The Journal of Experimental Biology 202, 3597–3603 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
JEB2222

TRIMETHYLAMINE OXIDE STABILIZES TELEOST AND MAMMALIAN LACTATE DEHYDROGENASES AGAINST INACTIVATION BY HYDROSTATIC PRESSURE AND TRYPSINOLYSIS

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Accepted 21 September; published on WWW 29 November 1999

Summary

Trimethylamine *N*-oxide (TMAO) is an organic osmolyte present at high levels in elasmobranchs, in which it counteracts the deleterious effects of urea on proteins, and is also accumulated by deep-living invertebrates and teleost fishes. To test the hypothesis that TMAO may compensate for the adverse effects of elevated pressure on protein structure in deep-sea species, we studied the efficacy of TMAO in preventing denaturation and enhanced proteolysis by hydrostatic pressure. TMAO was compared to a common 'compatible' osmolyte, glycine, using muscle-type lactate dehydrogenase (A_4-LDH) homologs from three scorpaenid teleost fish species and from a mammal, the cow. Test conditions lasted 1 h and were: (1) no addition, (2) 250 mmol l^{-1} TMAO and (3) 250 mmol l^{-1} glycine, in the absence and presence of trypsin. Comparisons were made at 0.1 and 101.3 MPa for the deeper occurring *Sebastolobus altivelis*, 0.1, 50.7 and 101.3 MPa for the moderate-depth congener *S. alascanus*, 0.1 and 25.3 MPa for shallow-living *Sebastes melanops* and 0.1 and 50.7 MPa for *Bos taurus*. Susceptibility to denaturation was determined by the residual LDH activity. For all the species and pressures tested, 250 mmol l^{-1} TMAO reduced trypsinolysis significantly. For all except *S. altivelis*, which was minimally affected by 101.3 MPa pressure, TMAO stabilized the LDH homologs and reduced pressure denaturation significantly. Glycine, in contrast, showed no ability to reduce pressure denaturation alone, and little or no ability to reduce the rate of proteolysis.

Key words: trimethylamine oxide, lactate dehydrogenase, trypsinolysis, deep sea, hydrostatic pressure, osmolyte.

Introduction

Most marine organisms are osmoconformers, maintaining cell volume with high levels of organic osmolytes, especially polyols, neutral amino acids and methylamines. These osmolytes are generally 'compatible' (Brown and Simpson, 1972) because, unlike most inorganic ions, they raise cellular osmolality without adversely affecting macromolecules. However, some osmolytes, including trimethylamine *N*-oxide (TMAO), are termed 'counteracting' (Yancey et al., 1982) because at physiological levels they enhance substrate binding and stability of proteins and offset adverse effects of salt ions, high temperature and, in cartilaginous fish, the perturbing osmolyte urea (Yancey, 1994). Although common in many marine taxa, TMAO is generally a minor osmolyte in shallow-water species except for squid and elasmobranchs (up to 200 mmol kg^{-1} fresh mass) (Hebard et al., 1982). The exception to the typical marine pattern of high levels of organic osmolytes is hypo-osmotic regulation, as exemplified by teleosts. With internal fluids at 300–400 mosmol l^{-1} (Lange and Fugelli, 1965), most teleosts have no need to accumulate large amounts of organic osmolytes. They generally contain TMAO, but, until recently, the reported concentrations were only 10–70 mmol kg^{-1} muscle (Hebard et al., 1982).

Recently, significant deviations from these patterns have been found in polar and deep-sea animals. Some polar teleosts have blood glycerol contents of up to 400 mmol l^{-1} and muscle TMAO contents up to 154 mmol kg^{-1} (Raymond, 1994; Raymond and DeVries, 1998). We have reported high levels of TMAO (up to 300 mmol kg^{-1} muscle) in deep-sea animals in the order abyssal>bathyal>shallow in osmoregulating teleosts of several families (Gillett et al., 1997; Kelly and Yancey, 1999), and in osmoconforming caridean shrimp, skates, brachyuran crabs, cuspidarid bivalves and anemones (Kelly and Yancey, 1999). In shrimp, osmoconformation was maintained as higher TMAO levels were offset by an equivalent decrease in glycine concentration (the dominant osmolyte of shallow species and typically a weaker protein stabilizer than TMAO; Yancey, 1994).

