Adaptations to Hydrostatic Pressure in Protein Structure and Organic Osmolytes in Deep-Sea Animals

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Abstract
Hydrostatic pressure inhibits protein functions involving positive volume changes. Some deep-sea proteins have evolved to reduce such effects; e.g., actins from deep-sea fish are less sensitive to pressure inhibition of filament assembly than actins from shallow fish. Just two amino acid substitutions yield this reduced sensitivity (studies by T. Morita). Still, these actins and many other deep-sea proteins retain some pressure sensitivity and seem incompletely adapted. Organic osmolytes such as TMAO (N-trimethylamine oxide), solutes in cells used to balance seawater's osmotic pressure, may help. Most shallow marine animals have low TMAO contents. We found that muscle TMAO contents increase with depth among species of shrimp, skates and 17 species (9 families) of teleost fishes, and within individual fish species from different depths. TMAO contents increase in a sigmoidal pattern (similar to pressure effects on proteins) between shallow and 1.4 km depth. TMAO counteracts the effects of pressure on a variety of systems including actin assembly, and does so better than other common osmolytes.

Keywords: Osmolyte, trimethylamine oxide, pressure

1. Introduction

The deep sea and the subsurface geosphere are the largest habitats on Earth, and yet, while life thrives in these realms, they remain the least explored. A major factor for life is high hydrostatic pressure, ranging in the oceans from 0 to about 110 MPa (roughly 1100 atm) in the deepest trenches. Pressure has broad effects on biochemistry, e.g., inhibiting protein folding and assembly and ligand binding, when there is a positive volume change involved. A positive change may result, for example, during binding and folding from the release and expansion of bound water molecules densely clustered around some protein surfaces and ligands. How biological processes operate properly under high pressure has not been fully elucidated, but some answers are emerging.

Some proteins from deep-sea animals exhibit less sensitivity to pressure effects than do homologues from shallow species. These findings indicate there are evolutionary differences in amino acid sequences that reduce the volume changes occurring in reactions (1). An example will be discussed later. Nevertheless, despite such adaptations in structure, many deep-sea proteins still exhibit significant pressure sensitivities and thus seem incompletely adapted. The solution to this problem may be found with organic osmolytes (2). Organic osmolytes are small solutes, primarily neutral amino acids, small carbohydrates, methylamine and methylsulfonium solutes, that accumulate in cells of many organisms to stop osmotic water loss. In marine organisms classified as osmoconformers (most marine organisms), these solutes accumulate in cells to balance the high osmotic pressure of extracellular fluids and seawater (about 1000 mosmol/kg). In marine invertebrates, cells contain high levels of neutral free amino acids such as...
taurine and glycine, and sometimes the methylamine glycine betaine (GB) or TMAO (trimethylamine N-oxide; the source of trimethylamine, which is the fish odor of many marine animals.) Among the vertebrates, osmoconformers include elasmobranch fishes (sharks, skates, rays), most of which use urea and TMAO as their major organic osmolytes. Typically elasmobranchs have about a 2:1 urea-TMAO ratio in their cells (2). Traditionally, only the higher vertebrates (bony fish to mammals) and a few crustacea have been classified as osmoregulators, that is, animals that do not use organic osmolytes, but rather maintain a consistent osmotic pressure (typically 300-400 mosmol/kg in vertebrates) considerably lower than that of seawater. However, some deep-sea fishes appear to be an exception, as will be discussed.

Organic osmolytes (except for urea) are often called "compatible solutes" because, unlike inorganic ions, many do not perturb protein structures or functions. Beyond simply compatibility, however, many osmolytes have been found to have "counteracting" properties; that is, they stabilize proteins and can offset effects of perturbants such as temperature and urea. Indeed, urea (a protein destabilizer) is high enough (300 mM or more) in elasmobranch fishes (as an osmolyte) and mammalian kidneys (as a waste product) to significantly perturb proteins, but methylamines in those animals (TMAO in elasmobranchs, glycerophosphorylcholine [GPC] and GB in kidneys) appear to counteract urea's effects, optimally at about a 2:1 urea-methylamine ratio (2). In our recent findings, some stabilizing solutes may also protect macromolecules from the effects of hydrostatic pressure.

