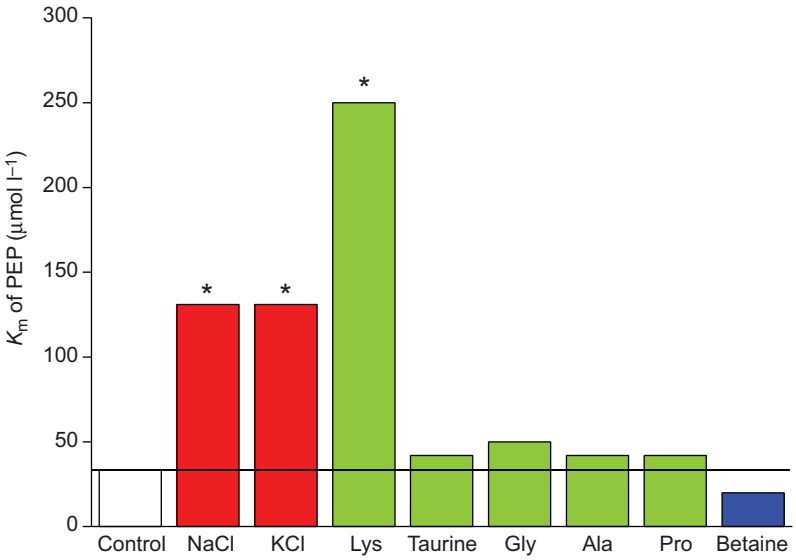

Fig. 3. Inhibitory and compatible solute effects on phosphoenolpyruvate (PEP) K_m of pyruvate kinase from a crab (*Pachygrapsus*). Data from Bowlus and Somero (1979). Molarities tested were: 100 mmol l^{-1} lysine; 400 mmol l^{-1}NaCl, KCl, taurine, glycine and betaine; 700 mmol l^{-1} alanine; 1 mol l^{-1} proline. Horizontal line represents control value. Note that NaCl and KCl are inhibitory (red) (*significantly inhibited as higher K_m values indicate weaker binding). Of the amino acids tested (green), lysine (a non-osmolyte) is highly inhibitory while all others show compatibility with no significant effect on K_m. The methylamine betaine (blue) tended to enhance binding.

normal, or to any hyperosmotic condition using impermeant solutes is deleterious, at least initially (Yancey et al., 1982; Yancey, 2005). Intracellular damage typically correlates with elevated intracellular ion concentrations (Burg et al., 2007). Often, up-regulation of chaperoning stress proteins is seen, indicating that cellular proteins are partly denatured by the high ion levels [e.g. salmon cells (Smith et al., 1999); nematodes (Lamitina et al., 2006); mammalian cells (Burg et al., 2007)].

In response to such stress, many cell types rapidly take up or release inorganic osmolytes to adjust cell volume, but usually only over a small osmotic range (Hoffmann et al., 2009; Voss et al., 2014). Cells that can adapt long-term usually switch to using organic osmolytes (Yancey, 2005; Burg and Ferraris, 2008). In part, this switch may be to minimize ion effects on membrane potentials. However, another reason is solute effects on macromolecules: Na^+, Cl^- and even K^+ at concentrations above normal are often quite disruptive of macromolecular functions (Fig. 3).

The first hypothesis for this switch was the 'compatibility' concept of Brown and Simpson (1972), extended by Clark and Zounes (1977), Wyn Jones et al. (1977), Bowlus and Somero (1979) and others. The hypothesis states that, in contrast to salts, organic osmolytes should not perturb macromolecules, even at high concentrations (Fig. 3). The perturbing effects of ions and the absence of negative effects of compatible osmolytes have usually been found to be similar with proteins from species and tissues with or without high levels of organic osmolytes. Thus, the effects of salts and organic osmolytes were proposed to be general features of protein–solute–water interactions, rather than intrinsic adaptations in proteins. Concentrations of compatible osmolytes can be varied with little effect on proteins. The use of taurine, glycine and other neutral amino acids in many marine animals has been explained by compatibility (Fig. 3).

High extracellular NaCl (500–1000 mosmol kg^{-1}) induces irreparable double-stranded breaks in DNA in cells from terrestrial nematodes and mammals, and marine flatworms, nemerteans, annelids, mollusks and crustacea clearly adapted to 1000 mosmol kg^{-1} (Dmitrieva et al., 2006). Breakage correlates with elevated intracellular inorganic ions, and cells that cannot compensate with organic osmolytes undergo apoptosis (Burg et al., 2007). Mammalian (renal) and marine invertebrate cells that use organic osmolytes thrive, even with unrepaired breaks (Dmitrieva and Burg, 2008), and it appears that they restrict breakage to 'gene deserts' – chromosomal regions with no genes. This may explain why osmolyte-using cells survive at high osmolalities despite persistent breaks (Dmitrieva et al., 2011). However, the mechanism for restricting breaks to these gene deserts is not known, nor is a causal connection with compatible osmolytes.

The compatibility hypothesis does not explain why there are dozens of different organic osmolytes. One reason is that they may not be fully interchangeable. Many cosolutes have potent and sometimes unique cytoprotective properties, such as antioxidation and stabilization of proteins, that go beyond simple compatibility. Micromolecules similar or identical to organic osmolytes are also accumulated by some organisms with numerous other environmental stressors (summarized in Tables 1 and 2). Conversely, osmolyes may be detrimental in the absence of an opposing perturbant (e.g. by overstabilizing proteins), making the term 'compatible' misleading. The complexity of potential and actual properties led Gilles (1997) to propose the term 'compensatory' rather than 'compatible' solutes.

Osmolyte properties: urea versus methylamines as counteracting solutes

The situation for urea seems to be different than for 'classic' compatible solutes. Firstly, while most osmolytes are much higher inside cells, partly as a result of low membrane permeability, urea concentrations are about equal in cells and ECFs (Fig. 2) because it equilibrates readily across most membranes via facilitated urea transporters (McDonald et al., 2006) or by simple diffusion through the phospholipids depending on composition (Poznansky, et al., 1976). Rapid equilibration may explain why urea osmotic shock does

Table 1. Cytoprotective roles of osmolytes and other cosolutes through stabilization of macromolecules and membranes

Stabilizing property	Cosolutes in nature
Counteract urea inhibition	Methylamines, especially TMAO
Increase thermostability (thermolytes)	Trehalose, anionic polyols, sorbitol, ectoines
Protect in freezing (cryoprotectants)	Carbohydrates, phosphodiesters
Preserve in anhydrobiotic (dry) state	Carbohydrates, especially trehalose
Counteract inorganic ion inhibition	Methylamines
Counteract hydrostatic pressure (piezolytes)	TMAO, see text for other possibilities

TMAO, *N*-trimethylamine oxide. Modified from Yancey (2005).

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Table 2. Other beneficial properties of cosolutes

Cytoprotective property	Cosolutes in nature
Antioxidation	Polyols, hypotaurine, taurine*
Redox/cofactor balancing	Proline, glycerol, β-alanine betaine
Sulphide detoxification/storage	Hypotaurine
Sulphate detoxification	Choline-*O*-sulphate
Energy reserve	Glucose, trehalose etc.
Predator repellent	DMSP, trans-hydroxyprolinebetaine
Ca^{2+} modulation	Taurine
Buoyancy	Methylamines, urea
Rapid transmembrane equilibration	Urea, glycerol

*Taurine protection from radicals may be due to enhancement of electron transport system, Jong et al. (2012).
DMSP, dimethylsulphoniopropionate. Modified from Yancey (2005).

not cause DNA breaks in mammalian cells (Kültz and Chakravarty, 2001; Burg et al., 2007), and may be one benefit of using it as both an intra- and extracellular osmolyte. Secondly, although urea is cytoprotective in some ways, it seems an odd evolutionary selection for an osmolyte because, at the concentrations found within kidneys and chondrichthyans' urea, it is not 'compatible': it destabilizes macromolecular structures and inhibits many functions (Fig. 4). Importantly, these negative effects are also seen with many (though not all) chondrichthyan and mammalian proteins (Yancey and Somero, 1979, 1980). These fishes and the mammalian kidney clearly survive indefinitely with high internal urea concentrations. There are three different hypotheses to explain this, each with supporting evidence. (1) Intrinsic urea resistance: some proteins appear to be insensitive to urea's effects; e.g. recently Feige et al. (2014) analyzed a shark immunoglobin (Ig) and found one more salt bridge and a larger hydrophobic core compared with a homologous human Ig, explaining the shark protein's greater resistance to urea destabilization. These features were used to engineer a human Ig that was also more urea-resistant. See Yancey (2005) for other examples. (2) Intrinsic urea requirement: at least a few chondrichthyan proteins