Thus, deep-sea osmoregulators and osmoconformers appear to use large amounts of TMAO. We have hypothesized (Gillett et al., 1997) that high levels of this osmolyte may stabilize

proteins under high hydrostatic pressure, which is known to affect protein activity and structure adversely (Siebenaller, 1987; Balny et al., 1997). In the ocean, pressure increases by 101.3 kPa (= 1 atm) for every 10 m depth increase (Saunders and Fofonoff, 1976). Some proteins in deep-sea organisms exhibit considerably greater resistance to high pressures than do shallow-water homologs, presumably due to protein structural adaptations. However, some deep-sea proteins in vitro are as sensitive to pressure as are shallow-water homologs, and even many pressure-resistant proteins exhibit some sensitivity (Siebenaller, 1987). For example, the cofactor Michaelis constant for muscle-type (A_4) lactate dehydrogenase (LDH) is less pressure sensitive in deep-living teleosts (Siebenaller and Somero, 1978), but still perturbed by pressures within the habitat range. A previous study showed that, for LDH of a deep-sea teleost, the cofactor Michaelis constant was increased 30 % by high pressure (30.4 MPa), but decreased to the control value at 0.1 MPa with the addition of 250 mmol l^{-1} TMAO (Gillett et al., 1997).

In addition to such effects on kinetics, the high pressures of the deep ocean can denature proteins and enhance proteolysis of a protein by altering the conformation and subunit aggregation state (Hennessey and Siebenaller, 1985, 1987a,b; Davis and Siebenaller, 1992). Previous studies have demonstrated differences in the pressure stability of A_4-LDH homologs between shallow- and deeper-occurring teleost fishes (Hennessey and Siebenaller, 1985, 1987a; Davis and Siebenaller, 1992). Of the species examined, on average the enzymes of deeper-occurring species were more pressure-stable (Hennessey and Siebenaller, 1985, 1987a). Nevertheless, deep-sea LDH homologs may not be fully resistant to habitat pressure. We have recently shown that the LDH homolog from a deep-living teleost loses significant activity at physiological pressure after prolonged (15 h) incubation, and that a physiological TMAO concentration can offset this loss (Fyfe-Johnson and Yancey, 1998).

The susceptiblity of LDH homologs to proteolytic inactivation has been shown to differ also, both at atmospheric and elevated pressures. At atmospheric pressure, the enzymes of shallower-occurring species were more rapidly denatured by the proteases trypsin and subtilisin. At 101.3 MPa pressure the proteolytic denaturation of LDH homologs from the shallower-occurring species increased to a greater extent than that of the homologs from the deeper species (Hennessey and Siebenaller, 1987a). However, for all deep and some shallow homologs, denaturation by proteolysis under pressure was higher than expected based on a simple combination of pressure-induced instability and proteolysis rate. This suggests that pressure alters the substrate proteins (including deep-sea homologs) in such a way as to make them more susceptible to proteolytic attack. Increased pressure does not expose new cleavage sites, but may increase proteolysis due to slight conformational changes in the substrate proteins (Davis and Siebenaller, 1992).

Thus, because the more pressure-resistant homologs of A_4-LDH may not be completely adapted to high habitat pressure as a result of their amino-acid sequences alone, in this study we examine the possible role of TMAO in countering pressure effects. Stabilizing and counteracting osmolytes appear to affect macromolecules through general solute, water and peptide-backbone interactions (Timasheff, 1992; Wang and Bolen, 1997). Consequently, one of the tenets of the counteracting-osmolytes hypothesis is that effects on macromolecules should be qualitatively universal, regardless of the source of protein (Yancey et al., 1982; Yancey 1994). In order to test our pressure-counteraction hypothesis further, in this study we analyzed the effects of 250 mmol l^{-1} TMAO on the structural stability of LDH homologs from related deep- and shallow-water teleosts and a mammal. Because we are interested in the potential of TMAO to stabilize proteins under deep-sea conditions, we employed high pressures and a concentration of TMAO on the high end of the range of values observed in deep-living species (Gillett et al., 1997; Kelly and Yancey, 1999). The LDH homologs were tested with the less stabilizing osmolyte glycine as a comparison. We used bovine trypsin to test for pressure-enhanced destabilization and exposure of the protein to digestion.

