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Muscle-type lactate dehydrogenase (M_4-LDH; EC 1.1.1.27), cytoplasmic malate dehydrogenase (MDH; EC 1.1.1.37) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) from several shallow- and deep-living marine teleost fishes were examined for susceptibility to inactivation by hydrostatic pressure and proteolysis. The molecular mass and subunit aggregation state of these enzymes are identical to those of other vertebrate homologues. The pressures required to half-inactivate the LDH homologues of six confamilial macrourid fishes are related to the pressures which the enzymes are exposed to *in vivo*. LDH homologues of deeper-living species tend to require greater pressures for inactivation than do shallow-occurring species. Fish MDH and GAPDH homologues are more resistant to inactivation by pressure than are LDH homologues from the same species. Incubation of fish enzymes for 1 h at 10°C and 1750 atm pressure results in 65 to 100% loss of LDH activity, 6 to 14% loss of MDH activity, and no loss of GAPDH activity. Pressures required to inactivate dehydrogenases *in vitro* are greater than pressures normally encountered in the habitat of the species. Among the homologues of M_4-LDH, MDH and GAPDH, resistance to inactivation by hydrostatic pressure appears to be determined by the subunit aggregation state of

the enzyme and the number of subunit interactions. Enzymes with more subunits and more subunit interactions tend to be less stable at high pressures. This is consistent with the model of Paladini and Weber (Biochemistry, 20:2587-2593, 1981) which predicts that susceptibility to dissociation of oligomeric proteins is controlled by the small "free volumes" at the intersubunit boundaries. Hydrostatic pressure, by affecting protein aggregation state and conformation, may increase susceptibility to proteolysis. At 10^{o}C and atmospheric pressure, the rates of inactivation by trypsin and subtilisin are three to four times greater for LDH homologues from shallow-living fishes than for homologues from deep-living fishes. Hydrostatic pressure (as low as 200 atm) has a greater influence on proteolytic inactivation of LDH homologues of shallow-living fishes than for homologues of deep-living fishes. Fish MDH homologues are less susceptible to proteolytic inactivation than LDH homologues from the same species. MDH homologues of shallow- and deep-occurring fishes are inactivated to a similar extent by proteases both at atmospheric pressure and at 1750 atm pressure. Under the same conditions fish GAPDH homologues are not inactivated by trypsin or subtilisin. Enzymes that are less susceptible to inactivation by pressure appear to be less susceptible to inactivation by proteases. Conformational perturbations in the structure of shallow-occurring LDH homologues that increase their susceptibility to inactivation by proteases at elevated pressure are not apparent in MDH and GAPDH homologues of the same species. The structural integrity of MDH and GAPDH homologues of marine fishes appear to be pre-adapted to the hydrostatic pressures of the deep-sea.

Biochemical Adaptation of Deep-Sea Fishes: Susceptibility of Dehydrogenases to Pressure-Inactivation and Proteolysis

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Introduction

Adaptation to hydrostatic pressure may be an important aspect of the biology of deep-living marine organisms (Somero et al. 1983). Hydrostatic pressure may disrupt protein structure and function, and has been used as a probe of protein conformation and aggregation state (see reviews by Heremans 1982; Jaenicke 1981, 1983; Morild 1981; Somero et al. 1983; Weber and Drickamer 1983). Penniston (1971) hypothesized that, because of the susceptibility of multimeric enzymes to inactivation by hydrostatic pressure, deep-sea organisms would utilize multimeric enzymes with strengthened protein-protein interactions or would use monomeric enzymes capable of catalyzing reactions requiring a multimeric enzyme in other organisms. The similarity of zymogram patterns of enzymes from shallow- and deep-living fishes (Siebenaller 1978, 1984a, b; Wilson and Waples 1983, 1984) suggests that the subunit aggregation states of enzyme homologues from shallow- and deep-living fishes are similar.

Perturbation of protein structure by hydrostatic pressure could have profound effects on the metabolism of deep-sea organisms. Shifts in the subunit aggregation equilibrium of multimeric enzymes could affect _in vivo_ activities of the enzymes. Pressure-induced changes in protein aggregation state and conformation could increase the susceptibility of proteins to _in vivo_ degradation. Proteins that are not protected, i.e. incorporated into the appropriate structure or

aggregation state and in the proper conformation, are more readily hydrolyzed by the basal system of proteases (Wheatley 1984).

In this dissertation, the effects of hydrostatic pressure on homologues of three structurally and functionally similar multimeric enzymes from shallow- and deep-living marine fishes are examined. The susceptibility of these enzymes to inactivation by pressure is determined, both from the effects of pressure on these multimeric proteins and from inactivation resulting from enhanced proteolysis of pressure-perturbed enzymes. The three enzymes studied, muscle-type (M_4) lactate dehydrogenase (LDH, EC 1.1.1.27; L-lactate: NAD^+ oxidoreductase), a tetramer, cytoplasmic malate dehydrogenase (MDH, EC 1.1.1.37; L-malate: NAD^+ oxidoreductase), a dimer, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12; D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase), a tetramer, have a number of structural similarities. The subunits of the three enzymes are of similar size and conformation. Each contains a nucleotide binding domain containing similar "supersecondary" structure (Rossmann and Argos 1978). Subunit interactions are almost identical along the Q-axis of LDH and MDH (Birktoft and Banaszak 1983). However, LDH and GAPDH are dissimilar in their subunit interactions and in the aggregation of their dimeric halves to form the native tetramer molecule (along the P- and R-axes); the GAPDH dimer-of-dimers is assembled backward relative to LDH (Moras et al. 1975; Buehner et al. 1975).

LDH homologues of 14 marine fishes and MDH and GAPDH homologues of five marine fishes were examined. These fishes occur from the

surface to 4693 m. Hydrostatic pressure increases 1 atm for every 10 m increase in depth, and thus these species cover a pressure range of 470 atm. Pressures as low as 68 atm double the K_m of coenzyme values for NAD-dependent dehydrogenases of shallow-living marine fishes (Siebenaller and Somero 1978, 1979; Siebenaller 1984a). Thus at elevated pressures the function of these enzymes is disrupted by their decreased affinity for coenzyme. Pressures of less than 500 atm inactivate multimeric enzymes from a variety of sources (Jaenicke 1981). Inactivation appears to be due to dissociation of the active enzyme multimers to inactive monomers. Hydrostatic pressure, by affecting subunit aggregation state and protein conformation, may influence protein turnover rates by increasing their susceptibility to proteolysis. For this reason two proteases, trypsin (EC 3.4.21.4) and subtilisin Carlsberg (EC 3.4.4.16), were used as probes of the effects of pressure on proteolytic inactivation.

Comparing the stability of homologous enzymes from shallow and deep-living fishes may reveal 1) the importance of enzyme inactivation by hydrostatic pressure in deep-sea organisms and its contribution to the low enzyme activity found in deep-sea animals, 2) whether patterns of pressure adaptation are similar in structurally and functionally related enzymes from the same deep-sea species, and 3) how much evolution of enzyme structure and function is necessary for colonization of the deep-sea.

BACKGROUND

Adaptation to hydrostatic pressure

The deep-sea is defined as that region of the ocean below 200 m (Hessler 1974; Hessler and Sanders 1967). It is characterized by low temperature (typically less than 5^{o}C), high hydrostatic pressure (up to 1100 atm) and reduced biomass relative to surface waters. High hydrostatic pressure and low ambient temperatures can affect biological processes by changing chemical reaction equilibria and by slowing reaction rates (Johnson et al. 1974; Morild 1981). Understanding the ways that biological systems have compensated for these conditions is important to our understanding of life processes in the deep-sea.

The effects of hydrostatic pressure on biological systems have been studied for almost 100 years beginning with the work of Regnard and Certes (Regnard 1891). Experiments testing the effects of pressure on proteins (Bridgman 1914; Larson et al. 1918; Bridgman and Conant 1929; Gidding et al. 1929; Bassett et al. 1933) led to the general conclusion that high hydrostatic pressure denatures proteins. Subsequent studies of the effects of pressure on proteins have shown that elevated pressure can denature, inhibit, stimulate or have no effect on the function of proteins (e.g. Johnson et al. 1974; Penniston 1971; Weber 1980; Jaenicke 1981, 1983; Heremans 1982).

The theoretical bases for pressure effects in biology and chemistry (Laidler 1951; Johnson and Eyring 1970; Johnson et al. 1974; Morild 1981) provide a mechanism by which pressure affects biochemical reactions, i.e. volume changes. For example, a conversion of

reactants to products in a chemical reaction may involve a change in the volume of the solvent-solute system. This volume change may be caused by changes in the volume of product molecules relative to reactant molecules or by rearrangement of the solvent molecules around the reactants and products. If an increase in volume accompanies a reaction, increased pressure will inhibit the reaction. If the volume decreases, pressure will stimulate the reaction. Only if there is no net volume change will a reaction be pressure insensitive.

Aggregation of protein subunits to form the native multimer produces an increase in the solvent-solute volume due to the formation of small free volumes at the intersubunit boundaries. Thus high hydrostatic pressure will tend to dissociate multimeric proteins (Paladini and Weber 1981).

Volume changes due to hydration changes (i.e. the ordering of water molecules or displacement of ordered water molecules surrounding metabolites and/or macromolecular catalysts) are the major contributor to system volume changes during biochemical reactions (Low and Somero 1975; Hochachka and Somero 1984). Two biochemical processes that are consistently accompanied by such volume changes are enzyme-ligand interactions during the course of reaction catalysis, and changes in enzyme conformation and subunit aggregation state during catalysis and regulation. If catalysis or regulation of catalysis is disrupted for even a small subset of the thousands of intracellular enzymes, metabolic imbalances can occur that might impair function of the organism. Thus colonization of the deep-sea may require that biochemical reactions that are pressure-sensitive evolve a pressure-

insensitive form, e.g. by decreasing the volume change accompanying the biochemical reaction (Siebenaller and Somero 1979).

The amount of evolutionary change required to convert a biochemical reaction from pressure-sensitive to pressure-insensitive appears to be very small. The pressure-sensitive M_4-LDH homologue of shallow-living Sebastolobus alascanus appears to have only minor differences in primary structure from the pressure-insensitive homologue of deep-living S. altivelis (Siebenaller 1984a). This implies that only minor changes in the primary structure of pressure-sensitive enzymes are required to adapt a shallow-living species to the deep-sea. However, such changes may affect other aspects of enzyme function (e.g. by decreasing catalytic efficiency [Somero and Siebenaller 1979]) resulting in decreased competitive ability of the species in its former habitat. As with the Sebastolobus congeners, which are thought to have evolved from a single, shallow-living ancestor, evolution of a new, deep-living species may result.

Pressure effects on protein structure and function

Investigations of the effects of hydrostatic pressure on protein structure and function provide two types of information. First, pressure is used as a variable to examine the intra- and intermolecular forces that stabilize proteins. Multimeric enzymes tend to be inactivated by elevated pressure (Penniston 1971). Many multimeric enzymes have been examined for susceptibility to inactivation by hydrostatic pressure. These include enzymes from fishes (ATPase and citrate synthase from Antimora rostrata and

Onchorhynchus sp. (Moon 1975; Hochachka et al. 1975), mammals (muscle- and heart-type lactate dehydrogenase from pig [Müller et al. 1981, 1982] and muscle-type LDH from rabbit [Schade et al. 1980]), birds (lysozyme from egg white [ZoBell and Kim 1972]) and microorganisms (e.g. phenylglucosidase and pronase from Streptomyces griseus [Berger 1958; ZoBell and Kim 1972], pyrophosphatase from Bacillus stenothermophilus [Morita and Mathemeier 1964], phosphoenolpyruvate carboxylase from Escherichia coli [Izui 1973], cellulase from Aspergillus niger and agarase from Pseudomonas atlantica [ZoBell and Kim 1972]). The pressures of half-inactivation for the above enzymes ranged from 300 atm to greater than 8000 atm pressure. Many of the enzymes studied are dissociated by pressures typical of the deep-sea (i.e. less than 1100 atm). This raises a question central to this thesis. Do the enzymes of organisms colonizing the deep-sea require modification to withstand deep-sea pressures? M_4-LDH is the most thoroughly studied enzyme in terms of the effects of pressure on enzyme kinetics (Somero et al. 1983) and the thermodynamics of inactivation/dissociation by hydrostatic pressure (Jaenicke 1983; Somero et al. 1985). LDH tetramers appear to be inactivated by pressure via a shift in the reaction equilibrium of active tetramer to inactive monomer (Müller et al. 1981, 1982). Thus LDH homologues are a logical focus for investigations of the effects of hydrostatic pressure on characteristics of deep-sea enzymes.

Hydrostatic pressure is also studied as an environmental factor affecting the metabolism and biochemistry of deep-sea fishes. Deep-sea fishes have different proximate composition of their bodies than

shallow-living fishes (e.g. Childress and Somero 1979; Somero and Sullivan 1980; Siebenaller et al. 1982; Siebenaller 1984b) as well as different structural and functional characteristics of their biochemical components, i.e. enzymes (Siebenaller and Somero 1978, 1979; Siebenaller 1984a), structural proteins (Swezey and Somero 1982, 1985) and membrane lipids (Cossins and Macdonald 1984).

The reduced enzyme activity levels in the white muscle tissue of deep-living fishes relative to shallow-living fishes is due primarily to depth-related differences in feeding and locomotory habits (Childress and Somero 1979; Sullivan and Somero 1980; Siebenaller et al. 1982). Scarcity of food in the deep-sea appears to dictate a more sedentary life style and thus a decreased need for metabolic activity and a decreased need for biochemical machinery to support that activity. Reduced enzyme activity may result from either a decreased concentration of active enzyme, a decreased efficiency of the enzyme or both (Somero and Siebenaller 1979). Another possibility, addressed in this thesis, is that high pressures in the deep-sea might cause decreased enzyme activity *in vivo* via a shift in the subunit aggregation equilibrium.