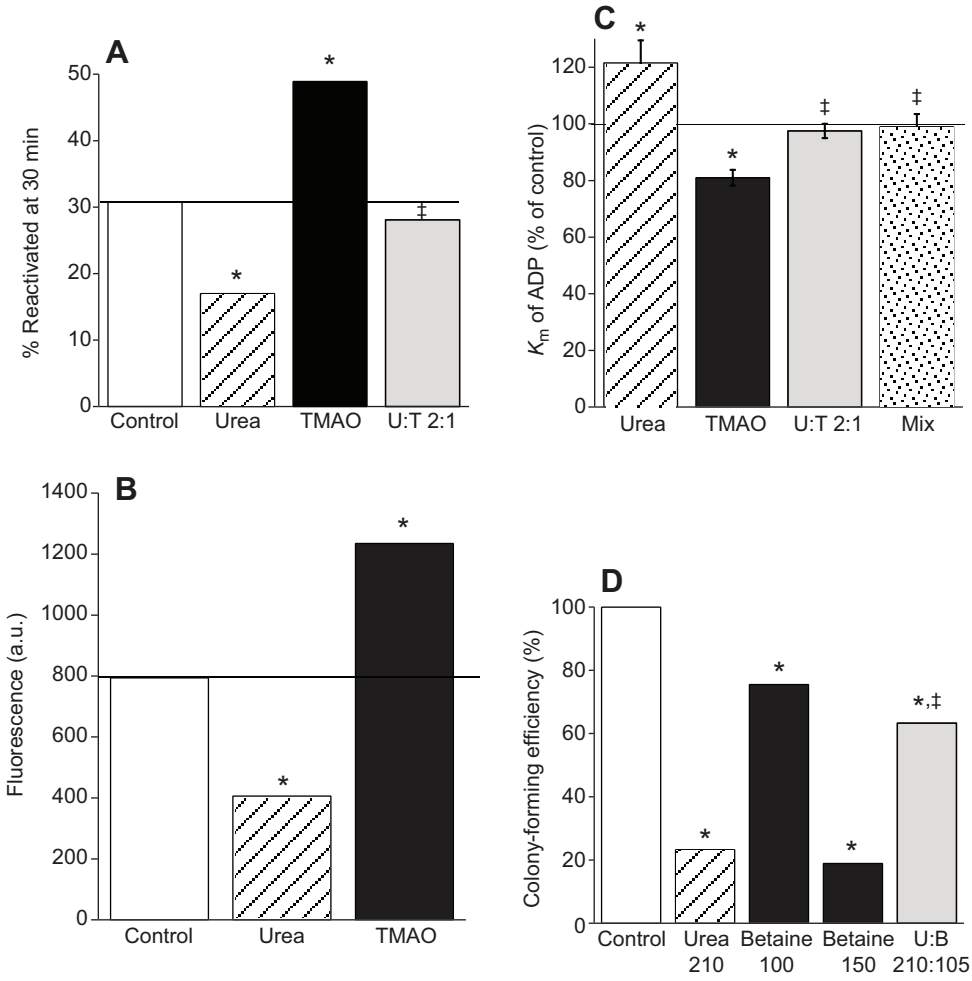

Fig. 4. Counteracting effects of urea and methylamines. Horizontal bars indicate control level. For all graphs, * indicates significantly different to control; ‡ indicates significant counteraction. (A) Original discovery of TMAO's chaperoning ability: extent of refolding at 30 min in a physiological buffer of white shark A_4-lactate dehydrogenase (LDH) after acid denaturation, without (control) and with 400 mmol l^{-1} urea and/or 200 mmol l^{-1} TMAO as indicated; U:T 2:1 was 400:200 mmol l^{-1}. Data plotted from Yancey and Somero, 1979. (B) Recent example of similar phenomenon: polymerization of mammalian (rabbit) actin measured by fluorescence of pyrene-labeled actin, without and with urea (500 mmol l^{-1}) or TMAO (250 mmol l^{-1}) as indicated; counteraction (not shown) was optimal at 2:1 urea:TMAO. Data plotted from Hatori et al., 2014. (C) ADP K_m values for pyruvate kinases (PK): from muscle of elasmobranch (*Urolophis haleri*) with 400 mmol l^{-1} urea, 200 mmol l^{-1} TMAO and/or 'Mix' [400 mmol l^{-1} urea, 65 mmol l^{-1} TMAO, 55 mmol l^{-1} sarcosine, 30 mmol l^{-1} betaine and 50 mmol l^{-1} β-alanine, the osmolyte composition of one skate muscle (King and Goldstein, 1983)]. Data from Yancey and Somero, 1980. (D) Growth of mammalian renal cells (Madin–Darby canine kidney), measured as colony-forming efficiency (CFE; the number of successful colonies relative to number of cells seeded in a growth flask) as % of control in normal medium (white bar). Other bars show growth with urea and/or betaine as indicated (values in mmol l^{-1}). Both betaine and urea alone were inhibitory, but the mixture (U:B) revealed counteraction. Data plotted from Yancey and Burg, 1990.

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appear to need urea for proper function. For example, K_m of pyruvate values for muscle A_4-lactate dehydrogenases (LDHs) in the absence of urea were lower for chondrichthyan homologues compared with those of other vertebrates. However, addition of 400 mmol l^{-1} urea increases the K_m to values similar to those for LDHs in other vertebrates, suggesting that the chondrichthyan enzymes have evolved intrinsically higher pyruvate affinities that urea reduces to normal values (Yancey and Somero, 1978). (3) Extrinsic counteracting osmolytes: the destabilizing effects of urea may be counteracted by osmolytes that stabilize proteins. This extrinsic adaptation in the cellular milieu may be the primary adaptation. While urea is destabilizing, other osmolytes typically promote folding of polypeptides into native states and subunit assembly of multimeric proteins. By doing so, stabilizing cosolutes can offset many effects of urea and other destabilizing agents (Table 1).

One of the first examples of physiological stabilization discovered was that of methylamines in chondrichthyans. Opposite effects of urea and methylamines on both protein structural stability and enzyme kinetics were found. Moreover, effects were additive such that they canceled each other, most effectively at about a 2:1 urea:TMAO ratio – similar to chondrichthyan cellular levels (roughly 400:200 mmol l^{-1}; Fig. 2). This ratio is so important that chondrichthyans maintain it in starvation (Treberg and Driedzic, 2006; Kajimura et al., 2008). Termed 'counteracting osmolytes', this phenomenon (like compatibility) manifests whether a protein is from a urea-accumulating organism or not (Yancey and Somero, 1979, 1980). Numerous studies continue to demonstrate urea–methylamine counteraction for proteins from many taxa, including bacteria. Counteraction occurs in more complex systems as well, such as muscle fiber contraction (Altringham et al., 1982) and renal cell growth (Yancey and Burg, 1990). Examples are shown in Fig. 4.

Stabilizing properties of cosolutes led to the term 'chemical chaperone', as a counterpart to the term 'molecular chaperone' for stress proteins such as heat-shock proteins (hsp); and led to proposals to use them to treat protein-folding diseases (Welch and Brown, 1996; Bennion et al., 2004; Zhao et al., 2007; Gong et al., 2009; Jia et al., 2009; Seeliger et al., 2013) and to enhance protein stability in biochemical and pharmaceutical preparations (e.g. Marshall et al., 2012). That protein conformation is key to many counteracting effects *in vivo* is revealed in studies on both chaperone types. For example, in dogfish-shark choroid plexus, hsp70 accumulation was reduced two-fold following acute heat stress in the presence of physiological TMAO concentrations (Fig. 5). Since hsp70 expression is induced by protein denaturation, this suggests that TMAO reduces such denaturation (Villalobos and Renfro, 2007).

TMAO is usually a stronger stabilizer than other methylamines including betaine. Trimethylamines, in turn, are stronger than di- and mono-methylamines (e.g. sarcosine). Fig. 6 illustrates this for thermal and chemical stresses on a model protein. TMAO's superb stabilizing abilities may explain why TMAO is the dominant non-urea osmolyte in most ureosmotic marine fishes. Moreover, some non-methylamine organic osmolytes are more compatible in the classic sense; for example, glycine, which is common in marine invertebrates but not urea-rich cells, can stabilize many proteins against temperature but shows no ability to counteract the effects of urea (Khan et al., 2013a) or pressure (Yancey and Siebenaller, 1999) on proteins. These widespread taxon-independent effects must involve universal protein–water–solute mechanisms.

The counteraction of urea and methylamine is not unique to chondrichthyans. The coelacanth also osmoconforms with urea: TMAO ratios at about 2:1 (Fig. 2). Methylamine osmolytes also

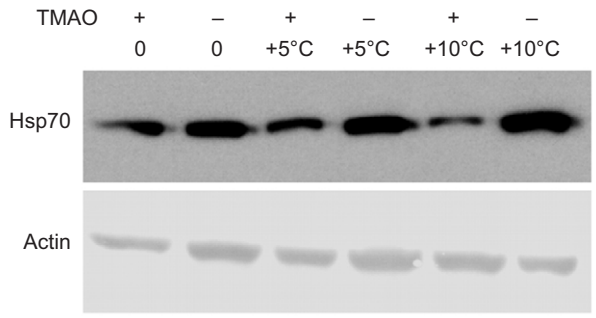

Fig. 5. Hsp70 expression (detected in immunoblots) in isolated shark (*Squalus acanthias*) lateral choroid plexus with and without heat shock and/or TMAO. TMAO line indicates presence (+) or absence (–) of 72 mmol l^{-1} TMAO; second line indicates incubation at 13.5°C for 7.5 h (0), at 18.5°C for 6 h plus recovery at 13.5°C for 1.5 h (+5°C), or 23.5°C for 1 h plus recovery at 13.5°C for 1.5 h (+10°C). Hsp70 immunoblots show induction of Hsp70 by heat shock that is reduced with TMAO present. Actin immunoblots, serving as a control, show no change in expression. From Villalobos and Renfro (2007).

occur with urea in the mammalian renal medulla: a discovery inspired by the chondrichthyan system (Bagnasco et al., 1986). Medullary cells, which often have high urea content as a result of the urinary concentrating mechanism, have close to a 2:1 ratio of urea to the methylamines glycerophosphorylcholine (GPC) and betaine (Fig. 1), with GPC regulated in parallel with urea (Peterson et al., 1992). These methylamines can counteract the effects of urea on proteins (Burg et al., 1996).