Materials and methods

Specimens

The three scorpaenid fish species used were *Sebastes melanops* (Girard, 1856), *Sebastolobus alascanus* (Bean, 1890) and *Sebastolobus altivelis* (Gilbert, 1893). As an adult, *S. melanops* is common at depths less than 150 m, with a maximum reported depth of 366 m; *S. alascanus* is commonly found at 100–850 m, with a maximum reported depth of 1524 m, and *S. altivelis* at 305–1755 m, with a maximum reported depth of 1755 m (Orr et al., 1998). The two *Sebastolobus* species overlap in their depth ranges, but *S. altivelis* is always more common at greater depths (Hubbs, 1926; Siebenaller and Somero, 1978; Wakefield and Smith, 1990; Lauth et al., 1997). Fishes were collected by otter trawl on cruises of the R/V Wecoma off the coast of Oregon, USA. Of the specimens used in this study, *S. melanops* was taken at 80 m, *S. alascanus* at 380–415 m and *S. altivelis* at 600–1030 m. Tissues were frozen on dry ice at sea and transported to the laboratory where they were maintained at –80 °C until used.

LDH purification

Muscle-type (A_4-) lactate dehydrogenase (LDH, EC 1.1.1.27, L-lactate:NAD^+ oxidoreductase) homologs were purified from white skeletal muscle by affinity chromatography on an oxamate amino-hexyl-Sepharose 4B column following the procedures of Spielmann et al. (1973) as modified by Yancey and Somero (1978). There was one additional modification: the A_4-LDH homologs were eluted with 10 mmol l^{-1} sodium pyruvate. The enzyme was concentrated by pressure filtration under N_2 with a PM-30 filter (Amicon Co., Lexington, MA) and stored at 4 °C as an ammonium sulphate precipitate. Each of these preparations had a single band when stained with 0.25 % Serva Blue R (Crescent Chemical Co., Hauppauge, NY, USA),

25 % 2-propanol and 10 % acetic acid, following electrophoresis in the presence of sodium dodecyl sulphate in 1.5 mm thick, 12.5 % polyacrylamide slab gels (Laemmli, 1970). Bovine A_4-LDH (Sigma Type XXVI) was from Sigma Chemical Co. (St Louis, MO, USA).

Trypsinolysis of A_4-LDH and inactivation at pressure

The loss of LDH activity at pressure was determined following the general protocol described in Hennessey and Siebenaller (1987a). A_4-LDH homologs were dialyzed overnight at 4 °C against 1000 volumes of 50 mmol l^{-1} Tris-HCl, pH 7.5 at 10 °C, 100 mmol l^{-1} KCl, 5 mmol l^{-1} 2-mercaptoethanol, 1 mmol l^{-1} EDTA. Tris was used because of the low sensitivity of the pK_A value to hydrostatic pressure (–0.019 pH units/101.3 MPa; Neuman et al., 1973). EDTA and 2-mercaptoethanol were included to protect sulphydryl groups because oxidation of sulphydryls has been implicated in the pressure-inactivation of rabbit LDH (Schmid et al., 1978). The LDH concentrations were determined by the Bradford dye-binding assay (Bradford, 1976). The LDH concentrations tested were in the range 0.5–3 μg ml^{-1}.

For incubations at elevated pressures, samples were transferred to polyethylene tubing with a wall thickness of 0.9–1 mm. Air bubbles were excluded and the tubing was sealed with a pipette heat sealer (SAMCO, San Fernando, CA, USA). Samples were incubated in water-filled, high-pressure vessels maintained at 10 °C in a refrigerated circulating water bath. The high-pressure vessels, pump and gauge are described in Hennessey and Siebenaller (1985) and are modeled after those of Zobell and Oppenheimer (1950). Incubations were for 1 h. No air bubbles were observed following the incubation.