Enzymes of deep-sea fishes function at low temperatures and high pressures where the function of some enzymes of shallow-living fishes may be impaired (Somero et al. 1983). NAD-dependent dehydrogenases of shallow-living *Sebastolobus alascanus* show an approximate doubling of their K_m of coenzyme values at 68 atm pressure relative to atmospheric pressure. Homologues from deep-living *S. altivelis* show little or no increase in K_m of coenzyme values at pressures of 68 atm or greater

(Siebenaller 1984a). This difference in the pressure-sensitivity of the K_m of coenzyme has been found in all M_4-LDH homologues of shallow- and deep-living fishes examined (Siebenaller and Somero 1978; 1979). Increased K_m of coenzyme reduces the affinity of the enzyme for coenzyme resulting in impaired catalytic function and an imbalance in metabolic control. Reduction of the pressure-sensitivity of the K_m of coenzyme in NAD-dependent dehydrogenases of deep-living fishes may thus be an important and ubiquitous feature of adaptation to the deep-sea (Siebenaller 1984a). Such adaptations to hydrostatic pressure could be essential for successful colonization of the deep-sea (Siebenaller and Somero 1978, 1979; Siebenaller 1984a). Are other structural and/or functional features of NAD-dependent dehydrogenases of deep-living fishes ubiquitously adapted to hydrostatic pressures of the deep-sea?

CHAPTER II

Pressure Inactivation of Tetrameric Lactate Dehydrogenase Homologues of Confamilial Deep-Living Fishes

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SUMMARY

The susceptibility to inactivation by hydrostatic pressure of the tetrameric (Figure II.1) muscle-type (M_4) lactate dehydrogenase homologues (LDH, EC 1.1.1.27; L-lactate: NAD^+ oxidoreductase) from six confamilial macrourid fishes was compared at $4^{\circ}C$. These marine teleost fishes occur over depths of 260 to 4815 m. The pressures necessary to half-inactivate the LDH homologues are related to the pressures which the enzymes are exposed to in vivo (Table I); higher hydrostatic pressures are required to inactivate the LDH homologues of the deeper-occurring macrourids. The resistance of the LDH homologues to inactivation by pressure is affected by protein concentration (Figure II.3). After an hour of incubation at pressure, the percent remaining activity approaches an asymptotic value (Figure II.2). The inactivation of the macrourid LDH homologues by pressure was not fully reversible. Assuming that inactivation by pressure was due to dissociation of the native tetramer to monomers, apparent equilibrium constants (K_{eq}) were calculated. Volume changes (ΔV) were calculated over the range of pressures for which plots of ln K_{eq} versus pressure were linear (Figure II.4). The ΔV of dissociation values of the macrourid homologues range from -219 to -439 ml/mol (Table II.1). Although the hydrostatic pressures required to inactivate the LDH homologues of the macrourid fishes are greater than those which the enzymes are exposed to in vivo, the pressure-stability of these enzymes may reflect the resistance of these to pressure-enhanced proteolysis in vivo.

INTRODUCTION

Adaptation to hydrostatic pressures in the marine environment may be an important aspect of the biology of deep-living organisms (Somero et al., 1983). Hydrostatic pressure may disrupt protein structure and function, and has been used as a probe of protein denaturation phenomena, for instance, conformational changes, polypeptide chain unfolding and subunit aggregation state (e.g., see reviews by Heremans 1982; Jaenicke 1981;, 1983; Morild 1981; Somero et al. 1983; Weber and Drickamer 1983). Disruption of protein structure is thus known to be an important locus of pressure perturbation.

Penniston (1971) has argued that, because of the susceptibility of multisubunit enzymes to pressure-inactivation, enzymes of deep-sea animals may be monomeric rather than multimeric, in contrast to homologous enzymes from other organisms. Zymogram patterns of enzymes from deep-sea fishes are similar to patterns observed in electrophoretic studies of shallow-living fishes (e.g., Siebenaller 1978, 1984a, b; Wilson and Waples 1983, 1984). This suggests that the subunit aggregation states of enzyme homologues from shallow- and deep-living species are similar.

Perturbation of protein structure could have profound influences on the metabolism of deep-sea organisms. For instance, are the depressed levels of enzymic activity in the white muscle tissue of deep-sea species (Childress and Somero 1979; Siebenaller and Somero 1982; Siebenaller et al. 1982; Sullivan and Somero 1980) the result of a pressure-induced shift in subunit aggregation equilibrium? To determine the importance of enzyme inactivation by hydrostatic

pressure, a study was undertaken of the effects of pressure on the tetrameric muscle-type (M_4) L-lactate dehydrogenase homologues (LDH, EC 1.1.1.27; L-lactate: NAD^+ oxidoreductase) of six confamilial marine fishes which occur over a depth gradient of 260 to 4815 m. These species of the deep-living family Macrouridae were chosen because they are phylogenetically close, experience similar temperatures, but have different depth distributions. Thus, the effects of pressure on the evolution of pressure-stability may be studied in the absence of other potentially confounding variables. Pressure increases approximately one apmosphere for every 10 m depth increase in the ocean, and thus these species experience pressures up to approximately 500 atm.

The bases of pressure effects are the volume changes which accompany biochemical processes. Pressure acts on the volume changes associated with biochemical equilibria. If a reaction proceeds with a positive volume change (the relevant volume change is that of the entire solute-solvent system), an increase in pressure will inhibit the reaction. A process with a negative volume change will be favored by increased pressure. Only if there is no net volume change will a process be pressure insensitive (Johnson et al. 1974)

MATERIALS AND METHODS

Specimens

Specimens were taken by otter trawl at their typical depths of abundance. The depths of abundance of these species are given in Table II.1. Coryphaenoides acrolepis was taken off the coast of Oregon, USA, on two cruises of the R/V Wecoma. C. leptolepis, C. carapinus, C. armatus, C. rupestris and Nezumia bairdii were taken on two cruises of the R/V Oceanus in an area south of New England, USA. Specimens were frozen on solid CO_2 at sea and transported to the laboratory where they were maintained at -85°C until used.

Chemicals

All chemicals, biochemicals and resins for chromatography were from Sigma Chemical Co. Proteins used as molecular weight standards and porcine M_4-LDH were obtained from Sigma. Water was processed through a Milli-Q water purification system (Millipore Corp.).

Enzyme purification

M_4-LDH from macrourid white muscle tissue was purified by affinity chromatography on an oxamate aminohexyl-Sepharose 4B column (1.5 x 12 cm) following the procedures of Spielmann et al. (1973) as modified by Yancey and Somero (1978). The enzyme was concentrated by pressure filtration under N_2 with a PM-10 filter (Amicon Co.) and stored at 4°C as a 90%-ammonium sulfate precipitate. Protein concentrations were determined using the Bradford (1976) Coomassie Blue (Serva Fine Biochemicals, Inc.) dye binding assay as given by Peterson (1983). Bovine serum albumin was used as the standard.

activity contained 100 mM Tris-HCl, pH 8.1 at 10°C, 20 mM $MgCl_2$, 0.4 mM oxaloacetate (neutralized with NaOH) and 150 μm NADH. The assay temperature was 5°C.

Pressure incubations

LDH aliquots were dialyzed overnight at 4°C against 2 changes of 1000 volumes of 20 mM imidazole-HCl, pH 6.98 at 20°C, 5 mM 2-mercaptoethanol and 1 mM EDTA. Imidazole was used as the buffer because of the low sensitivity of its pK_a to pressure (-0.020 pH units per 1000 atm [Marquis and Fenn 1969; Kauzmann et al. 1962]). EDTA and 2-mercaptoethanol were included to protect sulfhydryl groups. Oxidation of sulfhydryl groups has been implicated in pressure inactivation of rabbit LDH (Schmid et al. 1978). Dialyzed samples were centrifuge-filtered through a 0.45 μm nylon-66 filter (Rainin Instrument Co.) and diluted to the desired concentration with dialysis buffer.

The enzyme was incubated in 0.2 ml Microflex vials (Kontes Glass Co.) placed in a pressure bomb. These vials have an open-top cap with a teflon faced rubber liner which transmits pressure to the sample. The vials were filled with sample so as to exclude air bubbles. The vials were cleaned in 50% nitric acid, thoroughly rinsed and air dried between uses. The pressure vessels, described by ZoBell and Oppenheimer (1950), were maintained at the desired temperature (3-4°C) with a refrigerated water bath. Pressure was applied with an Enerpac model P-228 hydraulic pump filled with high grade mineral oil. The pressures used ranged from 1 to 1750 atm, the limit of our pressure apparatus. Pressure was monitored with a Marsh Masterguage

(model E4396B) with 50 atm subdivisions. The desired pressure was reached in 10 to 90 s. Temperature in the pressure vessels changed 1.5°C per 1000 atm during pressurization/depressurization. Thermal equilibrium was restored within a few minutes. Samples were incubated at pressure for 1 h and assayed for LDH activity after the 2 to 3 min necessary to remove the vials from the pressure vessel. Renaturation was negligible during this time interval. Samples were maintained at 4°C for 24 h after depressurization to determine whether the enzyme reactivated during this interval.

LDH activity was determined as described above. The pyruvate concentration used was in the optimal substrate range for each of the LDH homologues used. The assay temperature of 5 ± 0.2°C was maintained with a circulating refrigerated water bath. The change in absorbance at 340 nm was monitored in a Varian Techtron 634S spectrophotometer equipped with a Soltec model 1241 chart recorder.

Theory and data analysis

The enzyme activities of incubated samples were converted to percent activity remaining (ratio of the activity of the pressurized sample to a control incubated at atmospheric pressure).

The dissociation of tetrameric (M_4) LDH can be represented as:

M_4-LDH (active) <===> M_1-LDH (inactive) <===> M_n-LDH (inactive)

(Müller et al. 1982). M_1 is monomer and M_n is a high molecular weight aggregate. Apparent equilibrium constants (K_{eq}) were computed assuming that the two state system of active tetramer and inactive monomer were most important umder the incubation conditions employed.

Apparent equilibrium constants were computed as:

$$K_{eq} = \frac{[4C_o\ (1 - \alpha)]^4}{C_o\ (\alpha)} = \frac{[\text{monomer}]^4}{[\text{tetramer}]} = \frac{[\text{inactive LDH}]^4}{[\text{active LDH}]}$$

where C_o is the concentration (μM) of tetrameric LDH at the beginning of the incubation and α is the percent activity remaining after incubation at pressure. The value used for the molecular weight of M_4-LDH is 144,000. The apparent volume change of the dissociation of the LDH tetramer, ΔV, was determined from the slope of plots of ln K_{eq} against pressure. The pressure necessary to half-inactivate ($P_{1/2}$) LDH was determined from these regressions.

RESULTS

Subunit structure

The M_4-LDH homologues of the six macrourid species which occur over a depth gradient of 260 to 4815 m have subunit molecular weights and native molecular weights identical to the tetrameric LDH homologues of other vertebrates. The LDH homologues of the macrourid species elute at the same volume as catalytically active porcine M_4-LDH on the gel filtration column (Figure II.1). Each of the macrourid LDH preparations had a single protein band which comigrated with porcine M_4-LDH on polyacrylamide gels run in the presence of sodium dodecyl sulfate (Figure II.1). The tetrameric porcine M_4-LDH has a molecular mass of 144,000 Da and a subunit molecular mass of 36,000 Da (Kiltz et al. 1977; Everse and Kaplan 1973).

Pressure inactivation

An incubation time of 1 h was chosen after testing times from 10 min to 24 h. Inactivation approached an asymptote after approximately 1 h (Figure II.2).

A summary of the effects of hydrostatic pressure on the inactivation of the M_4-LDH homologues of the six macrourid species is presented in Table II.1. The pressures necessary to half-inactivate the LDH homologues ranged from 565 atm to 1715 atm. The depth at which a species is maximally abundant is related to the pressure necessary to half-inactivate its LDH homologue. Greater pressures are required to half-inactivate the LDH homologues of the deeper-occurring macrourid species than are required for the shallower-living species (Table II.1).

The concentration of LDH influences the susceptibility of the enzyme to inactivation by pressure. Each of the LDH homologues is more sensitive to inactivation at lower protein concentrations (Figure II.3). The addition of 0.1% bovine serum albumin does not affect the degree of pressure inactivation.

The volume changes (ΔV) associated with the inactivation of the LDH homologues were calculated under the assumption that the inactivation represents a pressure-induced change in the equilibrium between active tetramer and inactive monomer. The volume changes were calculated from least squares regressions of ln K_{eq} on pressure (Figure II.4). These plots were linear over the ranges of pressures where inactivation was detectable (i.e., values of α less than approximately 95% and greater than approximately 5%). The volume changes are given in Table II.1. The ΔV values range from -219 $\pm$ 30 ml/mol for the C. rupestris homologue to -439 $\pm$ 45 ml/mol for C. acrolepis.

The yields for reactivation of LDH after pressure inactivation were typically low, less than 40% for C. rupestris and C. acrolepis, and less than 15% for the other species. This contrasts with yields of 80% or more for reactivation of porcine M_4-LDH reported by Müller et al. (1982), and in our own hands under conditions identical to those employed with the macrourid homologues (data not shown). Reactivation of the macrourid LDH homologues was dependent upon the concentration of LDH and on the duration and magnitude of the pressure used (data not shown).

In denaturation experiments employing LDH concentrations greater

than 100 μg/ml, a flocculent precipitate was evident at the end of the pressure incubation. Large aggregates of LDH apparently formed, as has been reported for other dehydrogenases (Jaenicke and Rudolph 1983).

DISCUSSION

Penniston (1971) reasoned that because of the susceptibility of multisubunit enzymes to inactivation by pressure, enzymes of deep-sea animals must either be monomeric rather than multimeric or, if the enzymes were multimeric, subunit interactions would be more strongly stabilized by noncovalent interactions than the enzymes of other organisms. The data in Figure II.1 demonstrate that LDH homologues of the macrourid fishes are tetramers, as are the LDH homologues of other vertebrates. Other enzymes of deep-sea fishes appear to have subunit structures similar to shallow-living fishes (Siebenaller 1978, 1984a, b; Wilson and Waples 1983, 1984). The protein structures of deep-living organisms have not apparently adapted to pressure perturbation by evolving new subunit aggregation states.

The depths at which the macrourid species are maximally abundant are related to the pressures at which their enzyme homologues are half-inactivated (Table II.1). However, under the experimental conditions employed, these inactivating pressures are much greater than the pressures which the homologues are exposed to *in situ*. Studies of thermal denaturation of proteins have demonstrated an analogous result. Thermal denaturation temperatures are correlated with the cell temperatures, but these denaturation temperatures are much higher than the temperatures experienced *in vivo* (Alexandrov 1977; Hochachka and Somero 1984).

Pressure, by affecting aggregation state and conformation, may influence the rate of turnover of proteins by increasing their susceptibility to proteolysis. Basal proteolysis is constitutive and

is regulated by the availability of proteins as substrates. This is dependent in part on whether these substrates are protected, i.e., incorporated into the appropriate structure or aggregation state and in the proper conformation (Wheatley 1984). The relationship of $P_{1/2}$ with depth of maximal abundance (Table II.1) may reflect stabilization of the LDH homologues to pressure-enhanced proteolysis. Thermal stability may be a similar reflection of resistance to proteolysis (Goldberg and Dice 1974).