Finally, TMAO does not counteract urea's effects on all proteins (Yancey et al., 1982; Mashino and Fridovich, 1987). Such proteins in high urea concentrations may have evolved intrinsic urea requirements or resistance, as noted earlier. Or, they may receive urea protection by non-osmolyte extrinsic interactions *in vivo*; e.g. thornback-ray phosphofructokinase is inactivated *in vitro* by low urea but not protected by TMAO. It is, however, much less sensitive to urea when complexed with actin filaments (Hand and Somero,

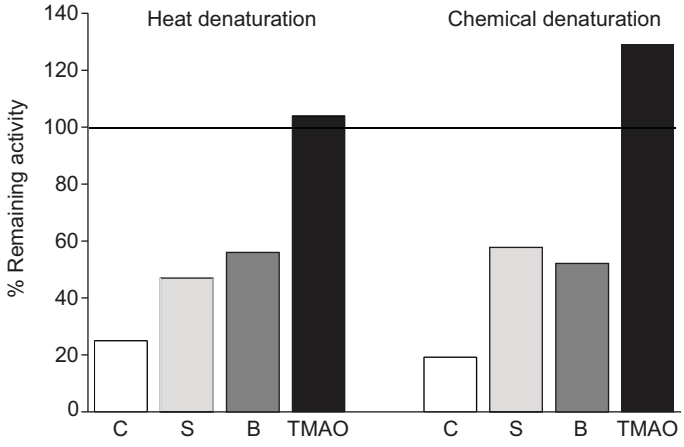

Fig. 6. Stabilizing and counteracting effects of methylamines against thermal and chemical denaturation. Horizontal bar at 100% indicates activity prior to treatment. Heat denaturation: trypsin was denaturated to zero activity by 10 min heating to 60°C alone or in the presence of 3.5 mol l^{-1} methylamines indicated, then given substrate for activity measurements. Chemical denaturation: trypsin was denatured to zero activity by 30% trifluoroethanol alone or in the presence of 3.5 mol l^{-1} methylamines as indicated, then given substrate for activity measurements. C, choline chloride; S, sarcosine; B, betaine; TMAO, *N*-trimethylamine oxide. Data plotted from Levy-Sakin et al. (2014).

1982). Similarly, activity of myosin ATPase from requiem shark (*Triakis scyllia*) is just as sensitive to urea as is carp myosin, but sensitivity is eliminated by actin binding (Kanoh et al., 1999). See Yancey (2005) for other examples.

Osmolyte properties: salts versus methylamines as counteracting solutes

Methylamines can sometimes offset perturbing salt effects, as discovered independently by Clark and Zounes (1977) with marine invertebrate enzymes and Pollard and Wyn Jones (1979) with plant enzymes, ranked by methylation: betaine(trimethylglycine) >dimethylglycine>sarcosine(methylglycine)>glycine.

Porcine LDH has weakened substrate binding in the presence of elevated KCl that can be counteracted by TMAO, which, however, has no effect on binding by itself (Desmond and Siebenaller, 2006). Counteraction occurs also with more complex systems; e.g. TMAO reverses salt disruption of barnacle muscle architecture (Clark, 1985) and inhibition of force generation in mammalian muscle fibers (Nosek and Andrews, 1998) more than betaine does.

Organic osmolytes as thermolytes

As chemical chaperones, most organic osmolytes stabilize proteins against thermal denaturation, although the degree varies by osmolyte type and often requires non-physiologically high concentrations. Those shown to work at physiological levels are sometimes called ‘thermolytes’ (Table 1). Such solutes, including charged phosphorylated sugars (Fig. 1), are best known in hydrothermal-vent Archaea and bacteria, and can also stabilize mammalian proteins at very high temperatures (Santos et al., 2011). Among Eukarya, there is little evidence for thermolytes beyond yeast trehalose and sorbitol in whiteflies (Wolfe et al., 1998). Regarding TMAO and hsp70 for shark tissue noted above (Fig. 5), it is not known whether such a thermolyte effect is important in a living chondrichthyan.

Co-evolution and the yin and yang of stabilization

An often-overlooked aspect of stabilization properties is that stabilization is not always beneficial. In particular, the term ‘counteracting’ applies to both stabilizers and destabilizers; e.g. while urea is clearly not compatible, many other cosolutes are also not compatible in the original sense. As Somero (2003) discusses, enzymes must not be too stable because they need to fluctuate among a range of conformations necessary for both binding and catalysis. Cosolutes and stressors, such as temperature, may have opposite effects on these functions. Protein structures therefore evolve not only to flex properly at an organism’s normal temperatures, but also in its cosolute milieu, which is also subject to evolutionary selection. Such co-evolution among macro- and micromolecules is still poorly understood.

Regarding counteracting solutes, high TMAO in the absence of urea may stabilize enzymes against denaturation while making them too rigid to flex properly, even leading to non-functional protein aggregates (Yancey et al., 1982; Devlin et al., 2001). Indeed, occasionally urea and TMAO exhibit ‘reversed’ effects: e.g. TMAO inhibition and urea enhancement of chondrichthyan and mammalian LDH kinetics (Yancey and Somero, 1980; Zhadin and Callender, 2011) and substrate binding by chondrichthyan 5′-monodeiodinase (Leary et al., 1999). The stability of rabbit actomyosin is enhanced by TMAO in opposition to urea as usual, but sliding motor activity and ATPase rate are suppressed by TMAO (Kumemoto et al., 2012).

Thermostabilization ability can also be detrimental. For a variety of fish LDHs, cosolutes including glycerol and TMAO enhanced thermostability while simultaneously reducing catalytic rates (Fields et al., 2001). In the shark study on TMAO versus hsp70 (Fig. 5), heat-stressed tissue suffered more functional inhibition with TMAO and thus low hsp70 induction, than in TMAO’s absence, with high hsp70 induction (Villalobos and Renfro, 2007). Trehalose (Fig. 1), another strong stabilizer, also reveals perturbing features. For shark ornithine transcarbamolase, trehalose protected the enzyme from thermal denaturation but simultaneously reduced its catalytic rate, an effect attributed to reduced protein flexibility (Bellocco et al., 2005). Similarly, high trehalose concentrations in yeast, induced by temperature stress, protect yeast enzymes at high temperatures, but inhibit them at room temperature. This was termed the ‘the yin and yang of trehalose’ (Singer and Lindquist, 1998), a phrase that applies equally well to urea and methylamines.

Negative effects of TMAO in the absence of a destabilizer may explain recent findings that an elevated level of TMAO in human blood is associated with cardiovascular disease (Tang et al., 2013). Indirect evidence suggests that TMAO in some unknown manner enhances atherosclerotic plaques, although TMAO may be correlative, not causative.

Physicochemical mechanisms of (de)stabilization

The compatible, (de)stabilizing and counteracting properties of inorganic ions (i.e. the Hofmeister ion series; Xie and Gao, 2013) and organic osmolytes are often universal. Therefore, they may involve broad water–solute–macromolecule interactions. These universal properties are only partly understood, but details continue to emerge on organic cosolutes.

Urea

Hypotheses for destabilization fall into two categories: direct binding and indirect effects through alteration of water structure. There is some evidence for both (Bennion and Daggett, 2004), but effects on water are weak and there is no consistent correlation between urea’s effects on water structure and its denaturing abilities (Pazos and Gai, 2012). Instead, most evidence favors the dominance of direct binding, specifically to peptide intra-backbone hydrogen bonds crucial to protein stability (Auton et al., 2007; Lim et al., 2009; Hua et al., 2008) and to some amino-acid side groups (Canchi and García, 2011). Salt ions also bind to charged amino acids in proteins. Binding leads to the phenomenon termed ‘preferential interaction’ (Timasheff, 2002), which will lead to unfolding of macromolecules as that maximizes favorable surface interactions (Fig. 7A, right).

Methylamines

Unlike urea, stabilizers exhibit a tendency to be excluded from a protein’s hydration layer, the shell of bound water molecules around the surface. This ‘preferential exclusion’ (Timasheff, 2002), creates an entropically unfavorable order of high and low solute concentrations and more- and less-ordered water. Proteins reduce this order by minimizing their exposed surface areas by folding more compactly (Fig. 7A, middle), especially α-helical secondary structure (Fig. 7B). Binding of ligands to active sites will also be favored if this involves loss of ordered bound water (Fig. 7A, middle).

Hypotheses continue to be proposed to explain the forces causing preferential exclusion, which may vary among cosolute classes (reviewed by Fields et al., 2001). Those regarding TMAO involve enhanced water structure, for which there is now considerable

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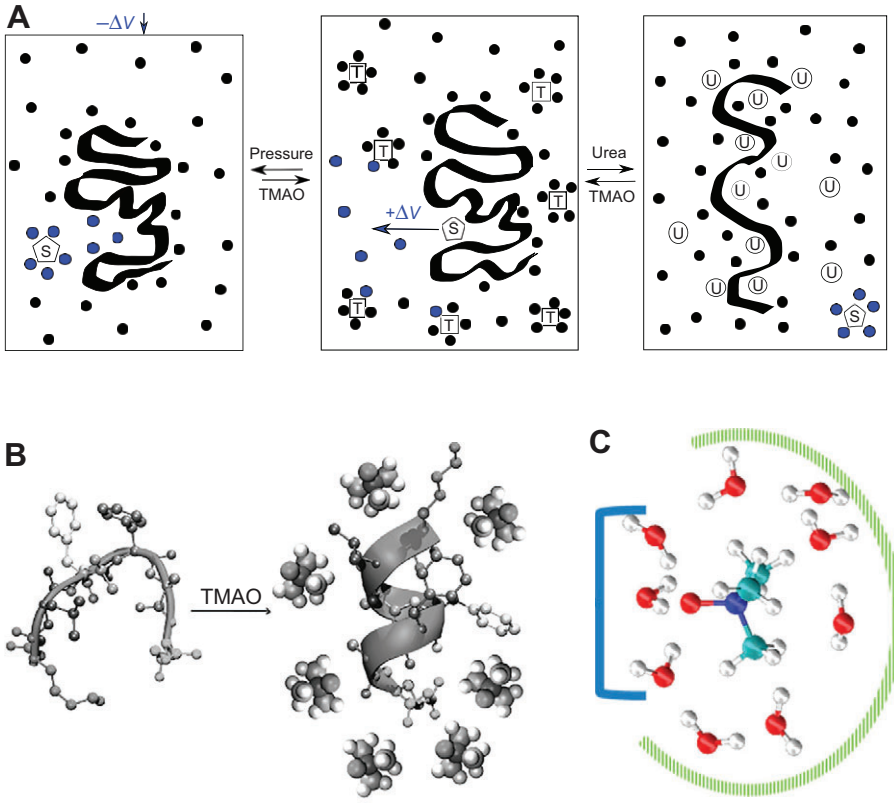