Proteolysis of each A_4-LDH with L-1-tosylamide-2-phenylethylchloromethyl ketone-treated bovine pancreas trypsin (TPCK-trypsin, EC 3.4.21.4) was performed at 10 °C and the indicated pressures. This temperature was chosen to maintain the native conformation of the LDH homologs from the cold-adapted fishes. Stock solutions of TPCK-trypsin and bovine serum albumin (BSA) were prepared in 1 mmol l^{-1} HCl, pH 3.3. At each pressure, the following conditions were tested in the presence and absence of TPCK-trypsin: (1) A_4-LDH with no added solute; (2) A_4-LDH plus 250 mmol l^{-1} TMAO; (3) A_4-LDH plus 250 mmol l^{-1} glycine. For incubations without TPCK-trypsin, an equivalent concentration of BSA was used. Each set of conditions was replicated 5–10 times. The final incubation conditions were 50 mmol l^{-1} Tris-HCl, pH 7.5 at 10 °C, 100 mmol l^{-1} KCl, 1 mmol l^{-1} EDTA, 5 mmol l^{-1} 2-mercaptoethanol, A_4-LDH, solute and TPCK-trypsin or BSA. Previous studies (Hennessey and Siebenaller, 1987a; Davis and Siebenaller, 1992) and preliminary experiments showed differences in the susceptibility of these enzymes to proteolysis, both at atmospheric and at elevated pressures, and to denaturation by pressure. Because our primary goal is to test the stabilizing effects of osmolytes, we chose pressures and LDH and trypsin concentrations for each species that would provide significant, but not complete, loss of LDH activity during an hour's incubation. For the *Sebastolobus* species and *Bos taurus*, 0.5 mg ml^{-1} trypsin was used; for *Sebastes melanops*, 0.1 mg ml^{-1} was used.

Samples were removed and LDH activity was determined by following the decrease in absorbance at 340 nm in an assay mixture containing 80 mmol l^{-1} imidazole-HCl, pH 7.06 at 15 °C (pH 6.98 at 20 °C), 4 mmol l^{-1} sodium pyruvate and 150 μmol l^{-1} NADH. Assays were conducted in a Perkin Elmer (Oak Brook, IL) Lambda 3B spectrophotometer at 15 °C and atmospheric pressure. Temperature was maintained by a Lauda RC6 refrigerated circulating water bath (Brinkman Instruments, Westbury, NY).

Tryptic activity

To evaluate the effects of 250 mmol l^{-1} TMAO and increased hydrostatic pressure, tryptic activity was assayed at 10 °C using the artificial substrate *N*-benzoyl-L-arginine ethyl ester (BAEE, Graßl and Walter, 1983). The assay mixture contained 50 mmol l^{-1} Tris-HCl, pH 8.0 at 10 °C, 20 mmol l^{-1} $CaCl_2$ and 0.96 mmol l^{-1} BAEE, with either 250 mmol l^{-1} or no added TMAO. TPCK-trypsin was prepared as a stock solution in 1 mmol l^{-1} HCl, pH 3.3. The absorbance change at 255 nm was followed in a 3 ml volume high-pressure optical cell, modeled after Mustafa et al. (1971), which was placed in the sample compartment of a Perkin Elmer Lambda 3B spectrophotometer. Temperature was maintained with a circulating refrigerated water bath. To prevent condensation on optical surfaces, air was passed through a bed of $CaSO_4$ and blown into the sample compartment.

Reagents

The chemicals, biochemicals, resins for column chromatography and trypsin were purchased from Sigma Chemical Co. Water was processed to a resistivity of 10–18 MΩ cm with a four-bowl Milli-Q filtration system (Millipore, Bedford, MA, USA).

Statistical analysis

Comparisons were made using a Student's *t*-test or analysis of variance (ANOVA) with a Tukey–Kramer multiple-comparisons test (Sokal and Rohlf, 1995; GraphPad Instat, version 3.00 for Windows 95, GraphPad Software, San Diego, CA, USA).

Results

Effects of TMAO and increased pressure on trypsin activity

Preliminary experiments assessed the effects of added solute (250 mmol l^{-1} TMAO) on trypsin using the artificial substrate BAEE (0.96 mmol l^{-1}). The absorbance change at 255 nm was followed. TMAO had no effect on the hydrolysis of BAEE (P>0.05, for three independent experiments of 3–5 replicates) compared to that with no added solute. The addition of TMAO to the reaction mixture did not significantly alter the pressure sensitivity of the reaction. Previous studies have indicated that hydrostatic pressure can affect tryptic activity, sometimes with a slight enhancement, but the sign and magnitude of the

pressure effects are dependent on the substrate and the pressure range (Kunugi et al., 1982; Hennessey and Siebenaller, 1987a; Groß et al., 1993). Thus, increased inactivation of LDH at increased pressure may result in part from stimulation of tryptic activity by pressure. However, because there are differential effects among species (Hennessey and Siebenaller, 1987a; Davis and Siebenaller, 1992), differences among the A_4-LDH substrates will influence the response to pressure.

