

**NITROGENOUS SOLUTES AS PROTEIN-STABILIZING
OSMOLYTES: COUNTERACTING THE DESTABILIZING EFFECTS
OF HYDROSTATIC PRESSURE IN DEEP-SEA FISH**

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Abstract

Hydrostatic pressure can inhibit protein functions. Many deep-sea proteins have evolved resistance to this, but not all. Recently we have found unusual osmolyte compositions in deep-sea fishes and other animals. The protein-stabilizer trimethylamine N-oxide (TMAO), under 80 mmol/kg wet wt in most shallow teleosts, increases with depth in white muscles of several families of teleosts (up to 288 mmol/kg at 2900 m). Similarly, deep-sea skates have more TMAO (and less urea) than do shallow relatives. Here we report that red muscle of deep-sea macrourids contains 93-118 mmol/kg wet wt TMAO (less than white muscle, but more than shallow species) plus large amounts of a possible (but unidentified) methylamine. We hypothesized that methylamines counteract effects of pressure on proteins, and have tested TMAO and pressure with lactate dehydrogenase, pyruvate kinase, and actin, all pressure-sensitive proteins. 200-250 mM TMAO (but not glycine) protected ligand binding, protein stability, and polymerization against pressure inhibition. Here, to test TMAO in living cells, we grew yeast under pressure. After 1 hr at 71 MPa, 3.5 hr at 71 MPa, and 17 hr at 30 MPa, 150 mM TMAO (but not glycine) generally doubled the number of cells that formed colonies. These results support the hypothesis.

Introduction

Most marine animals osmoconform with seawater at about 1000 mOsm, but their cells do not have high salt levels. Rather they prevent osmotic shrinking with intracellular organic osmolytes, which add up to about 600 mOsm to cells (the other 400 mOsm coming from basic inorganic and organic cell solutes). In invertebrates, organic osmolytes are neutral free amino acids (e.g., glycine, taurine), sometimes with lesser amounts of methylamines, especially glycine betaine and trimethylamine N-oxide (TMAO). In fishes, hagfish osmoconform using free amino acids, glycine betaine and TMAO, while cartilaginous fishes and coelacanths use urea, TMAO, and glycine betaine (Yancey 2001).

Except for urea, these solutes are probably used because, in contrast to common inorganic ions, they do not perturb proteins, and thus serve as "compatible" osmotic effectors (Brown and Simpson, 1972). But beyond this, many osmolytes—especially methylamines—enhance functional and structural properties of proteins such that they can offset negative effects of perturbants such as urea and NaCl. Such “counteracting” osmolytes have universal effects, working on proteins regardless of source, and are typically present at high levels only in cells subjected to protein perturbants. Well-studied marine examples are the elasmobranchs, with the protein destabilizer urea typically at 300-400 mM and TMAO at 150-200 mM in muscle cells (Yancey et al., 1982). At this ratio (about 2:1), urea’s inhibitory effects are often fully offset by TMAO’s stabilizing effects. Coelacanths have similar contents of these osmolytes. Another (non-marine) example is the mammalian kidney, where urea is the main nitrogenous waste, and methylamines (glycerophosphorylcholine, glycine betaine) are major cellular osmolytes. Like TMAO, renal methylamines can counteract urea’s effects on protein functions (Yancey, 1994, 2001).

In contrast to the common pattern of osmoconforming, teleosts are typically hypo-osmotic regulators. In most shallow marine teleosts, TMAO can be a major cellular solute, but at less than 80 mmol/kg (Hebard et al., 1982), leaving the animals hypo-osmotic—typically at about 350-400mOsm. An exception to this pattern are polar teleosts, some of which have glycerol up to 400 mM or TMAO up to about 150 mM (Raymond, 1994; Raymond and DeVries, 1998).

The above information is based on shallow-water organisms. Our recent studies have revealed striking differences in deep-sea animals, providing new insight into adaptation to this unique habitat. In particular, we discovered a linear increase in TMAO contents of white muscle with depth in several families of

teleosts (up to 288 mmol/kg wet wt in a morid cod from 2900m), as well as in some crustaceans and other invertebrates. In deep-sea skates, TMAO was also higher than in shallow elasmobranchs. In the osmoconformers, TMAO in effect replaced the major osmolytes of shallow species (glycine in shrimp, urea in skates). Interestingly, the urea:TMAO ratio in the deepest skates was about 1:2, a reversal of the typical shallow-water pattern (Gillett et al., 1997; Kelly and Yancey, 1999).