Fig. 7. Models of the effects of solute and pressure on protein folding. (A) Urea (U) binds to the peptide backbone and enhances unfolding (right box) since that maximizes favorable binding sites. Addition of TMAO (T), excluded from the protein hydration layer presumably because of its own structured water layers (spheres around T), favors folding and substrate (S) binding, since that reduces the total order (middle). Pressure can favor unfolding when this involves a decrease in volume ($-\Delta V$, left box), or, conversely, a volume expansion ($+\Delta V$, middle box) as water molecules are released from the folding protein into bulk water. Small spheres represent water molecules. Modified from Yancey (2005). (B) Model of unfolded protein driven to form an α-helix in TMAO solution. Reprinted with permission from Cho et al. (2011). (C) Model of water molecules around TMAO molecule (middle). Blue sphere, N; aqua spheres, C; red spheres, O; white spheres, H. Water molecules on the left, indicated by bracket, are held by hydrogen bonds with TMAO oxygen; those on the right, indicated by green 3/4 circle, are organized in a clathrate structure from TMAO's methyl groups. Reprinted from Larini and Shea (2013).

evidence. TMAO's osmotic coefficient, 1.19 (Robertson, 1989), is well above an ideal colligative value (1.0). Atomistic simulations indicate that not only does TMAO's oxygen hydrogen-bond more strongly to water than water–water hydrogen bonding (Rösgen and Jackson-Atogi, 2012; Doi et al., 2014), but the methyl groups of TMAO also induce a restricted water clathrate network (Fig. 7C). This enhances local water–water hydrogen bonding but, in turn, weakens water's hydrogen bonding to proteins (Ma et al., 2014).

How does enhanced water structure specifically affect protein folding? Bolen and colleagues have shown that unfavorable interactions between TMAO–water complexes and peptide backbones explain the strong exclusion of TMAO–water, and, therefore, enhancement of protein folding. In essence, the peptide backbone does not dissolve well in TMAO–water solutions compared to pure water, a phenomenon termed 'osmophobicity' (Bolen and Baskakov, 2001; Street et al., 2006). Many osmolytes exhibit osmophobicity, but TMAO is the strongest stabilizer compared with other common osmolytes (Fig. 8).

Importantly, the thermodynamics of peptide–osmolyte interaction reveal a ΔG for TMAO that is unfavorable for peptide backbone exposure to TMAO–water that is about double the favorable ΔG for urea (Fig. 8); in other words, TMAO's folding and urea's unfolding effects with respect to the peptide backbone cancel thermodynamically at about 2:1. This explains their universal (de)stabilizing roles; deviations from 2:1 are likely due to side-group interactions with different proteins (Auton et al., 2011).

A problem with preferential exclusion hypotheses is that different cosolutes can behave differently despite all exhibiting exclusion. For example, for the assembly and stability of HIV-1 capsid protein, methylamines greatly accelerated assembly but destabilized the protein during thermal denaturation, while polyols and sugars had opposite effects (Lampel et al., 2013). These effects are not understood.

Macromolecular crowding

Another biophysical issue about the cellular milieu is termed 'cellular' or 'macromolecular crowding': high concentrations of macromolecules reduce the availability of water for other cell molecules in the solution. This reduces diffusion, increases activity coefficients, favors inappropriate protein aggregations, and likely limits the total amount of solutes that can be packed into a cell (Levy et al., 2012). Osmolytes may help: for example, the mammalian renal osmolytes betaine, taurine and myo-inositol reduced crowding and thus formation of mRNA stress aggregates in osmotically stressed renal cells (Bounedjah et al., 2012). However, another study with a model amino acid suggests that TMAO stabilizes

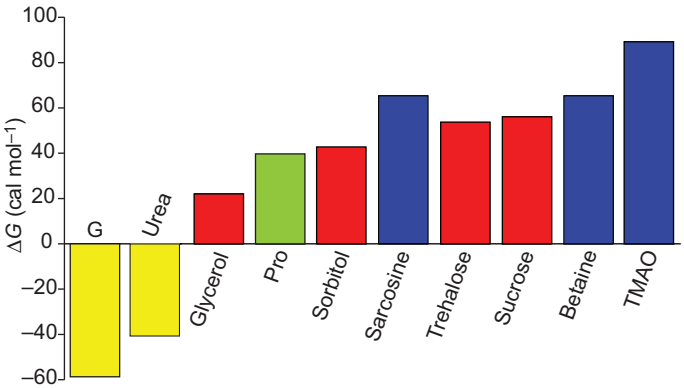

Fig. 8. Thermodynamic ΔG (cal mol^{-1}) of transfer of peptide backbone to 1 mol l^{-1} of indicated osmolyte. Yellow indicates denaturants (G, guanidinium); red, carbohydrates; green, amino acid (Pro, proline); blue, methylamines. Note TMAO's positive (unfavorable) value at +89 is about double the favorable negative value for urea –41 ΔG; i.e. TMAO's folding effects are twice as effective as urea's unfolding effects, thermodynamically canceling about 2:1. Values plotted from Street et al. (2006).

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proteins by increasing crowding (Ma et al., 2014). Thus, the role of cosolutes in crowding is unsettled.

Synergism

Although most studies show that the effects of methylamine and urea on proteins are independent (e.g. Marcelo et al., 2007; Holthauzen and Bolen, 2007), other studies suggest some synergy. Many brackish and euryhaline rays have lower TMAO and higher betaine, sarcosine and β-amino acid concentrations than in stenohaline marine chondrichthyans (King and Goldstein, 1983). Individually, the latter osmolytes can counteract urea but not as effectively as TMAO (Yancey and Somero, 1979, 1980). However, in a mixture there appears to be synergism (Fig. 4D, skate mix). A study with a model peptide revealed that betaine and urea interact synergistically through hydrogen bonding and van der Waals interactions, making betaine a stronger stabilizer and urea a weaker destabilizer in combination (Kumar and Kishore, 2013). Other studies showed synergistic interactions between amino acid and sugar stabilizers in thermal denaturation (Khan et al., 2013b) and between sucrose and glycine in pressure inactivation of an enzyme (Kidman and Northrop, 2005).

Metabolic and other protective properties

Organic osmolytes have other roles not related to broad protein (de)stabilization. Summarized in Table 2, these will only be covered briefly here. These 'non-chaperone' properties tend to be unique to each type, partly explaining the complex and different mixtures of osmolytes found among species and organs. Taurine is perhaps the most complex in terms of possible functions. It is thought to have many (poorly understood) functions: 'osmotic pressure, cation homeostasis, enzyme activity, receptor regulation, cell development and cell signaling' and 'indirect regulator of oxidative stress' (Schaffer et al., 2010). Some of these roles may explain why taurine concentration is often highest in vertebrate hearts and brains (Yancey, 2005).

Many other osmolytes are also thought to have antioxidant properties (Table 2). For example, hypotaurine (Fig. 1), at osmotically significant levels in mammalian semen, can react with oxygen radicals to become taurine (Holmes et al., 1992). Hypotaurine may also protect from sulfide toxicity: it is found along with thiotaurine at high levels in marine invertebrates at hydrothermal vents and cold seeps. Vents and seeps emit high quantities of H_2S, a gas that is toxic to animals but is a primary energy source for thiotrophic microorganisms. Initially, these taurine derivatives were found in animals (vestimentiferan tubeworms, vesicomyid clams) that house sulfide-oxidizing microbial endosymbionts. These solutes are osmolytes because they create much of the cell osmolality, effectively replacing common osmolytes (e.g. taurine, glycine). However, Pruski et al. (2000) hypothesized their primary role to be detoxification of sulfide radicals and/or for sulfide storage – for future use by symbionts – nontoxically, as follows: (hypotaurine) $^{+}NH_3$-CH_2-CH_2-SO_2^-+HS→$^{+}NH_3$-CH_2-CH_2-SO_2^--SH (thiotaurine).

As evidence for the storage function, hypotaurine is high in all tissues in these animals, but thiotaurine has been found only in non-trace amounts in symbiont-bearing tissues: gills in vesicomyid clams and trophosome in vestimentiferans. As evidence of a detoxification role, vent gastropods and *Paralvinellid* polychaetes without endosymbionts also have both hypotaurine and thiotaurine as major osmolytes. Both *in situ* and laboratory studies show that levels of thiotaurine in endosymbiont- and non-endosymbiont-bearing animals correlate well with environmental sulfide exposure (Rosenberg et al., 2006; Brand et al., 2007; Yancey et al., 2009).

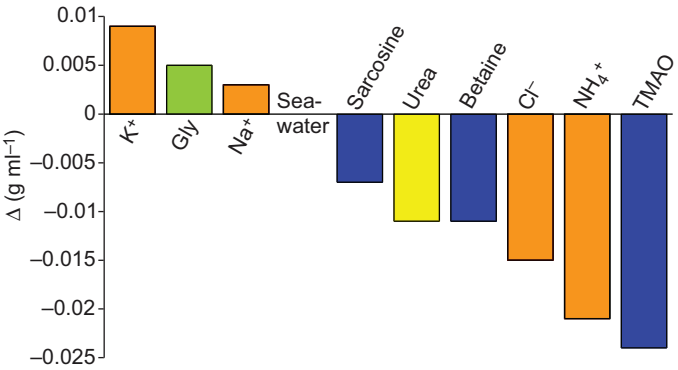

Fig. 9. Densities of 1 mol l^{-1} solutions of the indicated solutes as change (Δ) from average seawater at 1.024 g ml^{-1} (set at 0). Positive bars indicate lighter than seawater, negative bars indicate heavier; blue, methylamines; yellow, urea; orange, inorganic ions; green, amino acid (Gly, glycine). Data plotted from Withers et al., 1994a.