Effects of trypsinolysis and increased pressure on LDH activity

The results of these experiments are summarized in Fig. 1 and Table 1. For all species, incubation for 1 h at atmospheric pressure in the presence of bovine serum albumin did not result in any loss of LDH activity. To determine whether pressure caused an enhancement of proteolysis, we calculated the expected decrease in LDH activity as the product of the effects of pressure and proteolysis separately.

Sebastolobus altivelis

At atmospheric pressure without osmolytes, following a 1 h incubation, LDH activity decreased 13.2 % by trypsinolysis. This was not significantly affected by the addition of 250 mmol l^{-1} glycine; in contrast, the addition of TMAO at atmospheric pressure reduced proteolysis, with LDH activity declining only 7.3 %. Denaturation at 101.3 MPa, with no trypsin and in the presence of 0.5 mg ml^{-1} BSA, was minimal compared to the other species, with LDH activity decreasing 9 % during the 1 h incubation. This decline was not significantly affected by the addition of osmolytes. Tryptic digestion of LDH for 1 h at 101.3 MPa resulted in activity decreasing by 31.8 %. This was greater than the 21 % decline calculated from a combination of pressure-induced and proteolytic loss rates. While glycine had no effect on the loss from tryptic digestion at 101.3 MPa, the decrease was only 16.2 % in the presence of TMAO.

Sebastolobus alascanus

At atmospheric pressure with no osmolytes, trypsinolysis resulted in a 52–58 % decline in activity (two independent sets of experiments). The decline was 43–49 % in the presence of TMAO (an average 20.5 % increased stability). The effect of glycine was similar, with a decline in activity of 43–53 % (an average 15.4 % increased stability), but the effect was only significant in one of the two experiments. Denaturation in the presence of 0.5 mg ml^{-1} BSA, with no trypsin, increased with