2. Adaptations to Pressure

2.1 Intrinsic Adaptation: Evolution of Protein Structure

Many proteins appear to have evolved intrinsic (i.e., inherent structural) changes in amino-acid sequences that reduce volume changes (and thus pressure sensitivity). A landmark study that showed pressure-adaptive differences was that of Siebenaller and Somero (3), who studied muscle lactate dehydrogenases (LDH) in two congeneric fish (Sebastolobus spp). During anaerobic glycolysis in muscle, LDH binds and catalyzes pyruvate and NADH into lactate and NAD\(^+\); the latter is a necessary electron acceptor for glycolysis to continue. The researchers found that the Michaelis-Menten constant (K\(_{m}\)) for NADH was highly pressure sensitive in the LDH from the more shallow-dwelling species, but much less sensitive in the homologue from the deeper-living relative.

Swezey and Somero (4) found a similar pattern with actin, a major structural component of contractile cells. High pressure inhibits the assembly of actin units into the filamentous (F) form essential to muscle structure and contraction. Polymerization requires ATP hydrolysis and Ca\(^{2+}\) binding, which are pressure sensitive. However, actins from deep-sea fish are much less sensitive to pressure than those from shallow fish (4). Studies by T. Morita (5) on actins from abyssal macrourid fish (two Coryphaenoides species) show that just three amino acid substitutions are responsible for the reduced pressure sensitivity (when compared to carp actin). Two substitutions, Q137K (glutamine to lysine at position 137) and A155S (alanine to serine), reduce the volume change involved with ATP and Ca\(^{2+}\) dissociation. A V54A (valine to alanine) or L67P (leucine to proline) substitution (only one of which is found in each species' actin) reduces the volume change associated with the direct interaction of subunits undergoing polymerization. How the
amino acid differences reduce volume changes is not yet clear.

### 2.2 Extrinsic Adaptation: Organic Osmolytes as Pressure Counteractants

Despite the structural changes that reduce pressure sensitivity, many deep-sea proteins retain significant pressure sensitivities. For example, when measured at habitat pressures, deep-sea LDHs show a 25% increase in NADH $K_m$ (3), and polymerization of deep-sea actins a 20% inhibition (5). Thus, intrinsic adaptation does not appear to yield complete adaptation to pressure. It might be that deep-sea animals suffer from these pressure effects without ability to adapt fully. Alternatively, extrinsic factors such as organic osmolytes might help, given that some osmolytes can protect proteins from other perturbants like temperature and urea.

To test this idea, we initially analyzed organic osmolyte-type solutes in a variety of animals from shallow, 1.8 km and 2.9 km depths. In studies from 1997, 1999, and 2004, we found that TMAO contents increase linearly with depth in muscles of 12 species of teleost fishes in 6 families (Fig. 1, circles), as well as in shrimp (Fig. 1, triangles), crabs, skates and squid (6, 7, 8). Teleosts are osmoregulators, mostly reported to have internal osmotic pressures of 300-400 mosmol/kg, and with low amounts of TMAO (50 mmol/kg or less). However, the deep-sea species have much higher osmotic pressures, nearly 600 mosmol/kg in those from 2.9 km (6). TMAO thus may be serving as an osmolyte, to reduce osmoregulatory costs in the energy-poor deep sea. However, that hypothesis would not explain the pattern for osmoconformers. Skates, shrimp, crabs and shrimp are conformers, so if TMAO increases in content, there must be a decrease in other osmolytes to maintain osmotic balance. We indeed found this: in shrimp, there were decreasing contents of the common invertebrate osmolyte glycine with depth; in the skates, urea declined with depth (7). As noted earlier, skates and other elasmobranchs have about 2:1 urea-methylamine ratio, but that appears to hold only for shallow species. In the skates caught at 2.9 km depth, the ratio was about 1:2. Thus, the destabilizing solute is reduced and the stabilizing one increased.

![Fig. 1](image-url) TMAO contents of muscles in teleost fish and caridean shrimp. Most data are from 1997, 1999 and 2004, replotted from references 6, 7, 8 (circles, triangles). From our newest teleost data (9), three selected points (out of 23 total) are plotted as squares. Most symbols are for different species, except the two filled circles and filled square which are for the same teleost, the grenadier *Coryphaenoides armatus* (*C.arm*). These values are for tissue extracts; actual intracellular values would be considerably higher (6, 9). A linear fit is shown for each animal group. However, our new values (three of which are plotted as squares) suggest a sigmoidal increase between 0 and 1200 m (9). A possible fit for that range is shown.