Buoyancy

Another potentially important role, not involving (de)stabilization of macromolecules, is buoyancy: many organic osmolytes, particularly TMAO, betaine and urea, are less dense than seawater and common physiological ions such as Na^+ and K^+ (Fig. 9; Withers et al., 1994a). Density is a function of partial molal volume and molecular weight; TMAO, for example, has a positive molal volume that more than offsets the weight it adds to solution, while K^+ has the opposite effect. Withers et al. (1994a) proposed that urea and TMAO were selected as osmolytes, in part because these density properties provide buoyancy for chondrichthyans, which lack swimbladders. Overall, TMAO and urea were calculated to an estimated 6 g l^{-1} of lift in one shark (Withers et al., 1994b).

Hydrostatic pressure in the deep sea: intrinsic and extrinsic adaptations

We finish with a look at intrinsic and extrinsic adaptations to hydrostatic pressure. The deep sea and the subsurface biosphere are the largest habitats on Earth, and yet remain 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.

The physical environment has long been recognized as a critical factor for organisms of the deep ocean (Mills, 1983; Anderson and Rice, 2006). Ecologists subdivide the ocean into depth zones defined in Table 3, with the deep sea generally considered to start at 200 m. Across these zones there is a strong vertical pattern to the distribution of animals (e.g. Pearcy et al., 1982; Haedrich, 1997; Carney, 2005), and the physical parameters of these zones have been implicated in contributing to these distributions (e.g. Siebenaller and Somero, 1978; Carney, 2005). Pressure increases 1 atm (=0.101325 MPa) for every

Table 3. Benthic and pelagic zonation in the ocean

Benthic	Depth in meters	Pelagic	Depth in meters
Sublittoral	0–200	Epipelagic	0–200
Bathyal	200–3000	Mesopelagic	200–1000
Abyssal	3000–6000	Bathypelagic	1000–3000
Hadal	>6000	Abyssopelagic	3000–6000
		Hadopelagic	>6000

Approximate depth ranges are shown (Angel, 1997; Gage and Tyler, 1991 and Marshall, 1979). Pressure increases 0.101 mPa (=1 atm) for every 10 m increase in depth.

10 m depth increase (Saunders and Fofonoff, 1976). Pressures experienced by organisms range from 0.1 MPa at the surface to 111.5 MPa at 11,000 m (Table 3); at the average depth of the ocean (3800 m) the pressure is approximately 38.5 MPa. Deep-sea temperatures are approximately 2 to 4°C and temperatures are relatively constant at depths greater than 1000 m (Pickard and Emery, 1990). Depending upon its life history and depth distribution, a species or individual may experience not only high hydrostatic pressure, but also variable pressure.

Both temperature and hydrostatic pressure can affect ligand binding and the rates of catalysis, and will differentially affect the stabilities of the noncovalent chemical bonds that stabilize higher orders of macromolecular structure, including that of proteins and membranes (e.g. Johnson et al., 1974; Jaenicke, 1981; Somero, 1995; Mozhaev et al., 1996; Cossins and Macdonald, 1989). Problems may arise in regulating and coordinating metabolism because different components may be affected to different degrees or directions.

Intrinsic adaptations to pressure

The bases of pressure effects on biochemical reactions and equilibria are the volume changes of the solute–solvent system. The effect is dependent on the sign and magnitude of the volume change (Johnson et al., 1974; Siebenaller and Somero, 1989). Volume changes may derive, for example, from changes in the degree of hydration of the system components, alteration in the packing efficiency of acyl chains in membranes or the amino acid side chains in proteins, the electrostriction of water (e.g. Siebenaller, 1991) and at denaturing pressures, water penetration into the hydrophobic interior of proteins (e.g. Imai and Sugita, 2010). Membranes and membrane-associated processes are among the most sensitive to hydrostatic pressure (Siebenaller and Garrett, 2002). As few as tens of atmospheres of pressure can disrupt the function of enzymes from shallow-living species (Siebenaller, 1987). Pressures typical of the depths organisms inhabit in the ocean are capable of disrupting enzymatic reactions. Deep-sea species typically have enzymes and membranes that are more resistant to increased pressure than their shallow-water counterparts (Siebenaller, 2010). Below, we discuss examples of the effects of pressure in deep-living organisms.

Dehydrogenases

Skeletal muscle-type lactate dehydrogenase (A_4-LDH) orthologs isolated from white skeletal muscle of deep-sea (common at depths greater than 550 m) and shallow-living teleost fishes have been compared (Fig. 10). At 5°C, the apparent Michaelis constant (K_m) values of both the substrate (pyruvate) and coenzyme [reduced nicotinamide adenine dinucleotide (NADH)] (Siebenaller and Somero, 1978, 1979) are increased by moderate pressures (Fig. 10). The increase is greater in the orthologs from shallow-occurring species (Siebenaller and Somero, 1978, 1979). The K_m of the coenzyme is particularly sensitive to pressure perturbation in shallow-living species. Adaptation to the moderate environmental pressures characteristic of the bathyal habitat appears to have been selected. Convergent evolution is seen in A_4-LDH orthologs from four different fish families that have independently evolved resistance to pressure perturbation. At *in situ* pressures, the K_m values are conserved among species. The A_4-LDH orthologs of cold-adapted shallow-living species are not necessarily pre-adapted for function at high pressure. In contrast to A-type LDH homologs, recombinant heart-type LDH-B homologs from a deep- and a more shallow-living gadiform teleost, overproduced in *E. coli*, displayed no difference in the pressure sensitivity of apparent K_m values for NADH and pyruvate. The values, although differing between the species, were unaffected by 75 MPa of pressure at 5°C (Brindley et al., 2008).

A pattern similar to the to A-type LDH homologs is seen in the K_m values of the coenzyme for three other dehydrogenases, cytoplasmic malate dehydrogenase-1 (MDH-1), cytoplasmic malate dehydrogenase-2 (MDH-2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Siebenaller, 1984b, 1987) from deep- and shallow-living teleosts (Fig. 10). The orthologs from

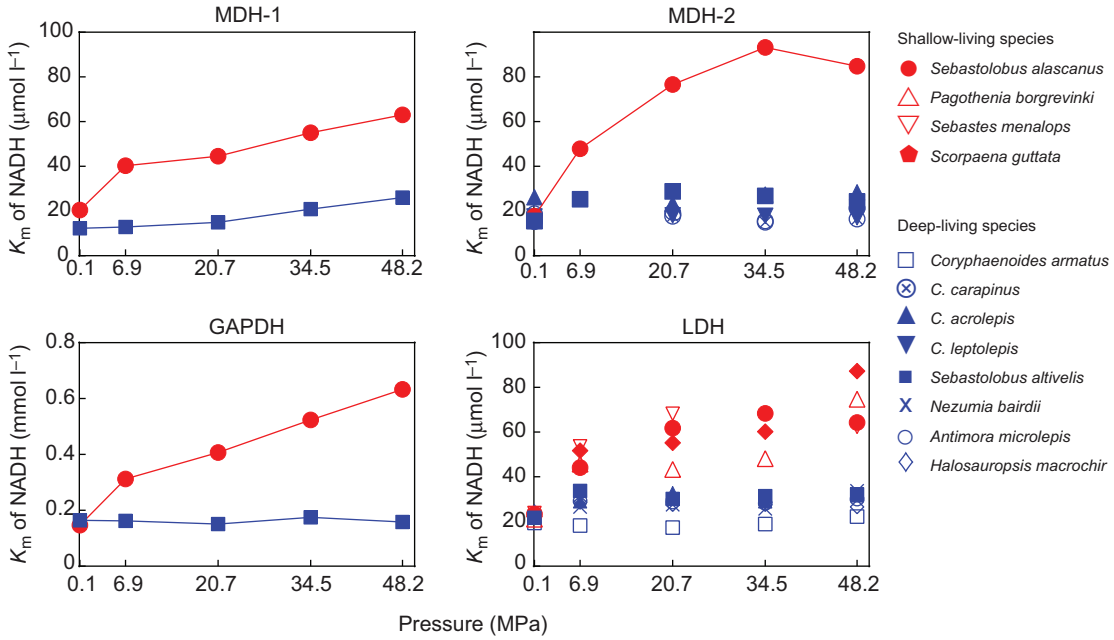

Fig. 10. Effects of pressure on K_m of coenzyme values for NAD-dependent dehydrogenases purified from white muscle tissue of deep- and shallow-living teleost fishes. Four families of deep-sea fish are represented. Determinations were made at 5°C. Species common at depths greater than 550 m are considered deep-living. The pressure range tested encompasses the pressures from the surface to 4750 m. Modified from Siebenaller (1987, 2010).

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the deeper-living species are more resistant to pressure perturbation. Dahlhoff and Somero (1991) found a similar pressure tolerance for cytosolic malate dehydrogenase orthologs from deep- and shallow-living invertebrates (Fig. 11). These dehydrogenases displayed a conservation of K_m of coenzyme values similar to that observed for fishes; deep-living species had enzymes that were minimally affected by elevated pressures.