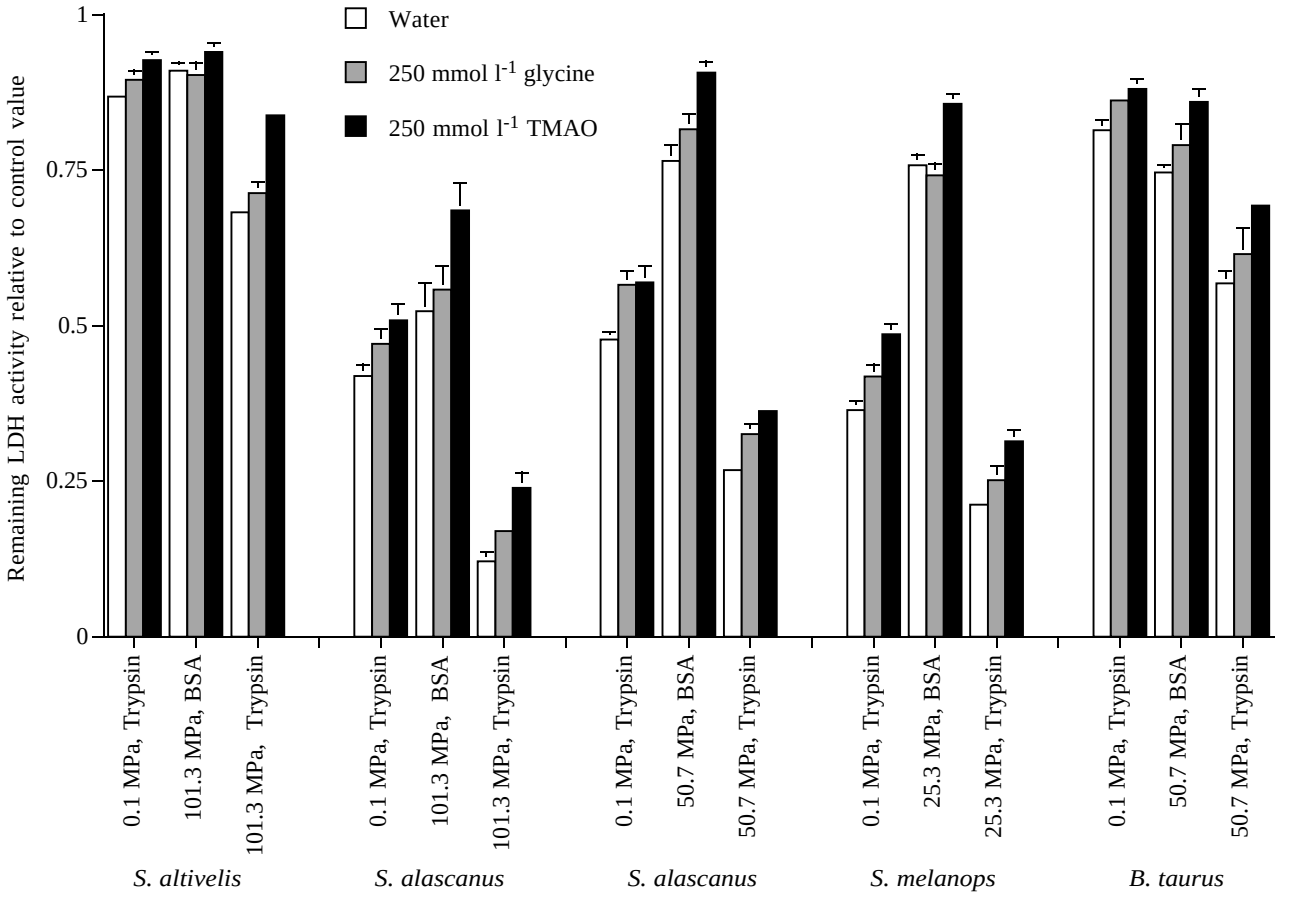

Fig. 1. The effects of added solutes at the indicated hydrostatic pressures, and of trypsin, on the denaturation of A_4-LDH homologs from three scorpaenid fishes, *Sebastolobus altivelis*, *S. alascanus* and *Sebastes melanops*, and a mammal, *Bos taurus*. For each species, the LDH activity is normalized to the activity at 0.1 MPa with no added trypsin. Values are means ± S.E.M.; each set of experimental conditions involved 5–10 replicates; see Table 1 for statistics. BSA, bovine serum albumin; TMAO, trimethylamine *N*-oxide.

Table 1. *Probability values for intraspecific comparisons of the effects of solutes at different pressures and additions of trypsin or bovine serum albumin*

	Trypsin		BSA
Sebastolobus altivelis	0.1 MPa	101.3 MPa	101.3 MPa
No addition *versus* glycine	n.s.	n.s.	n.s.
No addition *versus* TMAO	<0.01	<0.001	n.s.
Glycine *versus* TMAO	n.s.	<0.001	n.s.
Sebastolobus alascanus	0.1 MPa	50.7 MPa	50.7 MPa
No addition *versus* glycine	<0.05	<0.01	n.s.
No addition *versus* TMAO	<0.01	<0.001	<0.001
Glycine *versus* TMAO	n.s.	<0.05	<0.05
Sebastolobus alascanus	0.1 MPa	101.3 MPa	101.3 MPa
No addition *versus* glycine	n.s.	n.s.	n.s.
No addition *versus* TMAO	<0.05	<0.001	<0.05
Glycine *versus* TMAO	n.s.	<0.05	n.s.
Sebastes melanops	0.1 MPa	25.3 MPa	25.3 MPa
No addition *versus* glycine	n.s.	n.s.	n.s.
No addition *versus* TMAO	<0.001	<0.001	<0.001
Glycine *versus* TMAO	<0.05	n.s.	<0.001
Bos taurus	0.1 MPa	50.7 MPa	50.7 MPa
No addition *versus* glycine	<0.05	n.s.	n.s.
No addition *versus* TMAO	<0.01	<0.01	<0.01
Glycine *versus* TMAO	n.s.	n.s.	n.s.