Our newest studies on teleosts have extended the TMAO data to 17 species in 9 families...
over a much wider range of depths (0 to 4.9 km), and also to 8 individual species caught at different depths (9). TMAO contents increased with depth (Fig. 1, squares), similar to the older data comparing different species, and also increased within several species caught at different depths (one example, Coryphaenoides armatus, is shown in Fig. 1 as filled symbols). Perhaps significantly, TMAO increases in an apparent sigmoidal pattern between shallow and 1.2 km depth, then linearly at greater depths to 4.8 km (Fig. 1). The sigmoidal pattern resembles the effects of pressure on proteins (9), which typically are minimal over the equivalent of 0 to 0.5 - 1.0 km (1).

Given that pressure increases with depth, we hypothesized that TMAO might be serving as a pressure counteractant. In studies on a variety of systems, we indeed found that TMAO counteracts the inhibitory effects of pressure on kinetics and stability of shallow and deep enzymes including LDH (Fig. 2A), and assembly of F-actin from abyssal Coryphaenoides (Fig. 2B) (6, 8, 10, 11). Moreover, TMAO counteraction of pressure also worked with growth of yeast, which normally do not experience high pressures (12). Importantly, we found that TMAO is a better stabilizer than other common osmolytes (betaine, myo-inositol and glycine, Fig. 2B) (8, 10), suggesting why it is favored in the deep sea. Physicochemical considerations suggest that TMAO may aid the expansion of water molecules released during binding and assembly events, and/or may oppose the tendency of pressure to force water into the hydrophobic interior of proteins (reviewed in 2, 8).

What occurs in taxa that cannot make TMAO? Mollusks, cnidarians and echinoderms do not appear to do so, yet some can live at great depths. We analyzed a variety of species from different depths to address this issue. In these taxa, deeper-living species were found to have less of the common osmolytes (e.g., taurine, glycine), and more methylamines such as GB and GPC,
and a novel polyol, scyllo-inositol (8, 12). Whether these solutes are pressure counteractants is not yet known. We also analyzed a number of vesicomyid clams, which live at hydrothermal vents and gas seeps in the ocean from shallow to over 7 km depths. In species obtained from 6 depths between 0.5 and 6.4 km, we found that the common osmolytes taurine and glycine declined at greater depths, and were replaced (linearly with depth) by an unidentified methylamine and (more so) by an unidentified solute containing serine, ethanolamine and phosphate (13). Inorganic phosphate is one of the most effective protein-stabilizing anions of the Hofmeister series, and several phosphate-containing organic osmolytes such as GPC are strong protein stabilizers (reviewed in 2); thus, it is possible that the serine phosphate solute protects proteins from pressure. Recently, we obtained vesicomyid clams (*Vesicomya pacifica*) from 0.6 km depth off Oregon (using the *Alvin* submersible) and pressurized them to the equivalent of 0.6 and 2.2 km, the depth limit of this particular species. Tissue analysis revealed that the clams at the higher pressure, but not those kept at the lower pressure, had significant amounts of the serine phosphate solute (Andrell, Lee and Yancey, unpublished).

### 3. Conclusion

Hydrostatic pressure has significant inhibitory effects on biological function even at modest levels of 5 to 10 MPa. Some proteins have evolved structural (intrinsic) differences in amino acids that somehow compensate for positive volume changes that create pressure sensitivity. However, these differences are not sufficient in many cases, and extrinsic solution factors may be the key. In particular, organic osmolytes known to stabilize proteins against other physical and chemical perturbants may also counteract the effects of pressure. To test these ideas further, more analyses of more species from the deep sea are needed, including more studies on live animals. Tests on gene expression under pressure are also needed. Currently, we are investigating TMAO regulation at different pressures in living deep-sea fish in a pressure chamber developed by Drazen et al. [14]. These and other studies should provide more thorough tests of the osmolyte-pressure hypothesis.

### 4. References