Increased pressure affects the stability of teleost A_4-LDH (Hennessey and Siebenaller, 1985, 1987; Nishiguchi et al., 2008) and B_4-LDH (Brindley et al., 2008). The pressures that cause denaturation are much higher than *in situ* pressures. Nonetheless, orthologs of deeper-living species are more resistant to denaturation by high pressure than are the orthologs of more shallow-living teleosts. This difference in pressure stability is also reflected in susceptibility to proteolysis. The A_4-LDH homologs of deep-sea fishes are less sensitive to pressure denaturation (Hennessey and Siebenaller, 1985, 1987) and also less susceptible to proteolytic inactivation, both at atmospheric and 101.325 MPa pressure (10,000 m equivalence) (Hennessey and Siebenaller, 1987). Even 20.265 MPa of pressure significantly increased tryptic inactivation of the A_4-LDH of a shallow-living species by 14% in the absence of pressure denaturation (Fig. 12; Hennessey and Siebenaller, 1987). A HPLC peptide-mapping study indicated that although 13.78 MPa increased trypsinolysis of teleost A_4-LDH, pressure did not generate new cleavage sites. The same peptides were produced at atmospheric and elevated pressure. If there are different LDH conformers, pressure may alter the equilibrium to favor the more susceptible forms (Davis and Siebenaller, 1992). Pressure may induce conformational shifts of proteins from shallow-living species, resulting in increased susceptibility to proteolysis. More stable deep-sea enzymes may reduce turnover of proteins that otherwise might be too rapid and energetically wasteful in the food-poor deep-sea environment. A_4-LDH orthologs from deep-living species have lower k_{cat} values than the enzymes of cold-water shallow species; this decreased catalytic efficiency may be the cost of a pressure-stable enzyme (Somero and Siebenaller, 1979).

The differences in amino acid sequence between pressure-resistant and pressure-sensitive LDH homologs may be small. The two gadiform species B_4-LDH orthologs have only 21 amino acid differences, yet differ in both pressure and temperature stability. Chimeric combinations of these enzymes revealed that the N-terminal region of the enzyme contributed more to pressure tolerance (Brindley et al., 2008). A peptide-mapping comparison of A_4-LDH homologs from *Sebastolobus* fish species that differ in pressure sensitivity suggested that one, or at most a few, amino acid substitutions could account for the differences (Siebenaller, 1984a).

Actin

Actin from deep-living species displays adaptations to pressure. For example, the filamentous actin prepared from the skeletal muscle of the deep-living macrourid fish *Coryphaenoides armatus* has a low volume change of assembly (ΔV) and this ΔV is not affected by pressure. In contrast, for the F-actins of a shallower-occurring macrourid, *Coryphaenoides acrolepis*, and the warm-adapted chicken, polymerization volume changes were affected by pressure (Swezey and Somero, 1985). Recombinant α-actin monomers from two deep-living macrourids *C. armatus* and *Coryphaenoides yaquinae* were compared with monomers from *C. acrolepis*, as well as carp and chicken. The two deepest-occurring species had a lower polymerization volume change than the others and this ΔV, in contrast to the others, was not affected by increased pressure. The deeper species have a lower critical concentration of actin at high pressure (Morita, 2003).

Using molecular dynamics simulations at 0.1 and 60 MPa Wakai et al. (2014) modeled the pressure responses of *C. armatus* and *C. yaquinae* α-actin monomers in comparison to those of *C. acrolepis* and the shallow-living carp, as well as rabbit, human and chicken actins. These species have similar primary sequences; the two deeper species have a lysine as residue 137 near the active site; the other species have a glutamine. This position is important in controlling water molecules that behave as nucleophiles and attack ATP in polymer assembly. The two deep species also have two different amino acid substitutions distant from the active site and located on the surface which interacts with the neighboring F-actin protomer. These amino acid replacements are not present in the actins of the shallow-living fish or terrestrial organisms. Simulations indicated that the *C. armatus* and *C. yaquinae* actin had lowered conformation energies at high pressure relative to the

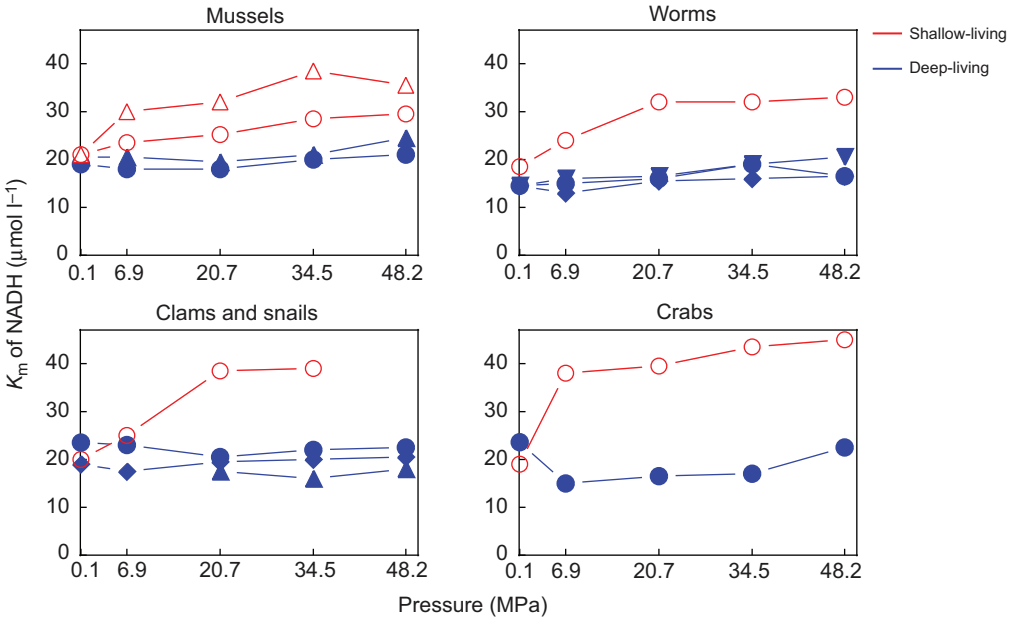

Fig. 11. Effects of pressure on K_m of NADH of partially purified cytosolic malate dehydrogenases from shallow- and deep-living invertebrates. Determinations were made at 5°C. Redrawn from Dahlhoff and Somero (1991). The pressure range tested encompasses the pressures from the surface to 4750 m.

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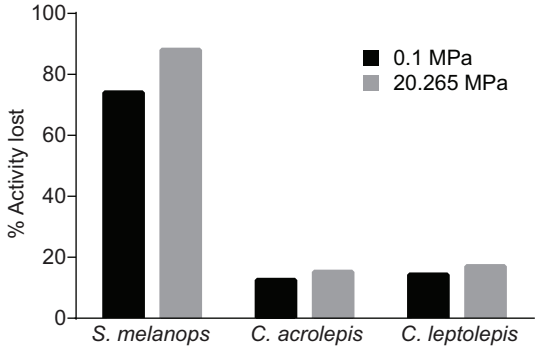

Fig. 12. Inactivation of A_4-LDH homologs by trypsin at 0.1 and 20.265 MPa pressure. LDH was isolated from white skeletal muscle of *Sebastes melanops* (abundant from 183 to 274 m), *Coryphaenoides acrolepis* (900 to 1300 m) and *C. leptolepis* (1900 to 3700 m). LDH was incubated for 60 min at 10°C with 0.5 mg ml^{-1} TPCK-trypsin at 0.1 MPa (black bars) and at 20.265 MPa (gray bars). There was no loss of LDH activity during incubations with 0.5 mg ml^{-1} albumin at 20.265 MPa. Redrawn from Hennessey and Siebenaller (1987).

other species that stabilized the actins. The deep-sea species had additional salt bridges between ATP and Lys137, which would be expected to stabilize ATP binding at elevated pressures. The deep-sea fish actins also had a greater total number of stabilizing salt bridges than the non-deep-sea species. The two amino acid replacements were sufficient to stabilize ATP binding and subunit arrangement by salt bridges.

G-protein-coupled signaling

As a last example, we examine the effects of deep-sea pressures on signal transduction processes in brain membranes of marine teleost fishes. Guanine-nucleotide-binding protein (G protein)-coupled signal transduction systems are influenced by hydrostatic pressure (Siebenaller and Murray, 1995). G proteins couple a superfamily of cell surface receptor proteins to a variety of effectors, such as adenylyl cyclase, ion channels and phospholipases (Spiegel et al., 1994). The subunits of the G proteins are designated α, β and γ. Receptors with bound agonist interact with heterotrimeric G proteins, promoting the binding of GTP in exchange for GDP on the α subunit. The binding of GTP evokes a conformational change resulting in the dissociation of the G protein into α•GTP and a βγ dimer (Coleman et al., 1994; Wall et al., 1995). The activated α•GTP subunit and the βγ dimer interact with the target effector. Signaling is terminated by the hydrolysis of bound GTP by the intrinsic GTPase activity of the α subunit and the subsequent reassociation of the α and βγ subunits (Gilman, 1995; Mixon et al., 1995). Using the A_1 adenosine–inhibitory G protein–adenylyl cyclase signaling complex in brain membranes of fishes which span a depth range of over 5000 m as a model, laboratory studies assessed the effects of pressure on the individual steps of signal transduction (Siebenaller and Murray, 1995; Siebenaller and Garrett, 2002). The binding of guanyl nucleotides to G proteins is an important component of pressure perturbation of transmembrane signaling (Siebenaller and Murray, 1994a,b; Murray and Siebenaller, 1993). Pressure alters the interactions of G proteins and receptors (Siebenaller et al., 1991; Murray and Siebenaller, 1993; Stevens and Siebenaller, 2000), and can decrease the efficacy of agonist activation of transmembrane signaling (Siebenaller and Murray, 1994a, 1999). Pressure also inhibits adenylyl cyclase activity (Siebenaller and Murray, 1990; Siebenaller et al., 1991; Siebenaller, 2000). Each of the steps in the signaling cycle is potentially susceptible to pressure perturbation and presents an adaptational challenge. Nonetheless, some, but not all, species living at depths greater than 2000 m may display 'complete adaptation' (Precht, 1958) to pressure (Siebenaller and Garrett, 2002).