The pressure-induced inactivation of the macrourid LDH homologues was not fully reversible under the conditions employed. Lack of full reversibility in subunit dissociation studies of dehydrogenases is not uncommon and may be due to formation of high molecular weight aggregates (Jaenicke and Rudolph 1983). We selected a 1 h incubation time to minimize the effects of such high molecular weight aggregation and have assumed that this short incubation permits an estimate of the tetramer and monomer concentrations at equilibrium (Figure II.2).

Calculation of the apparent equilibrium constants at pressure and the ΔV values are based on assumptions about the reversibility of pressure-inactivation and about the aggregation state of the LDH subunits. These assumptions are that only the tetramer is catalytically active, that inactivation is caused by subunit dissociation to the monomer with no accumulation of dimeric intermediates, and that the inactivation is reversible, but that under the conditions we used the yield of reactivation is poor. These assumptions are based on the extensive work on pressure-inactivation of mammalian LDH homologues (Schade et al. 1980; Schmid et al. 1978,

1979; Müller et al. 1981, 1982). Our findings that denaturation is related to LDH concentration (Figure II.3) and that the ΔV of dissociation values of the macrourid homologues are of the same order as the values for porcine enzyme reported by Müller et al. (1982) are consistent with our assumptions about the basis of the pressure inactivation.

The LDH homologues of six confamilial fishes which occur over a depth gradient of 260 to 4815 m display adaptations to pressure-inactivation which are correlated with the depths at which the species are most abundant (Table II.1). This pattern among the homologues of closely related species indicates that adaptation of protein structure to perturbation by pressure is an important evolutionary component of colonization of the deep sea. The pressures necessary to half-inactivate the LDH homologues are related to the pressures which the enzymes experience *in vivo*; higher hydrostatic pressures are required to inactivate the enzyme homologues of the deeper-occurring macrourids. Although the pressures required to half-inactivate these enzyme homologues are much greater than those which they experience in nature, the structural stability of these enzymes to pressure perturbation may reflect their resistance to pressure-enhanced degradation by the *in vivo* proteolytic system.

Table II.1. The pressures at which M_4-LDH homologues of six macrourid species are half-inactivated ($P_{1/2}$) and the ΔV of dissociation. $\pm$ standard error is given. The C. rupestris values are for 3°C; all others values are for 4°C. The depth distributions are from Siebenaller et al. (1982) and Sullivan and Somero (1980). The concentrations of the LDH homologues (μg/ml) are given in parentheses after the species.

SPECIES	DEPTH OF MAXIMAL ABUNDANCE (m)	DEPTH RANGE (m)	$P_{1/2}$ (atm)	ΔV (ml/mol)
Nezumia bairdii (13.8)	600	260-1965	840 ± 80	-256 ± 28
Coryphaenoides rupestris (22.6)	1000	550-1960	565 ±100	-219 ± 30
Coryphaenoides acrolepis (16.2)	1200	475-2825	770 ± 40	-439 ± 45
Coryphaenoides carapinus (18.3)	2000	1250-2740	1265 ± 90	-394 ± 57
Coryphaenoides armatus (16.6)	2900	1885-4815	1715 ± 95	-297 ± 37
Coryphaenoides leptolepis (19.5)	3500	2288-4639	1570 ± 20	-308 ± 20

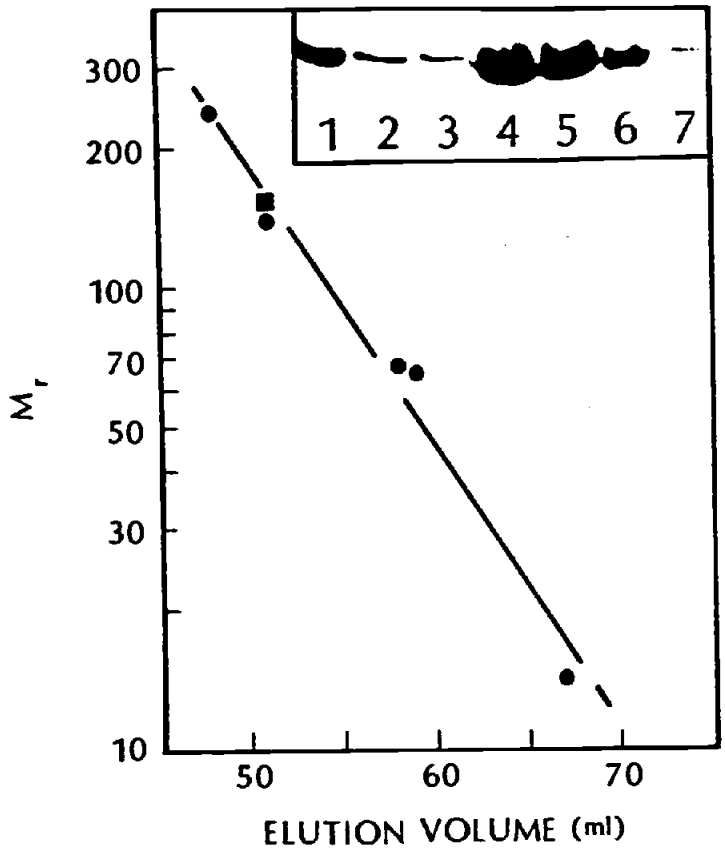


Figure II.1. Molecular weight calibration curve for native muscle-type LDH of six macrourid fishes. Calibration standards: ● ; macrourid LDH homologues: ■ . Inset. Sodium dodecyl sulfate polyacrylamide gel of M_4-LDH homologues of six macrourid fishes and pig. Lane 1: Coryphaenoides acrolepis; Lane 2: C. armatus; Lane 3: C. carapinus; Lane 4: C. leptolepis; Lane 5: C. rupestris; Lane 6: Nezumia bairdii; Lane 7: pig. Native porcine muscle-type LDH is a tetramer of molecular mass 144,000 Da, with a subunit molecular mass of 36,000 Da (Everse and Kaplan 1973; Kiltz et al. 1977).

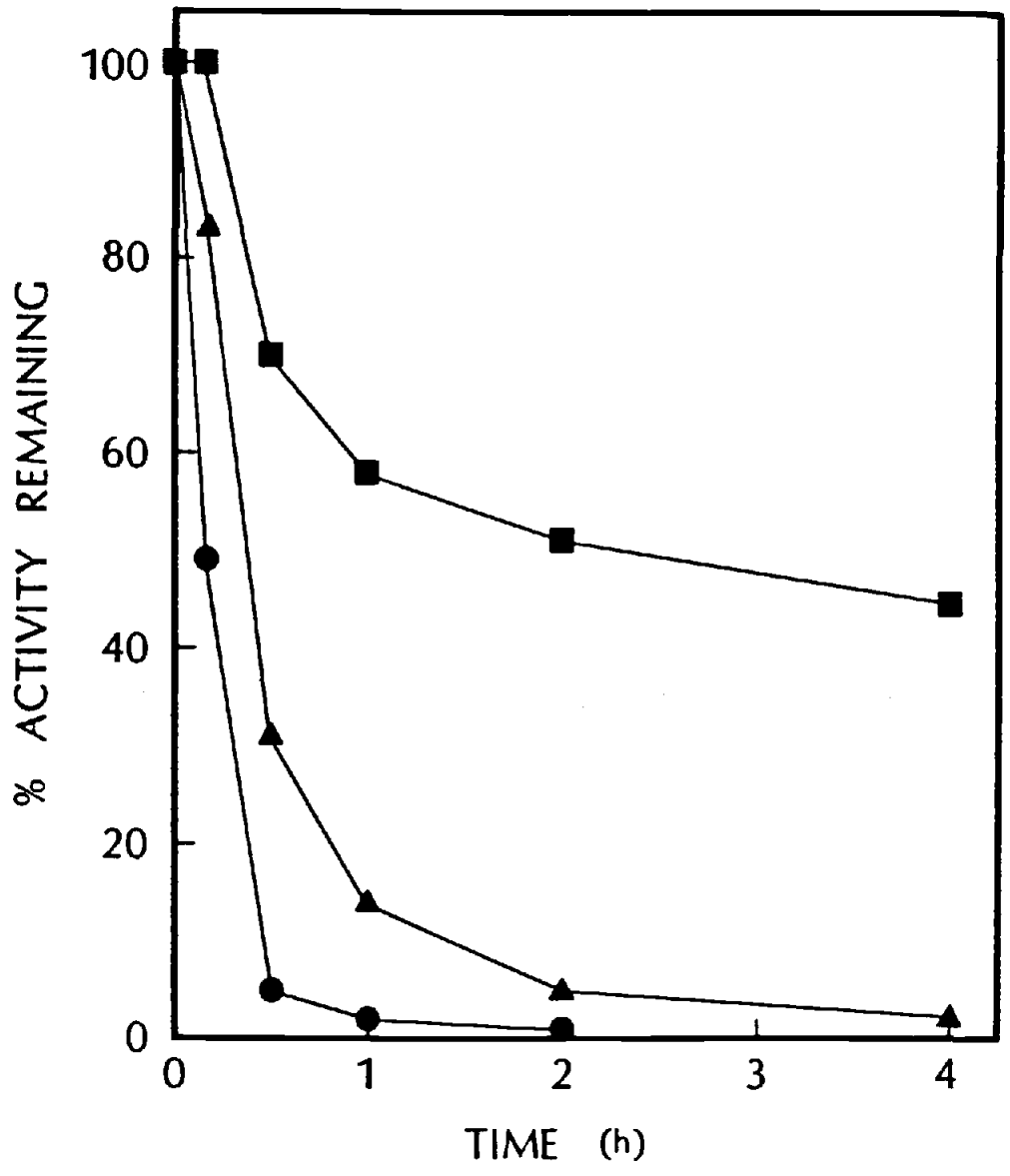


Figure II.2. The effect of time of incubation at pressure on percent activity remaining for the LDH homologue of Coryphaenoides rupestris. The incubations were at 5°C. 500 atm: ■ ; 900 atm: ▲ ; 1500 atm: ●.

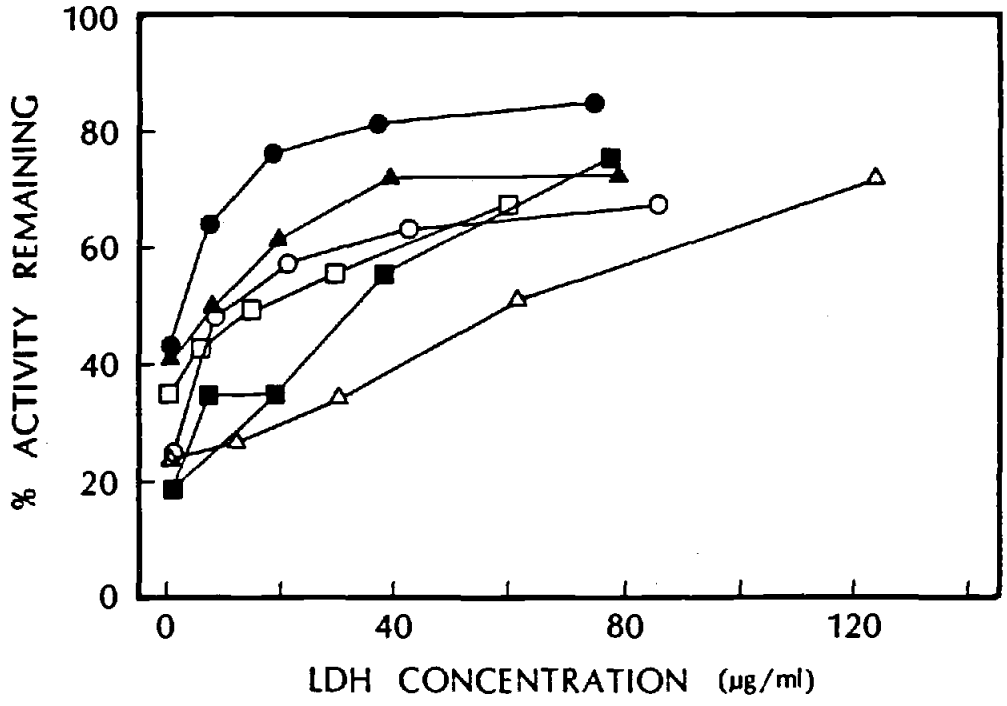


Figure II.3. The effect of LDH protein concentration on the inactivation of macrourid LDH homologues by pressure under the standard conditions at 4°C. Nezumia bairdii (750 atm): ○; Coryphaenoides rupestris (650 atm): ■; C. acrolepis (800 atm): △; C. carapinus (1100 atm): ●; C. armatus (1750 atm): □; C. leptolepis (1500 atm): ▲.