Our studies have focused on white muscle, the best studied tissue in terms of osmolytes in marine animals. Here we report on osmolytes in red muscles of macrourid teleosts, showing that they have high TMAO contents, but not as high as white muscle; additional osmotic pressure is due to other unusual osmolytes.

Why would methylamines be accumulated in the deep? The only environmental factor that is also linearly correlated with depth is hydrostatic pressure, which can inhibit protein folding and reaction kinetics. We hypothesized that TMAO (as a general protein stabilizer) might serve to counteract these effects (Gillett et al., 1997). Some deep-sea proteins have been selected to be less affected by high pressure than are homologues from shallow species (Siebenaller and Somero, 1989). But some deep-sea proteins exhibit significant pressure sensitivities and thus seem incompletely adapted. For cofactor (NADH) K_m , a lactate dehydrogenase (LDH) homologue from a deep-sea fish showed less pressure sensitivity than did a homologue from a shallower congener, but the deep homologue nevertheless had some sensitive (Siebenaller and Somero, 1978). We found that TMAO at appropriate levels can fully offset this inhibition, restoring K_m to the control (unpressurized) value (Gillett et al., 1997). Similarly, deep-sea LDH homologues had greater stability under prolonged pressure than did shallow-derived homologues, but the former still lost activity. They also had pressure-enhanced sensitivity to tryptic digestion, suggesting that subtle conformational changes occur under pressure. For all LDH homologues tested (from shallow and deep fish, and cow), TMAO but not glycine was able to counteract pressure destabilization (Yancey and Siebenaller, 1999).

We have also examined other deep-sea proteins that have sensitivity to pressure: actin in assembly of G- to F-form, and pyruvate kinase (PK) and its ADP K_m . Actin homologues in deep species are less sensitive, but not fully resistant to pressure (Swezey and Somero, 1982); deep homologues of PK show no pressure adaptation (Mustafa et al., 1971). We found that TMAO (but not glycine) partly or fully offset pressure effects on both systems (Yancey et al., 2001).

To test more directly the postulated protective effects of TMAO and the universality thereof, we chose a living model system--yeast cells grown under pressure. Other researchers have shown that yeast are protected from pressure by DMSO, D₂O (Komatsu et al., 1991), and trehalose (Iwahashi et al., 1998).

Materials and Methods

Fish Muscle Osmolytes

Deep-sea macrourid teleosts (*Coryphaenoides* species) were collected from 2900 m off Oregon and stored as previously described (Gillett et al., 1997). White and red myotomal muscles were analyzed for TMAO and other osmolytes by spectrophotometry and HPLC as previously described (Wolff et al., 1989; Kelly and Yancey, 1999). An unidentified solute detected by HPLC was further analyzed by ¹H-NMR as previously described (Yin et al., 2000) and by standard ninhydrin procedure.

Yeast

Yeast (*Saccharomyces cerevisiae*) were tested according to Komatsu et al. (1991), with minor modifications. Cells were grown in medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% g peptone, and 1.0% glucose (YM medium); 2% agar was added to the YM medium for plates. Cultures were grown in test tubes (with culture caps) in a shaking water bath at 25°C; after suitable growth, cell densities were determined with a hemocytometer. To determine if TMAO had any effects on yeast growth under standard conditions at 1 atmosphere, cultures were grown at 0, 50, 100, 150, and 200 mM TMAO, then serially diluted and plated. TMAO up to 150 mM had no effect on number of successful colonies, but was inhibitory at 200 mM. For pressure tests, cells were grown for 24 hr in YM medium with or without 100 or 150 mM TMAO, collected by gentle centrifugation, and resuspended at 10⁷ cells l⁻¹ in medium with or without TMAO. 70 µL samples of each culture (in quadruplicate for each test condition) were sealed in airless polyethylene tubes, then incubated at atmospheric and high pressures at 25°C in water-filled high-pressure steel cylinders. After varying times, they were rapidly depressurized, released from the tubes, and serially diluted by factors of 10, 100 and 1000. 50 µL aliquots were plated in quadruplicate, and resulting colonies were counted after 2-3 days at 26°C in an incubator. 150 mM glycine was tested similarly.