Extrinsic adaptations to pressure: Piezolytes

That intrinsic adaptation may not always be 'complete' is indicated by many proteins from deep-sea animals that are not fully pressure resistant [e.g. actin (Gibbs and Somero, 1989) and transmembrane signaling (Siebenaller and Garrett, 2002)]. Instead, they may also need extrinsic factors. One may be phospholipids for membrane proteins: Gibbs and Somero (1990) showed that Na/K ATPases from shallow- and deep-dwelling teleosts displayed pressure resistance in accord with homeoviscous adaptation (e.g. Chong and Cossins, 1983). The pressure response depended on the lipid environment in which the ATPase was embedded (Gibbs and Somero, 1990).

Another extrinsic adaptation may be 'piezolytes' (Martin et al., 2002), osmolyte-type solutes that counteract pressure effects. This idea arose in 1997 after it was found that TMAO contents in teleost muscles increase linearly with depth at least down to 2900 m. In those fish, ECFs had higher NaCl and thus osmolalities above the 350–400 mOsm kg^{-1} typically cited for teleosts (Gillett et al., 1997). Later studies confirmed and extended the pattern across numerous families and species of teleosts (Fig. 13A): TMAO contents of muscles increase both inter- and intraspecifically (e.g. abyssal grenadier *C. armatus* at many depths: * in Fig. 13A), from about 40 mmol kg^{-1} tissue in shallow species to 386 mmol kg^{-1} in a hadal species from 7000 m. The hadal species, the Kermadec Trench snailfish (uppermost square, Fig. 13A), was almost isosmotic at 991 mOsm kg^{-1} (Yancey et al., 2014).

What about marine osmoconformers, especially chondrichthyans, which in shallow waters have much higher TMAO levels than teleosts? Their osmolytes also show an intriguing pattern. Kelly and Yancey (1999) reported that TMAO increased with depth in some crustaceans, squid and two species of *Bathyraja* skates. Because all these animals are osmoconformers, an increase in TMAO must be offset with a decrease in other osmolytes. In skates, it was urea that declined as TMAO increased. Muscle urea and methylamine contents in shallow species ranged between 300–400 mmol kg^{-1} and 80–180 mmol kg^{-1}, respectively (near the 2:1 ratio), while a species from 2850 m had TMAO and urea values of 244 mmol kg^{-1} and 136 mmol kg^{-1}, respectively, yielding a 'reversed' ratio of nearly 1:2. The pattern has been extended for many shark and skate species from numerous depths; see Fig. 13B (Treberg and Driedzic, 2002; Laxson et al., 2011). Methylamines in the deep species were almost entirely TMAO (Laxson et al., 2011), in contrast to the complex mixtures found in some shallow skates (Fig. 4C). Note again that the depth trend occurs intraspecifically, at least for *Raja rhina* (* in Fig. 13B).

Why would TMAO increase with depth? Since pressure is the only environmental factor that increases linearly with depth, a pressure-counteraction role was hypothesized. In the Gillett et al. (1997) study, TMAO at concentrations measured in captured deep-sea fishes was found to fully counteract hydrostatic-pressure inhibition of deep-sea teleost LDH-cofactor binding; that is, TMAO acted as a piezolyte. Other hypotheses for high TMAO include increased osmolality to save energy, increased buoyancy and a byproduct of higher lipid production in the deep sea and in chondrichthyans in general (Seibel and Walsh, 2002). However, it is not obvious how these would explain the highly linear increase in TMAO with depth. Moreover, these hypotheses are not necessarily exclusive: higher TMAO could reduce osmotic costs, aid buoyancy and counteract pressure, and higher lipid production in deep-sea

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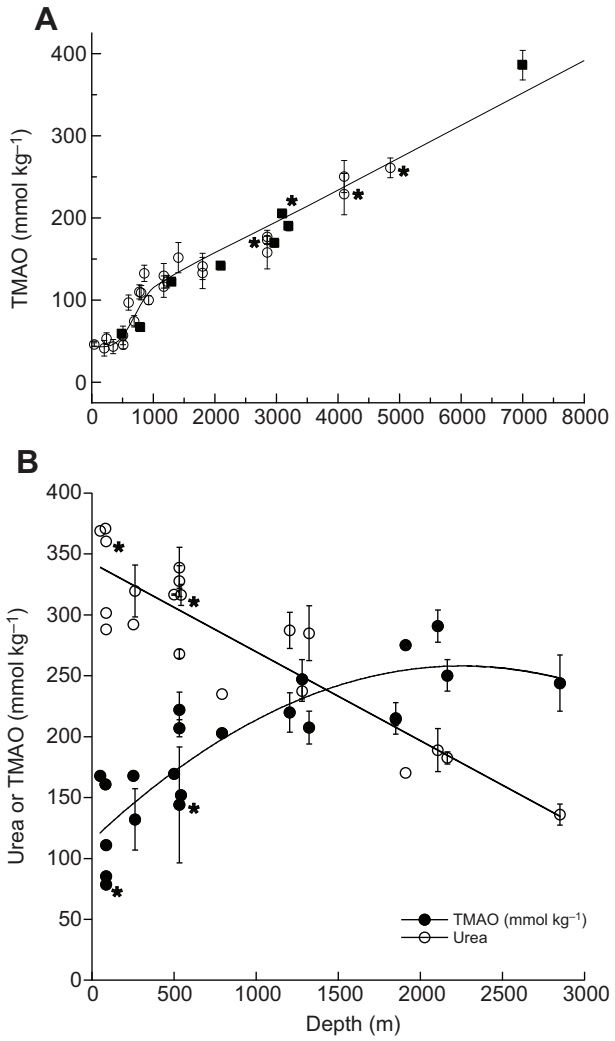

Fig. 13. Muscle osmolyte contents as a function of depth. Various analyses of tissue 'integrity', as described in Yancey et al. (2014), indicate that these values probably do not changed much (if at all) during fish capture. (A) TMAO in 19 species representing 9 families of teleosts. The hadal (trench) species, *Notoliparis kermadecensis,* is the uppermost square point. *Indicates same species, *Coryphaenoides armatus*. Circles, from Gillett et al., 1997; Kelly and Yancey, 1999; Yancey et al., 2004; Samerotte et al., 2007; squares, from Yancey et al., 2014. Curve fits: lower line for 1997–2007 data, upper line includes 2014 data. Figure from Yancey et al. (2014). (B) Urea and TMAO in 16 species of elasmobranchs (sharks, skates and other rays) and holocephalans; *, same species, *Raja rhina*. Reprinted with permission from Laxson et al. (2011).

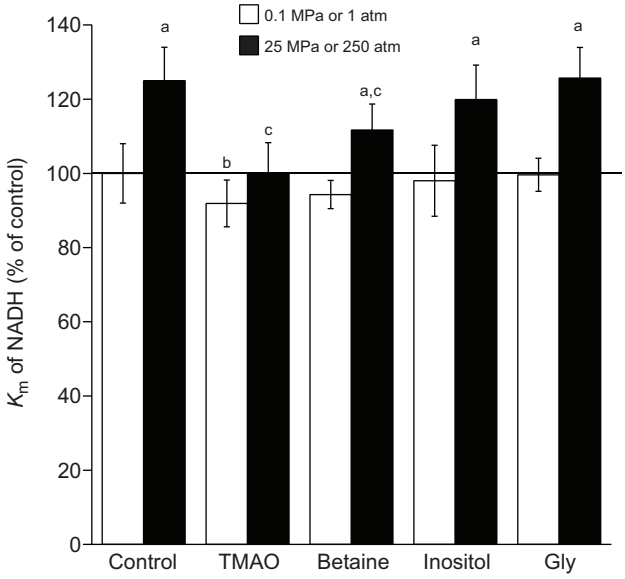

Fig. 14. Effects of osmolytes on NADH K_m of A_4-lactate dehydrogenase (LDH) from a teleost (*Coryphaenoides leptolepis;* ghostly grenadier) from 2900 m. Horizontal line represents control levels. Various osmolytes (indicated below the bars) were all tested at 250 mmol l^{-1}, with TMAO having the greatest counteracting effect. Gly, glycine. [a]Significantly inhibited from 1 atm control; [b]significantly enhanced relative to 1 atm control; [c]significant counteraction against pressure. Data from Yancey et al., 2004; plot modified from Yancey, 2005.

animals might be needed for making TMAO, a lipid by-product, as well as buoyancy (Samerotte et al., 2007).

As for the piezolyte hypothesis, counteracting effects of TMAO versus pressure have since been found work for a variety of 'incompletely adapted' proteins, including actin and pyruvate kinase (Yancey et al., 2001). Brindley et al. (2008) found that 250 mmol l^{-1} TMAO increased the pressure stability of B_4-LDH homologs from two gadiform species. TMAO was found to be the best pressure counteractant (compared with other osmolytes) for A_4-LDH from a deep-sea teleost (Fig. 14). Importantly, in a study of the effects of pressure and trypsinolysis on A_4-LDH homologs from a mammal and two shallow-living and one deep-living scorpaenid fish species, 250 mmol l^{-1} TMAO decreased pressure denaturation and proteolysis, both at atmospheric and elevated pressures (Fig. 15). Thus the effect of TMAO may be universal and independent of LDH intrinsic adaptations to pressure (Yancey and Siebenaller, 1999). Similarly, that piezolyte effects may be universal is also indicated by TMAO's ability to protect against pressure inhibition of yeast growth (Yancey et al., 2002) and a bacterial membrane channel (Petrov et al., 2012). With another model protein, staphylococcal nuclease (SNase) with TMAO studied by X-ray scattering, Krywka et al. (2008) reported: 'A drastic stabilization is observed for the osmolyte TMAO, which exhibits not only a significant stabilization against temperature-induced unfolding, but also a particularly strong stabilization of the protein against pressure.'

There appear to be no studies on chondrichthyan proteins under pressure, but the concomitant decline in urea with rising TMAO (Fig. 13B) was proposed to maintain osmoconformation while increasing pressure counteraction by TMAO while also reducing urea's destabilizing effects, which could compound those of pressure (Kelly and Yancey, 1999).