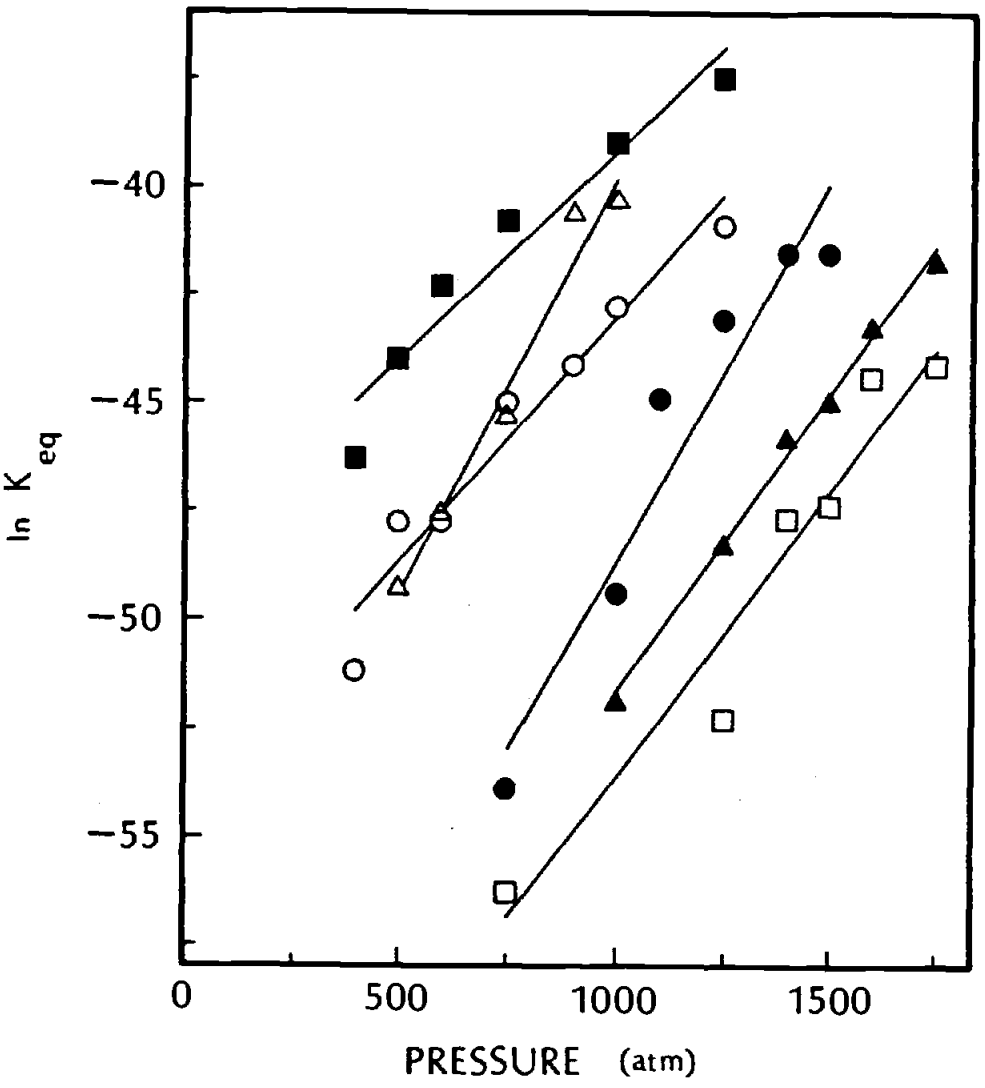


Figure II.4. The effect of pressure on ln K_{eq} of the M_4-LDH homologues of six macrourid species. Nezumia bairdii: ○ ; Coryphaenoides rupestris: ■ ; C. acrolepis: △ ; C. carapinus: ● ; C. armatus: □ ; C. leptolepis: ▲ . The C. rupestris homologue was incubated at 3°C; all other values were determined at 4°C. Values are plotted for the pressures over which ΔV was calculated.

CHAPTER III

Pressure-Adaptive Differences in Proteolytic Inactivation of M_4-Lactate Dehydrogenases from Marine Fishes

[The following manuscript has been submitted to the Journal of Experimental Zoology for publication.]

SUMMARY

Tetrameric muscle-type (M_4) lactate dehydrogenase (LDH, EC 1.1.1.27; L-lactate: NAD^+ oxidoreductase) homologues from five shallow-living and six deep-living teleost fishes were compared for susceptibility to inactivation by hydrostatic pressure and inactivation by the proteases, trypsin (EC 3.4.21.4) and subtilisin (EC 3.4.4.16), both at atmospheric pressure and at 1000 atm pressure. The native molecular mass and subunit structure of these LDH homologues are identical to those of other vertebrate LDH homologues. The pressures which inactivate the enzymes do not differ between the shallow- and deep-living fishes. However, inactivation pressures are much higher than the *in situ* pressures. Hydrostatic pressure, by affecting protein aggregation state and conformation, may increase susceptibility to proteolysis. At 10°C and atmospheric pressure, the enzymes of the shallow-living species are inactivated four times faster by trypsin and three times faster by subtilisin than are the homologues of the deep-living species. At 1000 atm pressure, the homologues of shallow species were inactivated 28 to 64% more than predicted from the summed effects of inactivation by 1000 atm pressure and tryptic inactivation at atmospheric pressure. The homologues of the deep-sea species were inactivated 0 to 21% more than expected. Inactivation by subtilisin at 1000 atm increased to a similar extent for the enzymes of the deep- and shallow-living species. However, at 1000 atm, the LDH homologues of the deep-sea species lost less activity (55.3%) than did the homologues of the shallow species (86.4%). Tryptic inactivation of the LDH of shallow-living

Sebastes melanops was increased 14% by 200 atm pressure. At 200 atm, no pressure-inactivation of the enzyme is evident. A pressure of 200 atm does not increase the tryptic inactivation of the enzymes of two deep-living macrourids. Increased structural stability of enzymes of deep-sea species may prevent too rapid protein turnover, which would be energetically costly in the food-poor deep-sea.

INTRODUCTION

Hydrostatic pressure, an important component of the environment of deep-living marine organisms, may disrupt protein structure and function (Heremans 1982; Morild 1981; Somero et al. 1983). Multimeric enzymes from terrestrial organisms are inactivated by hydrostatic pressure (cf. Jaenicke 1981). Penniston (1971) hypothesized that deep-sea organisms would utilize multimeric enzymes with strengthened protein-protein interactions or would use monomeric enzymes capable of catalyzing reactions normally requiring a multimeric enzyme. In a test of this hypothesis it was found that muscle-type L-lactate dehydrogenase (LDH, EC 1.1.1.27; L-lactate: NAD^+ oxidoreductase) homologues from six macrourid fishes were tetrameric, as are homologues from other vertebrates, and that the pressures which inactivate them are related to the depths at which the species occurred. Higher hydrostatic pressures were required to inactivate the LDH homologues from the deeper-occurring species (Hennessey and Siebenaller 1985. The hydrostatic pressures which inactivate these LDH homologues are greater than those which the enzymes are exposed to in vivo. This is analogous to the findings that thermal inactivation temperatures of proteins are much higher than the cell temperatures (Alexandrov 1977; Hochachka and Somero 1984). Thermal stability may be a reflection of resistance to proteolysis (Goldberg and Dice 1974; McLendon and Radany 1978; Daniel et al. 1982).

Hydrostatic pressure, by affecting protein aggregation state and conformation, may influence the rate of turnover of proteins by increasing their susceptibility to proteolysis in a manner similar to

temperature (Hennessey and Siebenaller 1985). For example, basal proteolysis is constitutive and is regulated by the availability of proteins as substrates. This depends in part on whether these substrates are protected, i.e., incorporated into the appropriate structure or aggregation state and in the proper conformation (Wheatley 1984).

To examine the importance of pressure in protein inactivation - via dissociation and/or proteolysis - in the deep-sea, we have undertaken a study of the effects of hydrostatic pressure on the inactivation of M_4-LDH homologues from 11 teleost fishes occurring from the surface to 4693 m. Hydrostatic pressure increases 1 atm for every 10 m depth increase in the ocean, and thus these species cover a pressure range of 470 atm. We have used two proteases, trypsin (EC 3.4.21.4) and subtilisin Carlsberg (EC 3.4.4.16), as probes of the effects of pressure on proteolytic inactivation. Do the enzymes of deep-living organisms display adaptations to proteolytic inactivation which may be mediated by the effects of pressure on conformation and aggregation state?

MATERIALS AND METHODS

Specimens

Fish were taken by otter trawl at their typical depths of abundance on cruises of the R/V Oceanus, R/V Wecoma and R/V Sacajawea. Antimora rostrata (Moridae), Coryphaenoides rupestris and C. leptolepis (Macrouridae) were taken in an area south of New England, USA. Microstomus pacificus, Parophrys vetulus, Platichthys stellatus (Pleuronectidae), Sebastes pinniger, S. melanops (Scorpaenidae), Antimora microlepis (Moridae), Anoplopoma fimbria (Anoplopomatidae) and Coryphaenoides acrolepis (Macrouridae) were taken off the coast of Oregon, USA. Specimens were frozen on solid CO_2 at sea and transported to the laboratory where they were maintained at -85°C until used. Specimens of Sebastes melanops, Parophrys vetulus and Platichthys stellatus were maintained at -20°C. For these studies, the species were grouped into two categories based on their depth distributions (Table 1).

Chemicals

The chemicals, biochemicals and resins for chromatography, subtilisin Carlsberg, L-1-tosylamide-2-phenylethylchloromethyl ketone treated bovine trypsin, porcine M_4-LDH and chicken egg albumin were purchased from Sigma Chemical Co. Water was processed through a Milli-Q purification system (Millipore Corp.).

Enzyme purification

M_4-LDH homologues were purified by affinity chromatography on an oxamate aminohexyl-Sepharose 4B column as described previously

(Hennessey and Siebenaller (1985). These preparations had a single band when stained for activity after electrophoresis in 12% starch gels following Harris and Hopkinson (1976).

The relative molecular mass (M_r) of native M_4-LDH homologues was determined by gel filtration on a calibrated Sephacryl S-300 (Superfine) column (1.5 x 40.5 cm) equilibrated with 50 mM potassium phosphate, pH 6.8, 5 mM 2-mercaptoethanol, 1 mM ethylenediamine-tetraacetic acid (EDTA) and 100 mM NaCl (Hennessey and Siebenaller 1985). Fractions from the column were assayed for protein content and LDH activity. Protein was detected using the Bradford dye-binding assay using Serva Blue G (Serva Fine Biochemicals, Inc.) as described by Peterson (1983). LDH activity was determined in an assay medium containing 80 mM Tris-HCl, pH 7.5 at the assay temperature of 10°C, 5 mM sodium pyruvate and 150 µM NADH. The decrease in absorbance at 340 nm was monitored in a Varian Techtron 634S spectrophotometer equipped with a Soltec model 1241 chart recorder. The assay temperature was maintained with a circulating refrigerated water bath. Each LDH homologue eluted as a single coincident peak of activity and protein.

The homogeniety and M_r of LDH subunits was determined by electrophoresis in the presence of sodium dodecyl sulfate in 1.5 mm thick, 12.5% polyacrylamide slab gels (Laemmli 1970). Each LDH preparation had a single protein band when stained with 25% 2-propanol, 10% acetic acid and 0.25% Serva Blue R (Serva Fine Biochemicals, Inc.).

Inactivation by hydrostatic pressure

The loss of LDH activity at pressure was determined following the procedures described by Hennessey and Siebenaller (1985). The enzymes were incubated in 0.2 ml Microflex vials (Kontes Glass Co.) placed in pressure vessels and maintained at 4°C with a refrigerated water bath. The enzymes were in 20 mM imidazole-HCl, pH 6.98 at 20°C, 5 mM 2-mercaptoethanol and 1 mM EDTA. LDH concentrations ranged from 6.2 to 22.0 μg/ml as determined by the Bradford dye-binding assay. Bovine serum albumin was used as the standard. In conjunction with proteolysis experiments, samples were in 50 mM Tris-HCl, pH 7.5 at 10°C, 100 mM KCl, 5 mM 2-mercaptoethanol and 1 mM EDTA (=Buffer A).

Imidazole-HCl and Tris-HCl were used as the buffers because of the low sensitivity of their pK_a values to hydrostatic pressure (-0.020 pH units per 1000 atm [Marquis and Fenn 1969; Kauzmann et al. 1962] and -0.019 pH units per 1000 atm [Neuman et al. 1973; Morild 1977] respectively).

Pressures were generated with an Enerpac model P-228 hydraulic pump filled with high grade mineral oil. Pressure was monitored with a Marsh Mastergauge (model E4396B).

Proteolysis

M_4-LDH homologues were dialyzed overnight at 4°C against 1000 volumes of Buffer A. Each homologue was diluted with Buffer A to a concentration of 0.7 to 1.2 μg/ml. The average LDH concentration did not differ between the shallow- and deep-living species (t-test, $P > 0.1$). Solutions of the proteases prepared in Buffer A were kept on ice and used within 30 min.

The rates of inactivation of LDH homologues by trypsin and subtilisin was determined at 10 $\pm$ 0.2^{o}C. Protease was replaced with an equivalent concentration of albumin in control tubes. Subsamples of 100 µl were removed at timed intervals to assay LDH activity.

To examine the effects of hydrostatic pressure on proteolytic inactivation of LDH, samples were incubated under the following conditions: 1) LDH plus protease at 1000 atm pressure, 2) LDH plus protease at atmospheric pressure, 3) LDH plus albumin at 1000 atm pressure, and 4) LDH plus albumin at atmospheric pressure. The vials were incubated at pressure for 30 min and assayed for LDH activity upon release of pressure. The paired samples incubated at atmospheric pressure were assayed for activity immediately afterward.

RESULTS

Subunit structure

The M_4-LDH homologues of the 11 fishes studied have subunit and native molecular weights identical to porcine M_4-LDH. The catalytically active LDH homologue of each species eluted from the gel filtration column in the same volume as active porcine M_4-LDH. The fish LDH homologues comigrated with porcine M_4-LDH when electrophoresed in the presence of sodium dodecyl sulfate on 12.5% polyacrylamide gels. Porcine M_4-LDH has a native molecular mass of 144,000 Da and a subunit molecular mass of 36,000 Da (Everse and Kaplan, 1973; Kiltz et al., 1977).

Inactivation by pressure

The pressures which half-inactivate ($P_{1/2}$) the M_4-LDH homologues are given in Table III.1. These values were calculated from incubations at six to eight pressures. The $P_{1/2}$ values for the homologues from the five shallow-living species ranged from 800 to 1350 atm. For homologues of the deep-living species, values ranged from 565 to over 1750 atm. The limit of our high pressure apparatus was 1750 atm. The $P_{1/2}$ values for the LDH homologues of shallow- and deep-living fishes are not significantly different (two-tailed Mann-Whitney test, $P > 0.38$). The $P_{1/2}$ values for LDH homologues of C. rupestris and C. acrolepis are the lowest values observed. The $P_{1/2}$ values of homologues of the other deep-occurring species are greater than or equal to the values for homologues from the shallow-living fishes (Table III.1).

Proteolysis at atmospheric pressure

The rates of proteolytic inactivation of M_4-LDH homologues at atmospheric pressure are given in Table III.1. Conditions were chosen so that samples of M_4-LDH incubated with albumin for 2 h had no detectable loss of activity. The concentrations of proteases were chosen to give limited inactivation in all samples. Inactivation rates were calculated from the slope of least-squares regressions of log percent LDH activity remaining versus time. Six to eight time points were used.

The rates of inactivation of LDH homologues of shallow-living fishes by both trypsin and subtilisin are significantly greater than the rates for the homologues of deep-living fishes (two-tailed Mann-Whitney test, $P < 0.01$, trypsin; $P < 0.01$, subtilisin). The rates of tryptic inactivation for LDH homologues from shallow-living fishes ranged from .230 to .744 h^{-1}. For the homologues of deep-living fishes the range of rates was 0.076 to 0.139 h^{-1}. The rates of inactivation by subtilisin ranged from 0.342 to 0.798 h^{-1} for homologues from the shallow species; 0.081 to 0.210 h^{-1} for homologues of the deep species.