Results

Red Muscle Osmolytes

In macrourids from 2900 m, red muscle differed somewhat from white. Unlike the latter, in which TMAO occurred at 170-183 mmol/kg wet wt, red muscle had 93-118 mmol kg/wet wt (Table 1) and 5-10 mmol/kg of scyllo-inositol. Over half of the rest of the osmotic pressure came from a solute that eluted near glycine betaine on the HPLC, but which did not match any know carbohydrate, methylamine or amino acid standard. It reacted weakly with ninhydrin, consistent with a mono-methylamine (this and amino acids react with ninhydrin, while di- and trimethylamines do not). In the NMR, this solute had a large singlet peak at 2.73 ppm, in the range of N-methyl hydrogens on mono-methylamines (e.g., N-methyl hydrogens of sarcosine and methyltaurine are at 2.71 and 2.79 ppm, respectively) (Agar et al., 1991; Yin et al., 2000). Another singlet peak was at 3.37 ppm and was unidentifiable. This solute was not seen in muscles of shallow-water cods (*Gadus macrocephalus*; macrourid relatives) in previous research.

Table 1. Contents of TMAO and an unidentified possible methylamine in white and red myotomal muscles of *Coryphaenoides* species from 2900 m off Oregon. Means and s.d. values are in mmol/kg wet wt, from 3 to 5 specimens each. Contents of the unidentified solute were estimated by refractive index detection (HPLC) using a refractive index typical of other methylamines (Wolff et al., 1989).

Species	TMAO		Unidentified (estimate)	
	White	Red	White	Red
<i>C. armatus</i>	179 ± 12	105 ± 21	< 1	62 ± 10
<i>C. filifer</i>	170 ± 9	93 ± 16	< 1	59 ± 7
<i>C. leptolepis</i>	183 ± 18	118 ± 9	< 1	34 ± 5

Yeast

Several combinations of time and pressure were used on yeast cultures. First, we tested for concentration effects of TMAO. As shown in Fig. 1A, baroprotection did occur and was dependent on concentration: survival was considerably higher in 150 mM compared to 100 mM TMAO. 50 mM had no significant effect (not shown).

Second, we tested 150 mM TMAO for 1 hr at 71 MPa (700 atm), 3.5 hr at 71

MPa, and 17 hr at 30 MPa (300 atm). TMAO improved survival of cells after pressurization in all cases, although not significantly in the 1 hr test (Fig. 1B). 150 mM glycine gave no significant protection (not shown). These results are similar to previous studies on DMSO and D₂O, which also gave baroprotection (Komastu et al., 1991).

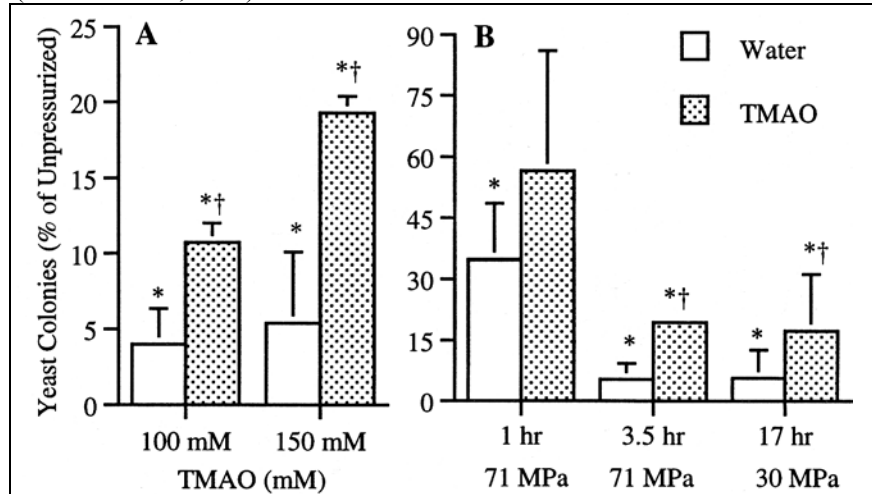


Figure 1. Effects of TMAO on yeast growth following pressurization for times and at MPa values indicated. A: Effects of 100 and 150 mM TMAO on growth after 3.5 hr at 71 MPa (700 atm). B: Effects of 150 mM TMAO on yeast grown under different pressure conditions and times. *significantly less than unpressurized controls; †significantly higher than pressurized samples without TMAO ($p < 0.05$).