Although TMAO may simultaneously serve as an osmolyte and piezolyte in marine animals, this is not always the case. Amphipods in Lake Baikal, the world's deepest (freshwater) lake (over 1600 m), revealed depth-correlated increases (down to 1200 m) in osmolality, NaCl in hemolymph and TMAO in muscle (Zerbst-Boroffka et al., 2005). Because organic osmolytes are detrimental to water balance in freshwater animals, TMAO must have another role. This pattern is in accord with the hypothesis that increasing TMAO with depth is selected in response to hydrostatic pressure.

TMAO is not the only potential piezolyte. Deep-sea bacteria exhibit numerous intrinsic adaptations, but may also use piezolytes. *Photobacterium profundum*, which grows optimally at 20–30 MPa, accumulated glutamate and betaine when grown at 0.1 MPa. However, when grown at optimal pressures, they increased concentrations of β-hydroxybutyrate and oligomers, while amino acid pools changed little (Martin et al., 2002). In non-deep-sea studies,

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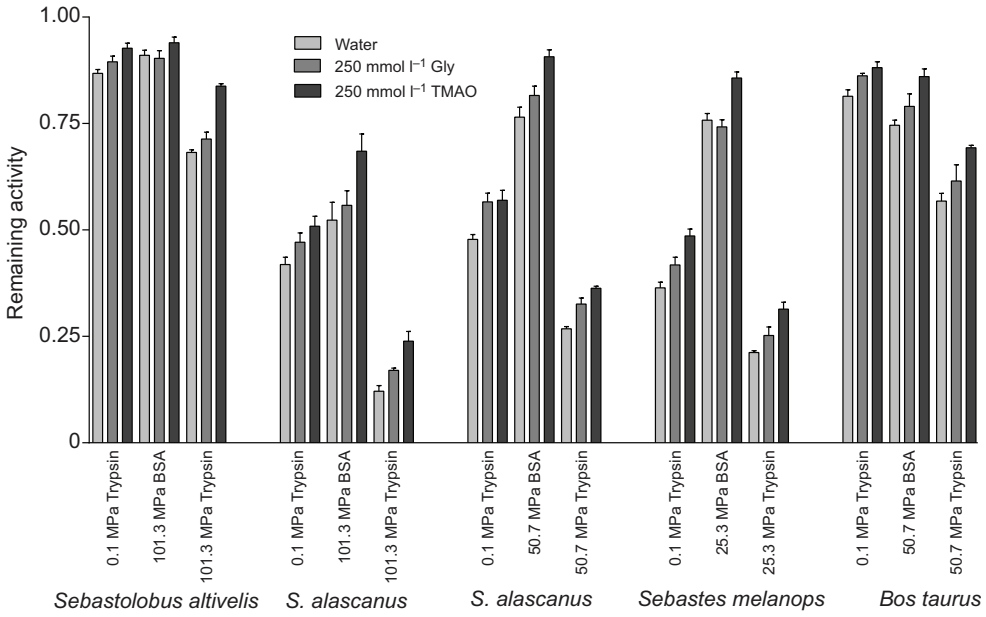

Fig. 15. Effects of added solutes at the indicated pressures, and of trypsin on the inactivation of A_4-LDH homologs from three sebastid fishes and a mammal. Three fish species: *Sebastolobus altivelis* (depth range 201 to 1757 m), *S. alascanus* (17 to 1600 m) and *Sebastes melanops* (0 to 366 m). For each species, the LDH activity was normalized to the activity at 0.1 MPa with no added TPCK-trypsin. BSA, bovine serum albumin. Modified from Yancey and Siebenaller (1999).

polyols were found to protect several proteins from pressure degradation (Athés et al., 1998; Ashie et al., 1999). Bovine mitochondrial ATPase complex was also protected by sugars, polyols, and betaine (Saad-Nehme et al., 2001). Some food-poisoning microbes can survive pressure sterilization if they are provided with the methylamines betaine or carnitine, but not other osmolyte-type cosolutes (Smiddy et al., 2004). This has implications for the food-processing industry, which is increasingly using high pressure to sterilize foods as a less-destructive treatment than heating or irradiating.

Piezolytes and depth limits

TMAO as a piezolyte may explain two curious depth distributions. The oceans descend to ~11,000 m in some trenches, where several invertebrate groups are found, but teleosts have been reported only to 8370 m, apparently absent from the greatest depths (Jamieson and Yancey, 2012; Priede and Froese, 2013). TMAO has been proposed to be the limiting factor, because as TMAO increases with depth, teleosts apparently become isosmotic at about 8200–8400 m, and greater depths could require more TMAO, higher internal osmolalities, and consequently a complete reversal in osmoregulatory systems. No fully marine teleosts are known that can handle reversed osmotic gradients (Yancey et al., 2014).

Chondrichthyans are even more restricted. Priede et al. (2006) and Priede and Froese (2013) thoroughly documented that Chondrichthyes species and abundances decline precipitously below 3000 m, with relatively few species between 3000 and 4156 m, and none deeper than that. The authors hypothesized that this absence of chondrichthyans in the abyss is due to their high metabolic needs, in part for the maintenance of enlarged lipid-rich livers.

As an alternative explanation, Laxson et al. (2011) hypothesized an osmolyte limit based on the pattern in Fig. 13B. First, the need for osmolytes in chondrichthyans coupled with the oligotrophic nature of the deep sea might result in the inability of these fish to accumulate high enough levels of TMAO to counteract both urea and hydrostatic pressure. The TMAO data in Fig. 13B hint at such a limit, but more species from greater depths are needed to test this. Second, it may be difficult for these fishes to reduce their urea content beyond a certain level. Euryhaline migratory stingrays and sharks moving into freshwater keep urea at levels well above osmoconformation levels, seeming to be unable to reduce urea. Although Fig. 13B does not show signs of a urea plateau at depth, no truly deep species (3000–4000 m) have been captured to analyze. If there is an inability to reduce urea below a certain level, it might be due to structural adaptations in the gill and kidney, which have evolved to retain urea. Or it might be due to urea-requiring proteins. Urea retention may have remained in deep-sea elasmobranchs (as it is in euryhaline species) so that urea can be readily increased in the event of vertical migration. Finally, of course, these osmolytes may have nothing to do with a depth limit for chondrichthyans.

If piezolytes were the primary mechanism of pressure adaptation, an individual that can regulate piezolytes with depth should be able to inhabit a wide range of depths, keeping proteins working. The *C. armatus* grenadier and *R. rhina* skate are likely examples (* in Fig. 13A,B). It may also be that high TMAO, if it cannot be down-regulated, would be detrimental at low pressures. Moreover, co-evolution of intrinsic and extrinsic adaptations may complicate this. For example, low pressure could 'stress' pressure-adapted proteins, preventing deep species from entering shallower waters. It may be that habitat limits in species with intrinsically adapted proteins may depend on whether there are mechanisms to regulate an appropriate (and perhaps complex) cytoprotectant mixture.

Piezolyte mechanism

Water-structuring and/or osmophobic effects could also explain TMAO's counteraction of hydrostatic pressure, as proposed by Yancey and Siebenaller (1999). Hydrostatic pressure inhibits release of hydration water from substrates and the folding and assembly of proteins in cases where volume expansion occurs (Fig. 7A, left). TMAO favors the opposite effect; i.e. its strong interactions with water may help remove hydration water (Fig. 7A, middle).

Biophysical studies by Sarma and Paul (2012, 2013) used model compounds with TMAO to explore pressure-counteraction mechanisms. Under high hydrostatic pressure, the model compound *N*-methylacetamide becomes increasingly hydrated and water around its non-polar methyl group becomes compressed. TMAO (in a dose-dependent manner consistent with the observed increase in TMAO with depth in fishes) prevents these hydration changes to the model compound. In terms

of protecting proteins from denaturation under pressure, the authors conclude: 'Solvation of TMAO molecules by water in the bulk prevents pressure-induced crowding of water molecules... This indirect effect of TMAO on water structure greatly reduces the need of water molecules to penetrate into the protein interior. In addition…TMAO also makes the water–hydrogen bond network stronger, which increases the penalty of transferring water molecules from its hydrogen bonded network to the interior of the protein' (Sarma and Paul, 2013).

Counteracting compression of water

A different hypothesis for piezolyte function is based on TMAO's prevention of pressure-induced water crowding (Sarma and Paul, 2013) and the water-expanding effects of methylamines, as evidenced in their partial molal volumes: TMAO's partial molal volume, for example, is +72.7 cm^3 mol^{-1} (Withers et al., 1994a,b). These properties might come into play in the deepest oceans where pressure compresses water volume up to 5% at 11,000 m. The presence of TMAO in cells could prevent this compression and thus protect cell volume (G.N. Somero, personal communication). This hypothesis and the protein-stabilizing one are not mutually exclusive, as both could occur.

Conclusion: co-evolution

Currently, we know virtually nothing about mechanisms for regulating TMAO (and urea) with pressure, and are only beginning to understand how intrinsic adaptations reduce volume changes under pressure (both major topics for future study). Moreover, it is apparent from the examples of adaptations to the deep sea that not all proteins or all species evolve in a similar manner to environmental stressors. For example, hadal amphipods have managed to go deeper than fish, down to 11,000 m. Future research will examine whether they have proteins with 'better' intrinsic adaptations to pressure. 'Complete' adaptation (Precht, 1958), however, may not often be achieved in protein evolution, and it may be that these deepest animals have different piezolytes than fish. The accumulation of cytoprotective cosolutes and the co-evolution of proteins in the cellular milieu may expand the potential range of environments that species can successfully inhabit. Clearly the study of protein evolution and bioinformatics predictions of protein folding must take into account the particular milieu of each species (Somero, 2003).

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Competing interests

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Author contributions

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