Proteolysis at increased pressure

Comparison of LDH activity lost due to increased pressure, proteolysis at atmospheric pressure and proteolysis at increased hydrostatic pressure are given in Figures III.1, III.2 and III.3. For the homologues of the shallow-occurring species, the percentage of LDH activity lost during incubation with trypsin at 1000 atm pressure was 28 to 64% greater than that predicted from the summed effects of

inactivation by trypsin at atmospheric pressure and inactivation by 1000 atm pressure (Figure III.1). At 1000 atm pressure homologues of the deep species were inactivated 0 to 21% more than expected (Figure III.1). This was significantly less than observed for the homologues of the shallow species (two-tailed Mann-Whitney test, $P < 0.01$).

Inactivation by subtilisin at 1000 atm increased to a similar extent for the enzymes of both the deep-and shallow-occurring species over that expected from the effects of 1000 atm pressure and subtilisin inactivation at atmospheric pressure (Figure III.2, two-tailed Mann-Whitney test, $P > 0.20$). Eleven to 53% more LDH activity was lost during incubation with subtilisin at 1000 atm pressure than expected for the homologues of the shallow species, and 14 to 35% more for the homologues of the deep species. At 1000 atm pressure, the M_4-LDH homologues of the deep-occurring species, however, lost less activity (mean 55.3 $\pm$ 18.5%) than did the homologues of the shallow species (mean 86.4 $\pm$ 8.6%; two-tailed Mann-Whitney test, $P < 0.05$).

Tryptic inactivation was investigated with a 60 min incubation at $10^{\circ}C$ at 1 and 200 atm using the M_4-LDH homologues from Sebastes melanops, Coryphaenoides acrolepis and C. leptolepis (Figure III.3). None of these enzymes are inactivated by 200 atm pressure. However, 200 atm pressure increased the tryptic inactivation of the S. melanops LDH 14% over the inactivation expected from the tryptic inactivation of the enzyme at atmospheric pressure (t-test, $P < 0.05$). At 200 atm pressure, tryptic inactivation of the C. acrolepis and C. leptolepis homologues was not increased over that observed at atmospheric pressure (t-test, $P > 0.4$, C. acrolepis; $P > 0.4$, C. leptolepis).

DISCUSSION

Subunit structure and inactivation by hydrostatic pressure

The muscle-type LDH homologues of the 11 fishes studied have subunit and native M_r values identical to those of the tetrameric muscle-type porcine LDH. Penniston (1971) found that multimeric enzymes of terrestrial organisms were inactivated by hydrostatic pressure and hypothesized that enzymes of deep-living organisms would either be monomeric or that their multimeric enzymes would be stabilized by stronger noncovalent interactions between subunits. The results of this and a previous study (Hennessey and Siebenaller 1985) indicate that LDH homologues of deep-living fishes retain the tetrameric structure typical of other vertebrate LDH homologues. There are no indications of altered quaternary structure of other enzymes from shallow- and deep-living fishes (Siebenaller 1978, 1984a, b; Wilson and Waples 1983, 1984).

Pressures much greater than those which are experienced *in situ* are required to inactivate the M_4-LDH homologues of both the deep- and shallow-occurring fishes (Table III.1). The average pressures necessary to half-inactivate the LDH homologues of the deep- and shallow-living species do not differ ($P > 0.38$; Table III.1). The deep-living species do, however, tend to have larger $P_{1/2}$ values (Table III.1).

Proteolysis

For six macrourid fishes occurring over 260 to 4815 m, the $P_{1/2}$ values for their M_4-LDH homologues were correlated with the

maximal depth of abundance of the species (Hennessey and Siebenaller 1985). These $P_{1/2}$ values were much larger than the pressures experienced by the species *in situ*. These results are analogous to studies which demonstrate a correlation of thermal inactivation temperatures, cell temperatures and susceptibility to proteolysis (Daniel et al. 1982; Goldberg and Dice 1974; McLendon and Radany 1978). Thus, since hydrostatic pressure, like temperature, can perturb protein aggregation state and conformation, pressure may affect the proteolytic inactivation of LDH and, *in vivo*, the rate of protein turnover.

Jeckel et al. (1973) have demonstrated that proteolysis of the apo-enzyme of LDH is directly correlated with loss of catalytic activity. Thus, at atmospheric pressure the inactivation of LDH is a measure of peptide bond hydrolysis. The rate of proteolysis will depend on the flexibility and exposure of the peptide bonds of the substrate. At atmospheric pressure the LDH homologues from the deep-occurring fishes are less susceptible to proteolysis both by trypsin, which hydrolyzes only peptide bonds involving the carboxyl groups of lysyl and arginyl residues (Kasper 1975), and subtilisin which has broader specificity (Harris and Roos 1959; Ottesen and Svendsen 1970). The M_4-LDH homologues of the shallow-living species are inactivated on average four times faster by trypsin and three times faster by subtilisin than are the enzymes of deep-sea species (Table III.1).

At 10°C, 1000 atm pressure increases the rate of hydrolysis of N-α-benzoyl-L-arginine ethyl ester by bovine trypsin and the hydrolysis of casein by subtilisin. Thus, some of the increase in proteolytic

inactivation of LDH at pressure might be due to increased activities of the proteases. Pressure also might induce conformational changes in the substrate and increase the accessibility of cleavage sites.

At 200 atm pressure, LDH of the shallow-living S. melanops is inactivated by proteolysis 88%; at atmospheric pressure proteolytic inactivation is 74%. There is no inactivation of the enzyme by 200 atm pressure (Figure III.3). In contrast, 200 atm pressure does not increase the rate of tryptic inactivation of the enzymes of Coryphaenoides acrolepis and C. leptolepis. The enzymes are inactivated by trypsin to the same extent at atmospheric pressure and 200 atm pressure. There is no pressure-enhancement of proteolytic inactivation of the LDH homologues from these deep-sea species by pressure similar to that of their habitat (Figure III.3).

Increased proteolysis of LDH at pressure may result from minor conformational perturbations of the LDH structure by pressure. Because the M_4-LDH of deep-living fishes are less susceptible to proteolytic inactivation both at atmospheric pressure and at increased hydrostatic pressure (Figures III.1, III.2, III.3), these enzymes might thus be structurally more stable than the homologues from cold-adapted shallow-living species. The disruption by hydrostatic pressure of the apparent Michaelis constant of coenzyme in dehydrogenase homologues of shallow-occurring fishes may result from minor pressure-induced conformational changes (Siebenaller and Somero 1978, 1979; Siebenaller 1983, 1984a, c). The lower catalytic capacity of LDH homologues of deep-sea fishes may reflect the ability of enzymes from deep-sea fishes to maintain their active (native)

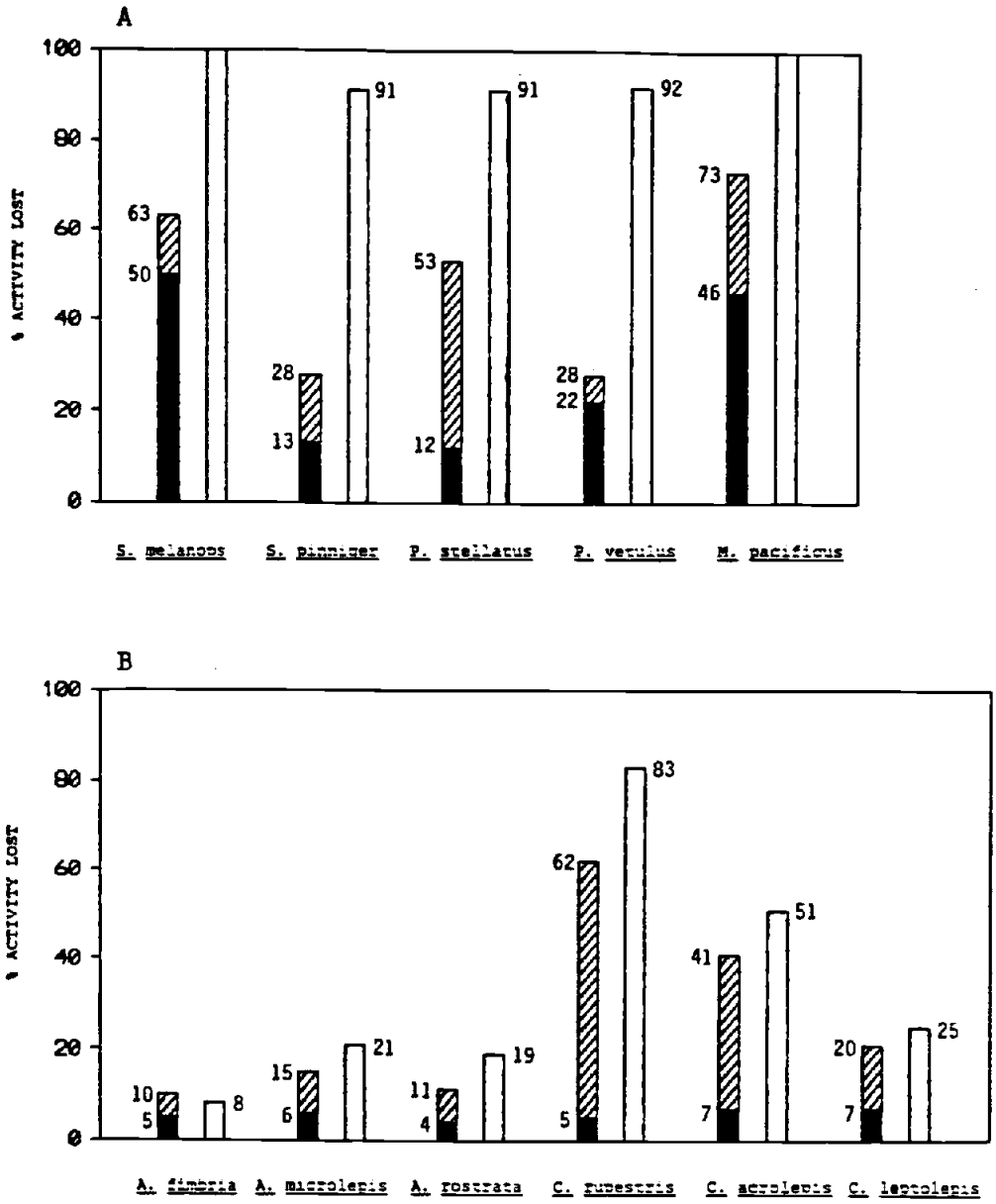
conformation at increased pressure (Somero and Siebenaller 1979; Somero et al. 1983). The increased structural stability of proteins of deep-occurring species preserves the catalytic and regulatory capacities of these enzymes and may prevent too rapid protein turnover, which would be energetically costly in the food-poor deep-sea environment.

Table III.1. The depths of distribution of the teleost fishes studied (taken from Siebenaller et al. [1982], Sullivan and Somero [1980] and Miller and Lea [1972]), the pressures at which the M_4-LDH homologues are half-inactivated ($P_{1/2}$) by incubation for 1 h at 4°C, and rates of inactivation of LDH at 10°C and atmospheric pressure by 0.5 mg/ml trypsin and 0.05 mg/ml subtilisin. ± standard error is given.

SPECIES	DEPTHS OF ABUNDANCE (m)	$P_{1/2}$ (atm)	RATE OF PROTEOLYTIC INACTIVATION (h^{-1}) TRYPSIN	SUBTILISIN
Shallow-living species				
Sebastes melanops	0- 100	1085 ± 60	0.744 ±0.047	0.342 ±0.005
Sebastes pinniger	0- 300	1085 ± 20	0.238 ±0.012	0.798 ±0.046
Platichthys stellatus	0- 300	800 ± 45	0.230 ±0.012	0.504 ±0.022
Parophrys vetulus	20- 330	1350 ± 70	0.395 ±0.017	0.524 ±0.024
Microstomus pacificus	30-1000	935 ± 55	0.708 ±0.032	0.351 ±0.008
Mean ± S.E.		1051 ± 27	0.463 ±0.033	0.504 ±0.025
Deep-living species				
Anoplopoma fimbria	200-1670	>1750	0.099 ±0.008	0.081 ±0.005
Antimora microlepis	400-3300	1330 ± 65	0.122 ±0.010	0.177 ±0.015
Antimora rostrata	825-2500	1620 ± 80	0.076 ±0.010	0.165 ±0.004
Coryphaenoides rupestris	550-1960	565 ±100	0.088 ±0.007	0.210 ±0.011
Coryphaenoides acrolepis	475-2825	770 ± 40	0.124 ±0.011	0.111 ±0.009
Coryphaenoides leptolepis	2288-4639	1570 ± 20	0.139 ±0.012	0.194 ±0.005
Mean ± S.E.		1171 ± 64	0.108 ±0.003	0.157 ±0.007

Figure III.1. Inactivation of M_4-LDH homologues of marine fishes by trypsin at atmospheric pressure and 1000 atm pressure. LDH homologues were incubated for 30 min at 10°C: () with 0.5 mg/ml trypsin at atmospheric pressure; () with 0.5 mg/ml albumin at 1000 atm pressure; () with 0.5 mg/ml trypsin at 1000 atm pressure. A) Shallow-living fishes. B) Deep-living fishes. For each species the difference between the height of the right bar and the height of the left bar represents pressure-enhanced inactivation by trypsin.