Discussion

Our results with red muscle show that TMAO contents are higher in red muscle of deep-sea macrourids than in tissues of shallow-living teleosts, but not as high as in white muscle. However, scyllo-inositol and another methylated amine not present in white muscle—and not one of the common biological amines—makes up the rest of the osmotic pressure expected. Thus, again an unusual osmolyte composition occurs in another tissue in the deep sea.

Our results with living cells add to our previous results with proteins in support of our hypothesis: that high levels of TMAO in some deep-sea animals have been selected to offset inhibitory effects of high hydrostatic pressure.

Accumulation of TMAO could facilitate adaptation to the deep sea by protecting cellular functions and perhaps decreasing protein turnover, reducing the need to evolve pressure-resistant proteins. However, since the latter clearly have evolved in some cases, protein evolution is probably necessary since TMAO is unlikely to counteract all effects of pressure completely. Similarly, TMAO does not counteract all effects of urea on proteins (Yancey, 1994). We have speculated elsewhere on the possible mechanism of TMAO in counteracting pressure (Gillett et al., 1997; Yancey et al., 2001). Briefly, high hydrostatic pressure inhibits reactions with a positive volume change, which can result from the release of water molecules densely bound to some ligands and protein side groups (Siebenaller and Somero, 1989). Stabilizing osmolytes tend to disfavor the formation of bound water (hydration water) around ligands and proteins, thus favoring protein folding and ligand binding (which reduce hydration water compared to unfolded and unbound states) (Timasheff, 1992; Wang and Bolen, 1997). The effects of pressure and TMAO may simply oppose each other in an additive manner.

It should be noted that, while TMAO was better than glycine at pressure counteraction in our tests, other solutes can offset pressure effects. Trehalose, DMSO and D₂O have been shown to protect non-marine microorganism growth from pressure (Komatsu et al., 1991; Iwahashi et al., 1998), and sugars and polyols can protect non-marine proteins from pressure degradation (Athés et al., 1998; Ashie et al., 1999). Thus there may be other osmolytes in deep-sea organisms that counteract pressure. Indeed, not all deep-sea animals contain high levels of TMAO. We have found high levels of the following in other deep-sea taxa: (1) scyllo-inositol in echinoderms (Yin et al., 1999); (2) this polyol plus glycine betaine and sarcosine (N-methylglycine) in gastropods, octopods, polychaetes and pycnogonids (Yin and Yancey, 2000); (3) N-methyltaurine and an unidentified methylated solute in vestimentiferans (Yin et al., 2000); and (4) a novel osmolyte made of serine, phosphate and ethanolamine in vesicomyid clams (from seeps in Pacific trenches) (Fiess et al., 2001). These solutes are markedly different from the common osmolytes of shallow invertebrates. They could relate to dietary and metabolic differences or to other environmental stresses such as sulfides and temperature, but may be adaptations to high pressure. The unidentified solute (if a methylamine) and/or scyllo-inositol in red muscle of macrourids may be as well.

There are other hypotheses for the occurrence of high TMAO in marine animals in addition to counteraction, including diet and counteraction of NaCl (Kelly and Yancey, 1999). Another hypothesis concerns buoyancy: TMAO solutions are

less dense than seawater and solutions of other common osmolytes (Withers et al., 1994). However, this property would not explain why TMAO increases linearly with depth in shrimp, since shallow shrimp should benefit as much as deep shrimp from TMAO's buoyancy properties (Kelly and Yancey, 1999). Another explanation concerns lipid metabolism. Seibel and Walsh (2002) have shown a correlation between acylglycerol metabolism and TMAO production, proposing that the latter arises as a byproduct in proportion to the former. However, even if this is correct, such metabolism and/or retention of TMAO could still be selected for in species that use its counteracting properties, so this proximate hypothesis is not mutually exclusive with an evolutionary one.

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