Analysis of variance with a Tukey–Kramer multiple-comparisons test among solutes was performed for the indicated pressures and protein additions. Each set of experimental conditions involved 5–10 replicates. n.s., not significant at the P=0.05 level. The means and S.E.M. are displayed in Fig. 1.

TMAO, trimethylamine *N*-oxide; BSA, bovine serum albumin.

increasing incubation pressure. After 1 h with no osmolytes, LDH activity decreased 23.5 % at 50.7 MPa and 47.7 % at 101.3 MPa. TMAO but not glycine was effective in stabilizing LDH activity. LDH activity after incubation with 250 mmol l^{-1} TMAO decreased only by 9.3 % (18.6 % higher than control) at 50.7 MPa, and only by 31.5 % (31.0 % higher than control) at 101.3 MPa. Trypsinolysis with no osmolytes resulted in 73.2 % and 87.9 % decreases in activity at 50.7 and 101.3 MPa, respectively. Again, the decline was greater than would be due to a simple combination (65.7 % and 73.2 %, respectively). With TMAO, the decreases were 63.7 % at 50.7 MPa (35.5 % higher than controls) and 76.1 % at 101.3 MPa (97.5 % higher than controls). Glycine was effective only at 50.7 MPa, with a 67.4 % decrease in activity (21.6 % higher than controls).

Sebastes melanops

At atmospheric pressure without osmolytes, activity of this LDH was decreased by 63.6 % with trypsin, but only by 51.4 % with TMAO (33.5 % higher); glycine had no effect. Denaturation of LDH at 25.3 MPa, with 0.1 mg ml^{-1} BSA and no trypsin, resulted in a decrease of 24.2 % without osmolytes, but only 14.3 % with 250 mmol l^{-1} TMAO (13.1 % higher); 250 mmol l^{-1} glycine was again ineffective. Trypsinolysis at 25.3 MPa resulted in a decrease of 78.8 % without osmolytes (compared to an expected 72 % based on a simple combination), but only 68.6 % with 250 mmol l^{-1} TMAO (48.1 % higher); glycine was not effective in reducing tryptic digestion.

Bos taurus

At atmospheric pressure without osmolytes, activity of this mammalian LDH was decreased by 18.6 % by trypsin. Both glycine and TMAO were effective in reducing this inactivation, to 13.8 % and 11.9 %, respectively (6–8 % higher), and with no significant difference in the efficacy of the two osmolytes. However, only TMAO significantly decreased denaturation at 50.7 MPa pressure in the presence of 0.5 mg ml^{-1} BSA and no trypsin: pressure resulted in a decrease of 25.4 % without osmolytes, but only 14 % with TMAO (15.3 % higher). At 50.7 MPa, again TMAO but not glycine was effective in reducing the proteolytic inactivation of LDH: pressure and trypsin resulted in a decrease of 43.2 % without osmolytes (compared to an expected 39 % based on a combination), but only 30.7 % with TMAO (22 % higher).

Discussion

We have coupled proteolysis with pressure to probe the protective effects of osmolytes on enzymes perturbed by high hydrostatic pressure. This combination of factors has proved to be a sensitive method for studying structural perturbation (Hennessey and Siebenaller, 1987a; Davis and Siebenaller, 1992). TMAO increased the measures of LDH stability that we examined, i.e. susceptibility to proteolytic inactivation, inactivation by high pressures and the two factors combined. For all the species except *S. altivelis*, which was minimally affected by 101.3 MPa pressure, TMAO stabilized the LDH homologs and reduced denaturation by pressure alone. The osmolyte also reduced the rates of proteolysis for all of the LDH homologs tested, with the protective effect apparent at all the pressures tested (Fig. 1, Table 1). In contrast to TMAO, the ‘compatible’ amino acid glycine did not decrease pressure denaturation in any case, and was generally less effective than TMAO in reducing the rate of proteolysis. These results support the hypothesis that the high TMAO levels accumulated in deep-sea animals may counteract those adverse effects of pressure not fully eliminated by protein structural evolution (Gillett et al., 1997). Also, dissociated monomers of multisubunit enzymes may ‘drift’ into conformations which render reassembly unlikely or which lead to loss of activity (King and Weber, 1986; Weber, 1986). The stabilizing effects of TMAO may counteract such losses. It is noteworthy that although the elevated pressures used in the present study (25.3–101.3 MPa) were greater than those typically encountered by these fishes in situ (up to 17.8 MPa), TMAO was still able to provide significant protection from pressure-induced destabilization both in the absence and presence of a proteolytic enzyme.