Figure III.1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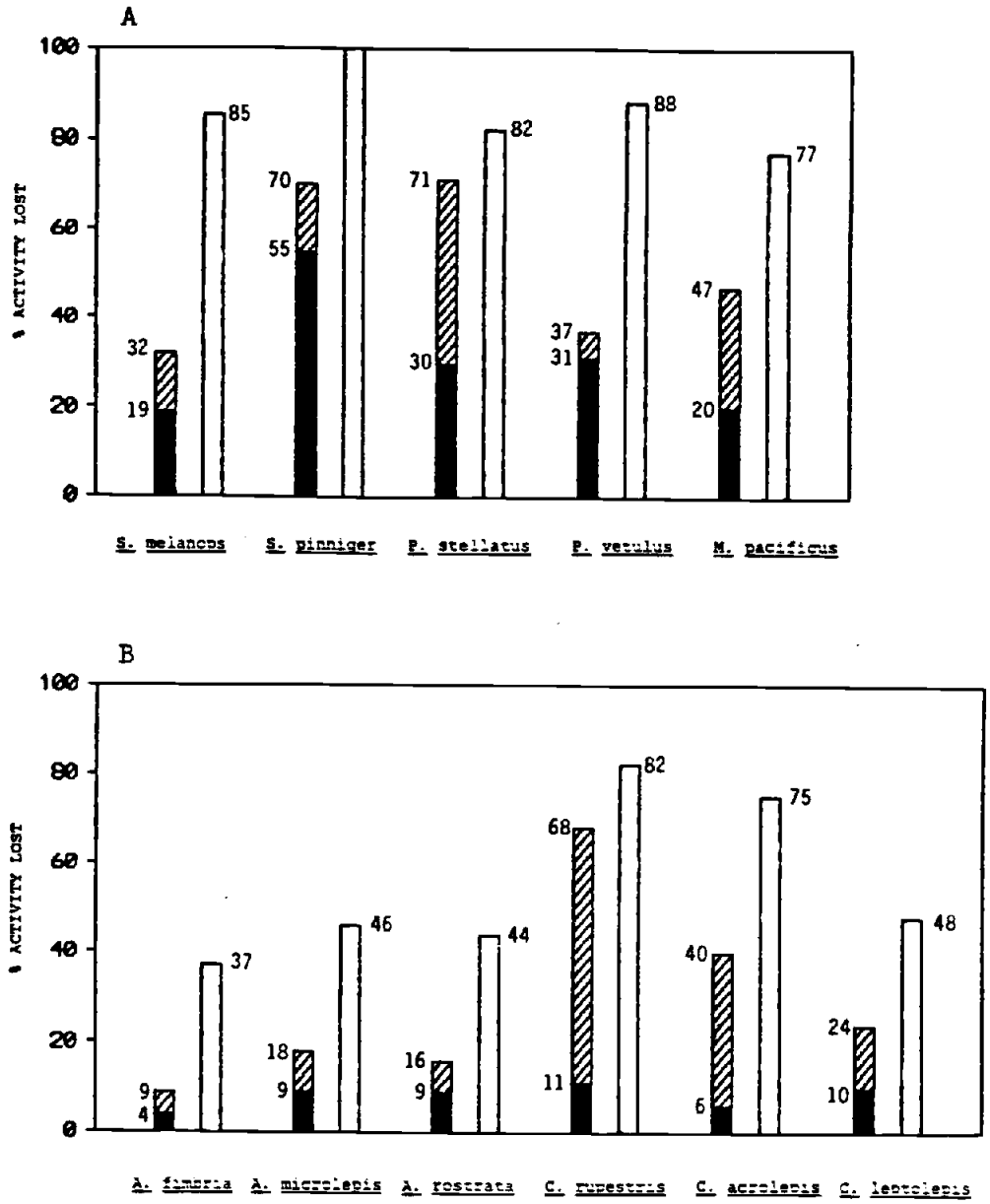
6
21
11
4
19
62
5
83
41
7
51
20
7
25
A. fimbria
A. macrolepis
A. rostrata
C. rubescens
C. acrolepis
C. leptolepis

Figure III.2. Inactivation of M_4-LDH homologues of marine fishes by subtilisin at atmospheric pressure and 1000 atm pressure. LDH homologues were incubated for 30 min at 10°C: () with 0.05 mg/ml subtilisin at atmospheric pressure; () with 0.05 mg/ml albumin at 1000 atm pressure; () with 0.05 mg/ml subtilisin at 1000 atm pressure. A) Shallow-living fishes. B) Deep-living fishes. For each species the difference between the height of the right bar and the height of the left bar represents pressure-enhanced inactivation by subtilisin.

Figure III.2


A
% ACTIVITY LOST
100
80
60
40
20
0
32
19
85
70
55
71
30
82
37
31
88
47
20
77
S. melanops
S. pinniger
P. stellatus
P. vetulus
M. pacificus
B
% ACTIVITY LOST
100
80
60
40
20
0
9
4
37
18
9
46
16
9
44
68
11
82
40
6
75
24
10
48
A. fimbria
A. microlepis
A. rostrata
C. rupestris
C. acrolepis
C. leptolepis

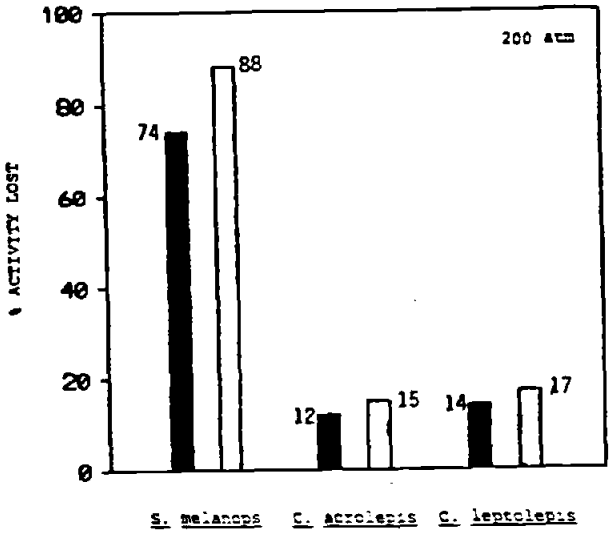


Figure III.3. Inactivation of M_4-LDH homologues of Sebastes melanops, Coryphaenoides acrolepis and C. leptolepis by trypsin at atmospheric pressure and 200 atm pressure. LDH homologues were incubated for 60 min at 10°C: (▮) with 0.5 mg/ml trypsin at atmospheric pressure; (▯) with 0.5 mg/ml trypsin at 200 atm pressure. There was no LDH activity lost during incubations of LDH with 0.5 mg/ml albumin at 200 atm pressure. For each species the difference between the height of the right bar and the height of the left bar represents pressure-enhanced inactivation by trypsin.

CHAPTER IV

Pressure-Stability and Proteolytic Susceptibility of NAD-Dependent Dehydrogenases from Shallow- and Deep-Living Fishes

SUMMARY

Cytoplasmic malate dehydrogenase (MDH, EC 1.1.1.37; L-malate: NAD^+ oxidoreductase) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12; D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase) homologues from two shallow-living and three deep-living fishes were compared for susceptibility to inactivation by hydrostatic pressure and inactivation by the proteases, trypsin (EC 3.4.21.4) and subtilisin (EC 3.4.4.16). The molecular mass and subunit aggregation state of native fish MDH and GAPDH homologues are identical to those of other vertebrate MDH and GAPDH homologues. At 1750 atm and 10°C, MDH homologues from shallow- and deep-living fishes lost 6 to 14% of their activity. GAPDH homologues of these five species show no loss of activity under these conditions. At atmospheric pressure and 10°C, the rate of tryptic inactivation of MDH homologues from shallow-living fishes is greater than for homologues of deep-living fishes. The rate of inactivation of MDH homologues by subtilisin was similar for both shallow- and deep-living fishes. At 1750 atm pressure and 10°C, inactivation of MDH homologues by trypsin was 1 to 21% greater than predicted from the summed effects of inactivation by 1750 atm pressure and tryptic inactivation at atmospheric pressure. Inactivation by subtilisin at 1750 atm pressure was 16 to 40% more than expected. Inactivation of GAPDH homologues by 1 mg/ml trypsin or subtilisin was not detectable at 10°C and atmospheric pressure or at 1750 atm pressure. The structural integrity of MDH and GAPDH homologues of deep-living fishes appears to be pre-adapted to the high hydrostatic pressures of the deep-sea. The

pre-adapted pressure-insensitivity of MDH and GAPDH homologues of shallow-living fishes may arise from conservation of minor features of enzyme primary structure that were selected by other influences on the structural integrity of the enzymes.

INTRODUCTION

Hydrostatic pressures encountered in the deep-sea may be sufficient to affect protein structure and function. This may be an important influence on the biology of deep-living organisms (Somero et al. 1983). Adaptations to hydrostatic pressure have been documented for enzymes of deep-sea fishes, particularly for tetrameric muscle-type (M_4) lactate dehydrogenase (LDH, EC 1.1.1.27; L-lactate: NAD^+ oxidoreductase) (Somero et al. 1985). Adaptation of enzymes to high hydrostatic pressure enables deep-living organisms to successfully inhabit regions of the ocean that are unsuited for organisms not adapted to high pressure. Adaptive differences between M_4-LDH homologues of shallow- and deep-living fishes provide a model for studies of adaptations to pressure in other enzymes of deep-sea organisms by revealing the focal points of these adaptations. The K_m of coenzyme values for M_4-LDH homologues of shallow-living fishes increase significantly at pressures typical of the deep-sea environment. This results in a decrease in the affinity of the LDH homologues for coenzyme and impaired catalytic function. Homologues of deep-living fishes show little or no increase in K_m of coenzyme at these pressures (Siebenaller and Somero 1978, 1979). Thus the function of M_4-LDH homologues of shallow-living fishes, but not deep-living fishes, would be impaired at deep-sea pressures which would upset metabolic homeostasis in the muscle tissue. The same pattern of adaptation to pressure is seen in homologues of three other NAD-dependent dehydrogenases from shallow-living Sebastolobus alascanus and deep-living S. altivelis. These two species have been used as a

model to investigate adaptive differences between shallow- and deep-living organisms (see Somero et al. 1983). The K_m of coenzyme for two isozymes of cytoplasmic malate dehydrogenase (MDH-1 and MDH-2, EC 1.1.1.37; L-malate: NAD^+ oxidoreductase) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12; D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase) of shallow-living Sebastolobus alascanus increase at pressures typical of the deep-sea. There is no increase of the K_m of coenzyme for homologues of deep-living S. altivelis (Siebenaller 1984a). X-ray diffraction analyses of NAD-dependent dehydrogenases from other species show that these enzymes have similar "supersecondary" structures in their nucleotide-binding domains (Rossmann and Argos 1978). Increases in the K_m of coenzyme of NAD-dependent dehydrogenases from S. alascanus by pressures typical of the habitat of S. altivelis and the insensitivity of S. altivelis homologues to these pressures demonstrate that reduced sensitivity to pressure of coenzyme binding of NAD-dependent dehydrogenases may be an important and ubiquitous adaptation to the deep-sea (Siebenaller 1984a). Such adaptations to pressure may be essential for successful colonization of the deep-sea (Siebenaller and Somero 1978, 1979; Siebenaller 1984a). The tertiary structure of M_4-LDH, MDH and GAPDH from other species are also similar (Rossmann 1983). Given these similarities between NAD-dependent dehydrogenases, are differences in the pattern of response to pressure for shallow- and deep-living fishes similar in MDH and GAPDH to that in M_4-LDH?

Hydrostatic pressure may disrupt protein subunit interactions thus affecting the subunit aggregation equilibrium of multimeric

proteins (Heremans 1982; Morild 1981; Somero et al. 1983). Penniston (1971) hypothesized that deep-sea organisms would utilize multimeric enzymes with strengthened protein-protein interactions or would use monomeric enzymes capable of catalyzing reactions normally requiring a multimeric enzyme. In a test of these hypotheses muscle-type LDH homologues of 14 marine fishes occurring from the surface to 4693 m were found to have the tetrameric structure typical of the LDH homologues of other vertebrates (Hennessey and Siebenaller 1985; Chapter III). The pressures required to inactivate M_4-LDH homologues of six confamilial macrourid fishes increases with increasing maximum depth of abundance of the species (Hennessey and Siebenaller 1985). In general, higher pressures are required to inactivate M_4-LDH homologues from deeper-living fishes than homologues from shallow-living fishes (Chapter III). The hydrostatic pressures which inactivate these LDH homologues are greater than those which the enzymes are exposed to in vivo. This is analogous to the findings that thermal inactivation temperatures of proteins are correlated with cell temperatures, but that these temperatures are much higher than the cell temperatures (Alexandrov 1977; Hochachka and Somero 1984). Thermal stability may be a reflection of resistance to proteolysis (Goldberg and Dice 1974; McLendon and Radany 1978; Daniel et al. 1982).

Hydrostatic pressure, by affecting protein aggregation state and conformation, may influence protein turnover rates by increasing their susceptibility to proteolysis (Hennessey and Siebenaller 1985). At atmospheric pressure the rates of proteolysis by trypsin and

subtilisin of M_4-LDH homologues of shallow-living fishes are three to four times the rates for homologues of deeper-living fishes. At high hydrostatic pressure, tryptic inactivation of LDH homologues of deep-living fishes does not increase as much as it does for homologues of shallow-living fishes. Thus LDH homologues of deep-occurring fishes might be structurally more stable than homologues of shallow-occurring species. Increased structural stability of enzymes of deep-sea species may prevent too rapid protein turnover, which would be energetically costly in the food-poor deep-sea (Chapter III).

To determine whether MDH and GAPDH homologues of shallow and deep-living species display patterns of adaptation to pressure, similar to M_4-LDH, we have examined the effects of hydrostatic pressure on enzyme activity and susceptibility to proteolytic inactivation of MDH and GAPDH homologues from two shallow-living and three deep-living marine teleost fishes. These fishes occur from the surface to 4693 m (Table IV.1). Hydrostatic pressure increases 1 atm for every 10 m depth increase; thus, these fishes cover a pressure range of 470 atm. To examine the effects of pressure on proteolytic inactivation of these enzymes we have used two proteases, trypsin (EC 3.4.21.4) and subtilisin Carlsberg (subtilopeptidase A, EC 3.4.4.16).

MATERIALS AND METHODS

Specimens

Specimens were taken by otter trawl at their typical depths of abundance on cruises of the R/V Oceanus, R/V Wecoma and R/V Sacajawea. Coryphaenoides acrolepis, Sebastes melanops and Parophrys vetulus were taken off the coast of Oregon, USA. C. rupestris and C. leptolepis were taken in an area south of New England, USA. Specimens were frozen on solid CO_2 at sea and transported to the laboratory where they were maintained at -85°C. Specimens of S. melanops and P. vetulus were maintained at -20°C.

Chemicals

Chemicals, biochemicals and resins for chromatography were purchased from Sigma Chemical Co. Subtilisin Carlsberg (EC 3.4.4.16), L-1-tosylamide-2-phenylethylchloromethyl ketone treated trypsin from bovine pancreas (EC 3.4.21.4) and chicken egg albumin were obtained from Sigma. Water was processed through a Milli-Q purification system (Millipore Corp.).