Again, it is worth re-emphasizing that the A_4-LDH homologs of cold-adapted, shallow-living teleosts are more

susceptible to proteolysis than are the orthologous enzymes of deep-living species (Fig. 1; Hennessey and Siebenaller, 1987a). Even non-denaturing pressures increase the rate of proteolysis of the A_4-LDH homolog of *Sebastes melanops* (Hennessey and Siebenaller, 1987a). Nevertheless, the greater-than-expected declines in LDH activity with proteolysis at elevated pressures indicate that pressure can enhance proteolytic denaturation, even in the more resistant homologs (Fig. 1; Hennessey and Siebenaller, 1985, 1987a; Davis and Siebenaller, 1992). This may result from pressure altering the conformation and aggregation state of the substrate proteins (Hennessey and Siebenaller, 1987a; Davis and Siebenaller, 1992). As determined by reverse-phase high-pressure liquid chromatography (HPLC) peptide mapping, pressure appears not to expose new cleavage sites, but to increase the accessibility of those cleavage sites exposed at atmospheric pressure (Davis and Siebenaller, 1992).

Although we have not examined interspecific differences in the present study, inspection of the data suggests differences between the *Sebastolobus* species (Fig. 1). The amino acid compositions and reverse-phase HPLC mapping of tryptic peptides from these LDH homologs do not reveal any differences in tryptic cleavage sites (Siebenaller, 1984). The apparent differences in proteolysis and pressure sensitivity may result from posttranslational modifications. Also, the greater stability of the mammalian homolog in comparison to homologs from shallow-living teleosts (Fig. 1) is a phenomenon that has been noted for other mammalian proteins, and has been suggested to be a consequence of high-temperature adaptation (e.g. Somero and Siebenaller, 1979; Swezey and Somero, 1982, 1985; Siebenaller and Somero, 1989).

That TMAO had a pressure-protective effect on both a mammalian and on teleost enzymes (Fig. 1, Table 1) suggests a universal effect. Our pressure-counteracting hypothesis is based on the well-documented protein-stabilizing capabilities of TMAO that appear to be universal for other denaturants. For example, with proteins from several taxa, TMAO (up to 200 mmol kg^{-1}) can offset the destabilizing effects of the main and noncompatible osmolyte in elasmobranchs, namely urea (Yancey, 1985, 1994). Recently it has also been shown that in the absence of a denaturant, TMAO can rescue misfolded mammalian proteins such as the cystic fibrosis transmembrane conductance regulator (Welch and Brown, 1996). How stabilization effects might be universal (Yancey et al., 1982) was first indicated by the work of Timasheff (1992) and colleagues, who showed that stabilizing osmolytes are preferentially excluded from the hydration layer of proteins. As a result, any tendency of a protein to expose more hydrated surface area (e.g. subunit dissociation, peptide unfolding, ligand release) will be entropically unfavorable in the presence of these osmolytes. Molecular dynamic simulations indicate that TMAO tightly coordinates water molecules (Noto et al., 1995), perhaps explaining its inability to interact with water around proteins. Also, recent work by Wang and Bolen (1997) and Baskakov and Bolen (1998) has demonstrated that unfavorable interactions between TMAO and peptide backbones explain the strong exclusion of methylamines (although this would not explain binding effects). All these studies suggest that counteraction between TMAO and pressure may simply be additivity of independent stabilizing and perturbing forces. However, how TMAO affects protein structure and function under pressure remains speculative.

The accumulation of TMAO by deep-living animals may be an important adaptive mechanism complementing the evolution of pressure-resistant proteins in order to allow successful colonization of the deep ocean habitat. High intracellular TMAO concentrations may reduce protein turnover, lowering metabolic costs. Based on the observation that deep-living vertebrates and invertebrates accumulate this compound (Gillett et al., 1997; Kelly and Yancey, 1999), phylogenetically unrelated groups may have independently discovered the proposed pressure-counteracting effects of TMAO.

This work was supported by National Science Foundation Grant IBN–9407205. We thank Julie A. Spoelker for assistance in the laboratory.

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