Enzyme purification

Frozen white muscle tissue was homogenized in a Waring blender in 50 mM potassium phosphate, pH 6.8, 5 mM 2-mercaptoethanol and 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was stirred for 1 h at 4°C and centrifuged at 6,000 g (0-4°C) for 30 min. LDH was removed from the supernatant by affinity chromatography as described previously (Hennessey and Siebenaller 1985). LDH-free eluants were brought to 50% saturation with solid ammonium sulfate (300 g/l),

allowed to stand for 30 min and centrifuged at 15000 g for 30 min. The pellet was discarded and the supernatant brought to 90% saturation with solid ammonium sulfate (300 g/l), allowed to stand for 1 h and centrifuged at 15000 g for 30 min. The pellet was redissolved in a minimal volume of 20 mM Tris-HCl, pH 8.2 at 5°C, 5 mM 2-mercaptoethanol and 1 mM EDTA (buffer A) and dialyzed exhaustively against buffer A. MDH and GAPDH were isolated from the dialyzed solution by pseudo-affinity chromatography on a 2.5 x 8.5 cm reactive blue 2-agarose column equilibrated with buffer A. The column was washed with 2 l buffer A (0.5 ml/min) to remove non-adsorbing proteins. GAPDH was eluted by washing the column with 1.0 mM NAD in buffer A. Fractions containing GAPDH activity were retained for electrophoretic analysis of purity. MDH was eluted by washing the column with 0.3 mM NADH in buffer A. S. melanops and P. vetulus had multiple isozymes of MDH. The MDH-2 isozyme was separated by ion-exchange chromatography as described by Siebenaller (1984a).

Enzymes were concentrated by ultrafiltration under N_2 with a PM-10 filter (Amicon Co.), dialyzed against 90% ammonium sulfate, 5 mM 2-mercaptoethanol and 1 mM EDTA and stored at 4°C. EDTA and 2-mercaptoethanol increased the stability of the purified MDH and GAPDH preparations.

Electrophoresis of purified enzyme homologues in the presence of sodium dodecyl sulfate in a 1.5 mm thick, 12.5% polyacrylamide slab gel (Laemmli, 1970) resulted in a single band of M_r 34,000 for MDH homologues and M_r 36,000 for GAPDH homologues. The gels were stained in 25% 2-propanol, 10% acetic acid and 0.25% Serva Blue R (Serva Fine

Biochemicals, Inc.). GAPDH homologues produced a single band when stained for activity after starch gel electrophoresis by the method of Harris and Hopkinson (1976). Starch gel electrophoresis and activity staining of MDH homologues by the methods of Harris and Hopkinson (1976) produced a single band in the three species of Coryphaenoides. MDH homologues of S. melanops and P. vetulus exhibited a second minor band, the MDH-1/MDH-2 heterodimer. The MDH heterodimer band was less than 25% of the staining intensity of the MDH-2 homodimer band.

The relative molecular masses (M_r) of native MDH and GAPDH homologues were determined by gel filtration on a calibrated Sephacryl S-300 (Superfine) column (1.5 x 40.5 cm) equilibrated with 50 mM potassium phosphate, pH 6.8, 5 mM 2-mercaptoethanol, 1 mM EDTA and 100 mM NaCl (Hennessey and Siebenaller 1985). Fractions from the column were assayed for protein content and MDH or GAPDH activity. Protein was detected by a dye-binding assay (Bradford 1976) using Serva Blue G (Serva Fine Biochemicals, Inc.) as described by Peterson (1983). MDH activity was determined in a medium containing 100 mM Tris-HCl, pH 8.1 at the assay temperature of 10°C, 20 mM $MgCl_2$, 0.4 mM oxaloacetate and 150 µM NADH. The GAPDH assay medium contained 80 mM Tris-HCl, pH 8.5 at 10°C, 38 mM sodium arsenate, 4 mM L-cysteine, 1.7 mM D-glyceraldehyde-3-phosphate and 1.5 mM NAD. Oxaloacetate and D-glyceraldehyde-3-phosphate solutions were prepared every 3 - 4 h. Activity, measured by the change in absorbance at 340 nm, was monitored in a Varian Techtron 634S spectrophotometer equipped with a Soltec model 1241 chart recorder. The assay temperature was maintained with a circulating refrigerated water bath. Each enzyme

homologue eluted as a single peak of coincident protein and enzyme activity.

Pressure inactivation

The inactivation of dehydrogenases by hydrostatic pressure was determined using methods described previously (Hennessey and Siebenaller 1985). Enzyme homologues were dialyzed overnight against 1000 volumes of 50 mM Tris-HCl, pH 7.5 at 10°C, 100 mM KCl, 5 mM 2-mercaptoethanol and 1 mM EDTA then centrifuge-filtered through a 0.45 μm nylon-66 filter (Rainin Instrument Co.) and diluted to 5 μg per ml. Protein concentration was determined by the Bradford dye-binding assay using bovine serum albumin as the standard. Tris-HCl was used as the buffer because of the low sensitivity of its pK_a to hydrostatic pressure (-0.019 pH units per 1000 atm [Neuman et al. 1973; Morild 1977]). MDH and GAPDH homologues were incubated at 1750 atm and 10°C.

Pressures were generated with an Enerpac model P-228 hydraulic pump filled with high grade mineral oil. Pressure was monitored with a Marsh Mastergauge (model E4396B).

Proteolytic inactivation

Dehydrogenases were dialyzed overnight at 4°C against 1000 volumes of 50 mM Tris-HCl, pH 7.5 at 10°C, 100 mM KCl, 5 mM 2-mercaptoethanol and 1 mM EDTA and centrifuge-filtered. Enzymes were diluted to 5 μg protein per ml with dialysis buffer. Trypsin and subtilisin stock solutions were prepared every 30 min with dialysis buffer. MDH and GAPDH homologues were incubated at 10°C. Solutions were maintained at their incubation temperatures ± 0.2°C throughout

the experiment with a circulating refrigerated water bath or heating block. Aliquots of 50 to 100 µl were removed periodically during the 2 h incubation to determine the enzyme activity remaining. Protease was replaced with an equal concentration of albumin in control incubations. Rates of inactivation were calculated from the slope of the regression of log percent activity remaining versus time.

Proteolysis at elevated hydrostatic pressure was performed in 0.2 ml Microflex vials (Kontes Glass Co.) placed in a pressure vessel (Hennessey and Siebenaller 1985). Four vials were incubated for each enzyme homologue: 1) enzyme plus protease at 1750 atm pressure, 2) enzyme plus protease at atmospheric pressure, 3) enzyme plus albumin at 1750 atm pressure, and 4) enzyme plus albumin at atmospheric pressure. Vials were assayed for enzymatic activity upon release of pressure. The paired samples incubated at atmospheric pressure were assayed immediately afterward.

RESULTS

Subunit structure

NAD-dependent dehydrogenases from the fishes used in this study have native and subunit molecular weights similar to mammalian homologues. Catalytically active fish GAPDH and cytoplasmic MDH-2 homologues eluted from the gel filtration column in the same volume as rabbit GAPDH and pig mitochondrial MDH respectively. Fish GAPDH and cytoplasmic MDH-2 comigrated with rabbit GAPDH and pig mitochondrial MDH, respectively, in sodium dodecyl sulfate polyacrylamide gels. Rabbit GAPDH, a tetramer, has a native molecular mass of 146,000 Da and a subunit molecular mass of 36,000 Da (Harris and Perham 1965; Harrington and Karr 1965). Pig mitochondrial MDH, a dimer, has a native molecular mass of 68,000 Da and a subunit molecular mass of 34,000 Da (Banaszak and Bradshaw, 1975).

Pressure-inactivation

Incubation of MDH homologues for 30 min at 1750 atm and 10°C resulted in 6 to 14% loss of activity (Figure IV.1). MDH homologues of shallow- and deep-living fishes were inactivated to the same extent under these conditions (two-tailed Mann-Whitney test, $P > 0.4$). Incubations at 1500 atm pressure resulted in no detectable loss of MDH activity.

GAPDH homologues showed no loss of activity after a 24 h incubation at 1750 atm pressure and temperatures less than 25°C. Incubation of GAPDH homologues for 30 min at 30°C and 1750 atm resulted in 9 to 85% loss of activity (see Figure A.4). At 35°C (but not 30°C) significant inactivation of homologues of shallow-occurring

species occurred at atmospheric pressure (data not shown).

Proteolytic inactivation

The rates of inactivation of MDH homologues at 10°C by 1 mg/ml trypsin were 0.167 to 0.377 h^{-1} (Table IV.1). MDH homologues from shallow-living fishes are more susceptible to tryptic inactivation than homologues from deep-living species (two-tailed Mann-Whitney test, $P < 0.01$). The mean rate of inactivation for MDH homologues of the two shallow-living fishes was 50% greater than the mean rate for homologues of the three deep-living fishes.

GAPDH homologues were not inactivated by 1 mg/ml trypsin at 10°C after a 24 h incubation. At 30°C the rate of tryptic inactivation ranged from .002 to .040 h^{-1} (data not shown).

The rate of inactivation of MDH homologues by 0.5 mg/ml subtilisin ranged from 0.202 to 1.422 h^{-1} (Table IV.1). Rates of inactivation for homologues of the two shallow-occurring species were not significantly different than for homologues of deep-occurring species (two-tailed Mann-Whitney test, $P > 0.4$).

Under the conditions used, GAPDH homologues were not inactivated by 1 mg/ml subtilisin at 10°C after a 24 h incubation. At 30°C and 1 mg/ml subtilisin GAPDH inactivated at rates ranging from 0.006 to 0.054 h^{-1} (data not shown).

Loss of MDH activity due to 1750 atm pressure, proteolytic inactivation at atmospheric pressure and proteolytic inactivation at 1750 atm pressure is shown in Figure IV.1. Tryptic inactivation of MDH homologues at 1750 atm resulted in a loss of 1 to 21% more activity than expected from the combined effects of proteolysis at

atmospheric pressure and inactivation at 1750 atm. Inactivation of MDH homologues by subtilisin at 1750 atm resulted in 16 to 40% greater loss than expected. Proteolytic inactivation at 1750 atm pressure of MDH homologues did not differ between shallow- and deep-living species (two-tailed Mann-Whitney test, $P > 0.4$, trypsin; $P > 0.4$, subtilisin; Figure IV.1).

DISCUSSION

Subunit structure

The MDH and GAPDH homologues of the five fishes studied have subunit and native M_r values identical to those of porcine mitochondrial MDH and rabbit GAPDH, respectively. Penniston (1971) hypothesized that, because multimeric enzymes of terrestrial organisms were inactivated by hydrostatic pressure, enzymes of deep-sea organisms either would be monomeric or would be stabilized by stronger non-covalent interactions between subunits of multimeric enzymes. The result of this and other studies (Siebenaller 1978, 1984a, b; Wilson and Waples 1983, 1984; Hennessey and Siebenaller 1985; Chapter III) indicate that several enzymes of deep-living fishes maintain the multimeric structure typical of their respective homologues in other vertebrates.

Inactivation by hydrostatic pressure

Under the conditions used in this study, MDH and GAPDH homologues of marine fishes were far less susceptible to inactivation by hydrostatic pressure than M_4-LDH homologues from the same species (cf. Chapter III, Figures IV.1). After 1 h at 1750 atm pressure and 10°C, fish MDH homologues lost 6 to 14% activity; fish GAPDH homologues lost no activity. LDH homologues from these same species lost 65 to 100% activity under identical conditions (Hennessey and Siebenaller 1985; Chapter III). The decreased susceptibility of MDH to pressure-inactivation, relative to LDH, may be attributed to its subunit aggregation state. MDH is a dimer while LDH is a tetramer. MDH and LDH have similar secondary and tertiary structures,

particularly in the nucleotide-binding domain of each subunit, and similar Q-axis intersubunit contacts (Birktoft and Banaszak 1983; Rossmann et al. 1975; Rossmann 1983). When comparing related enzymes of different subunit aggregation states, small multimers (i.e. dimers such as MDH) are more pressure-stable than large multimers (e.g. M_4-LDH) (Jaenicke 1983). This stems from the greater number of intersubunit contacts (58 per subunit for LDH versus 32 for MDH [Birktoft and Banaszak 1983]) and the greater surface area of the subunit interfaces in large multimeric proteins. Consequently the free volume occurring along these interfaces is greater in larger multimers. Greater free volume in these interfaces results in greater overall compressibility of the molecule which appears to increase susceptibility to dissociation by hydrostatic pressure (Paladini and Weber 1981).

GAPDH homologues are much less susceptible to inactivation by hydrostatic pressure than are M_4-LDH homologues of the same species (cf. Chapter III). This is evident from both the higher pressures and the higher temperatures required for significant GAPDH inactivation. GAPDH and LDH have structurally and functionally similar nucleotide-binding domains located in different regions of their respective polypeptide chains (Rossmann and Argos 1978). However, there are substantial differences in their tertiary and quaternary structures. Stabilization of LDH quaternary structure is primarily dependent on subunit interactions along the Q-axis and N-terminal "tail" of the LDH subunits (Holbrook et al. 1975). GAPDH tetramers are assembled back-to-back relative to LDH homologues (see Buehner et al. 1975). This

results in most of the subunit interactions occurring along the R- and P-axes and very few interactions along the Q-axis of GAPDH (Moras et al. 1975). The decreased susceptibility of fish GAPDH homologues to inactivation by hydrostatic pressure, relative to M_4-LDH homologues, may be attributed to a decrease in the total number of subunit interactions in GAPDH (38 per subunit [Moras et al. 1975]) relative to LDH (58 per subunit [Birktoft and Banaszak 1983]) as well as to the dissimilarity of subunit interactions resulting from the back-to-back assembly of GAPDH relative to LDH.

GAPDH and MDH are similar in their number of intersubunit contacts (38 versus 32 [Moras et al. 1975; Birktoft and Banaszak 1983]). However, they differ substantially in their susceptibility to inactivation by hydrostatic pressure and in the type and location of their subunit interactions (Harris and Waters 1975). Thus the type as well as the quantity of the subunit interactions appears to be important in determining susceptibility to perturbation by pressure (see Swezey and Somero 1982, 1985). Analogous to the interpretation of Torgerson et al. (1979, 1980) on deformation of ligand-binding sites by hydrostatic pressure in ligand-protein complex formation, the "hard" (i.e. incompressible) versus "soft" (i.e. compressible) character of the subunit interactions will determine their susceptibility to perturbation by hydrostatic pressure. This is consistent with Paladini and Weber's (1981) model which predicts that susceptibility to dissociation of oligomeric proteins is controlled by the small free volumes at the intersubunit boundaries.

Pressures used in this study to partially inactivate MDH and GAPDH homologues are greater than pressures needed to partially inactivate M_4-LDH homologues from the same species and much greater than the pressures these enzymes are exposed to in vivo. The relationship between resistance to pressure inactivation and species depth of maximum abundance that was observed for M_4-LDH (Hennessey and Siebenaller 1985; Chapter III) is not apparent for MDH or GAPDH homologues. This suggests that the structural features of LDH homologues of shallow-living fishes that are responsible for their susceptibility to inactivation by pressure (e.g. number of subunit interactions, subunit aggregation state) are not present or are pre-adapted in MDH and GAPDH homologues of the same species.

Proteolysis

As shown in Table IV.1, proteolytic inactivation rates of fish MDH homologues are much less than those of LDH homologues from the same species (cf. Table III.1). The difference in tryptic inactivation rates for MDH homologues of shallow- and deep-living species (a difference of 50% or less) is small compared to that for LDH homologues of shallow- and deep-living species (a four-fold difference). The small differences in susceptibility to tryptic inactivation between shallow- and deep-occurring homologues of MDH at 1750 atm pressure may indicate that conformational perturbations in LDH structure (of shallow-occurring homologues) that increase their susceptibility to inactivation by trypsin at pressure are not found in MDH homologues of the same species. Similarly, GAPDH homologues of shallow-occurring species show no increase in susceptibility to

proteolytic inactivation at 1750 atm pressure. This may indicate that, for deep-living fishes, hydrostatic pressure is not as strong an influence on susceptibility to proteolytic inactivation of MDH and GAPDH as it appears to be for M_4-LDH.

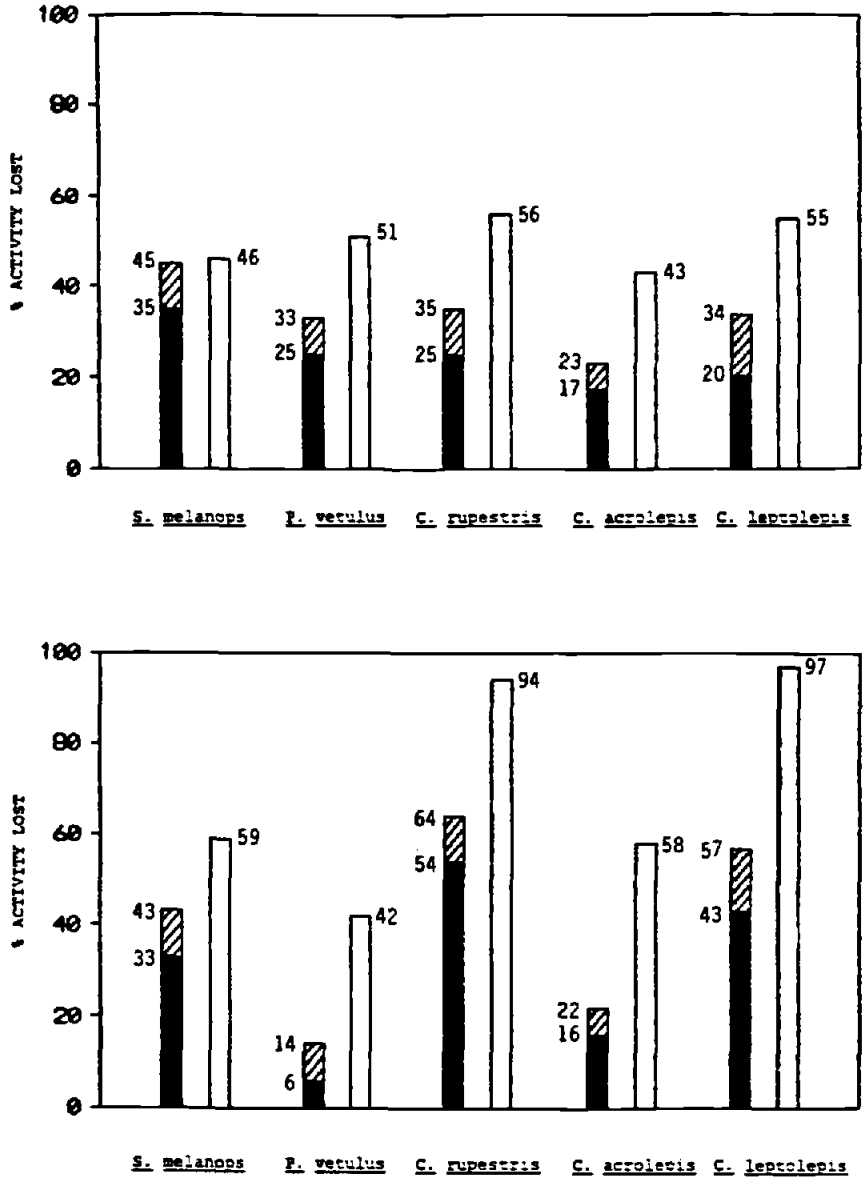
The structural integrity of MDH and GAPDH homologues of deep-living fishes appears to be pre-adapted to the high hydrostatic pressures of the deep-sea. However, the K_m of coenzyme values of these two enzymes are not pre-adapted in shallow-living fishes (Siebenaller 1984a). This is in contrast to M_4-LDH homologues of deep-living fishes which appear to require modification of both structural stability (e.g. subunit interactions, susceptibility to proteolysis [Hennessey and Siebenaller 1985; Chapter III]) and functional parameters (e.g. K_m of coenzyme and K_m of pyruvate [Siebenaller and Somero 1978, 1979], and catalytic efficiency [Somero and Siebenaller 1979]). Minor changes in the primary structure appear to differentiate the M_4-LDH homologue of a shallow-living fish from the homologue of a deep-living congener (Siebenaller 1984c). The MDH and GAPDH homologues examined show differences in the functional parameters but not in their structural stability. The pre-adapted pressure-insensitivity of MDH and GAPDH homologues of shallow-living fishes may also arise from conservation of minor structural features that were selected by other influences on the structural integrity of the enzymes. This is consistent with the contention that the evolutionary change needed to convert a pressure-sensitive enzyme to a pressure-insensitive enzyme may be relatively small (Hochachka and Somero 1984).

Table IV.1. Depths of abundance of the marine fishes studied (taken from Siebenaller et al. [1982] and Miller and Lea [1972]) and rates of proteolytic inactivation of the malate dehydrogenases. MDH homologues were incubated at 10°C and atmospheric pressure with either 1.0 mg/ml trypsin or 0.5 mg/ml subtilisin. ± standard error is given.

SPECIES	DEPTH OF ABUNDANCE (m)	INACTIVATION RATE (h^{-1})	
		TRYPSIN	SUBTILISIN
Shallow-living species			
Sebastes melanops	0-100	0.377 ±0.007	0.933 ±0.075
Parophrys vetulus	20-330	0.253 ±0.009	0.202 ±0.011
Deep-living species			
Coryphaenoides rupestris	550-1960	0.251 ±0.012	1.169 ±0.044
Coryphaenoides acrolepis	475-2825	0.167 ±0.013	0.262 ±0.006
Coryphaenoides leptolepis	2288-4693	0.198 ±0.014	1.422 ±0.056

Figure IV.1. Inactivation of MDH homologues of marine fishes by hydrostatic pressure and proteolysis. A) 1.0 mg/ml trypsin. B) 0.5 mg/ml subtilisin. MDH was incubated for 30 min at 10°C: () with protease at atmospheric pressure; () with albumin at 1750 atm; () with protease at 1750 atm. The difference in height between the bar on the right and the bar on the left represents pressure-enhancement of proteolytic inactivation of MDH homologues.

Figure IV.1


% ACTIVITY LOST
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S. melanops
P. vetulus
C. rupestris
C. acroleipis
C. leptolepis
% ACTIVITY LOST
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42
64
54
94
22
16
58
57
43
97
S. melanops
P. vetulus
C. rupestris
C. acroletis
C. leptolepis

Chapter V

Conclusions

INACTIVATION BY HYDROSTATIC PRESSURE

The difference in hydrostatic pressure between shallow- and deep-ocean environments appears to be important in the evolution of the biochemistry of deep-sea organisms (Somero et al. 1983). In this research hydrostatic pressure was used as a probe to investigate the relative stabilities of NAD-dependent dehydrogenases of shallow- and deep-living fishes. The pressures necessary to half-inactivate M_4-LDH homologues of confamilial deep-living fishes are related to the pressures which the enzymes are exposed to *in vivo*; higher hydrostatic pressures are required to inactivate the LDH homologues of the deeper-occurring species. In general, M_4-LDH homologues of deeper-occurring fishes tend to require higher hydrostatic pressures for half-inactivation than do homologues of shallow-living fishes. Susceptibility to inactivation by hydrostatic pressure of the fish MDH homologues examined is similar to that of LDH homologues of deeper-occurring fishes. Fish GAPDH homologues are much less susceptible to inactivation by hydrostatic pressure than any of the LDH or MDH homologues examined.

Under the conditions used in this study, the pressures necessary for partial inactivation of NAD-dependent dehydrogenases of marine fishes are greater than the pressures experienced by the enzymes *in vivo*. Saturation of coenzyme binding sites, similar to *in vivo* conditions (Atkinson 1977), increases the stability of these enzymes (Table A.3; Schade et al. 1980; Müller et al. 1982) as does inclusion of phosphate at *in vivo* concentrations (20 mM) (personal observations). Thus *in vivo* stability of NAD-dependent dehydrogenases

is probably greater than it was in these experiments and enzyme activity *in vivo* is not likely affected by pressure-dependent shifts in the subunit aggregation equilibrium (i.e. active multimer <===> inactive monomer). However, given the similarity of intracellular conditions in muscle of different fishes (e.g. pH [compensated for temperature differences; Yancey and Somero 1978a], ionic strength [Yancey et al. 1982], substrate concentrations [Yancey and Somero 1978b]), these results are a measure of the relative stability of the enzymes studied, both between different enzymes and between homologues of the same enzyme. Relative stability to inactivation by hydrostatic pressure may reflect the resistance of these enzymes to pressure-enhanced degradation by *in vivo* proteases.

PROTEOLYTIC INACTIVATION

Hydrostatic pressure, by affecting protein aggregation state and conformation, may increase susceptibility to proteolysis. Two proteases, trypsin and subtilisin, were used as probes of 1) the relative susceptibility of enzymes homologues of shallow- and deep-living fishes to proteolytic inactivation, and 2) the effect of hydrostatic pressure on the relative susceptibility of these enzyme homologues to proteolytic inactivation. The susceptibility to proteolytic inactivation of M_4-LDH homologues of shallow-living fishes is greater at atmospheric pressure and increased more by high hydrostatic pressure than for homologues of deeper-occurring fishes. The rate of proteolytic inactivation *in vitro* is a relative indicator of the proteolytic inactivation rate *in vivo* (Goldberg and Dice 1974). Thus it appears that M_4-LDH homologues of deeper-occurring fishes are degraded slower than are homologues of shallow-occurring fishes. This may represent one method of energy conservation in deeper-living fishes inhabiting the food-poor deep-sea.

Fish MDH and GAPDH, which are structurally similar to M_4-LDH, do not show similar relationships between susceptibility of homologues to inactivation by hydrostatic pressure and species depth of abundance, or between susceptibility of homologues to proteolytic inactivation and species depth of abundance. There does, however, appear to be a relationship for these three enzymes between susceptibility to inactivation by hydrostatic pressure and susceptibility to proteolytic inactivation. The enzyme least susceptible to inactivation by hydrostatic pressure (GAPDH) is also the least susceptible to

proteolytic inactivation. LDH, the most susceptible to inactivation by hydrostatic pressure (at least for homologues of shallower-occurring species), is also the most susceptible to proteolytic inactivation. Thus the lack of differences in the structural integrity of MDH and GAPDH homologues of shallow- and deep-living fishes may indicate that the structural features of the shallow-occurring enzymes which determine their susceptibility to inactivation by hydrostatic pressure are pre-adapted to the high hydrostatic pressures of the deep-sea.

Pre-adaptation of one feature of an enzyme does not imply pre-adaptation of all features of an enzyme. For LDH, MDH and GAPDH homologues of deep-living Sebastolobus altivelis the K_m of coenzyme is pressure-insensitive. For homologues of these enzymes from S. alascanus the K_m of coenzyme is pressure-sensitive (Siebenaller and Somero 1978; Siebenaller 1984a). Thus pressure-insensitivity of structural stability should not imply pressure-insensitivity of functional stability. Pre-adaptation of structural stability for MDH and GAPDH of shallow-living fishes does not appear to influence pressure-sensitivity of their kinetic parameters. This suggests that structural adaptations in LDH homologues of deep-living fishes (e.g. in subunit interactions and susceptibility to proteolysis) are not necessarily linked with functional adaptations in these homologues (e.g. for K_m of coenzyme) and vice versa.

The amount of evolutionary change necessary to convert a pressure-sensitive enzyme to a pressure-insensitive enzyme appears to be relatively small. The difference between pressure-sensitive M_4-LDH

of S. alascanus and pressure-insensitive M_4-LDH of S. altivelis appears to result from minor changes in the primary structure of the enzyme (Siebenaller 1984c). These minor changes are responsible for both the structural and functional differences between these LDH homologues. Adaptive differences between MDH and GAPDH homologues of shallow- and deep-living fishes may require lesser alterations in enzyme primary structure. Thus pre-adapted pressure-insensitivity of MDH and GAPDH homologues of shallow-living fishes may arise from minor primary structure features that were selected by other influences on the structural integrity of the enzymes.

BIBLIOGRAPHY

Alexandrov V Ya (1977) Cells, molecules and temperature. Springer-Verlag, New York.

Atkinson D E (1977) Cellular energy metabolism and its regulation. Academic Press, New York.

Banaszak L J, Bradshaw R A (1975) Malate dehydrogenases. The Enzymes 11:369-398.

Basset J, Macheboeuf M, Sandor G (1933) Etudes sur les effets biologiques des ultrapressions. Action des pressions très élevées sur les protéides. Compt Rend 197:796-798.

Berger L R (1958) Some effects of pressure on a phenylglucosidase. Biochim Biophys Acta 30:522-529.

Birktoft J J, Banaszak L J (1983) The presence of a histidine-aspartic acid pair in the active site of 2-hydroxyacid dehydrogenases. J Biol Chem 258:472-482.

Bradford M M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem 72:248-254.

Brewer G J (1970) An introduction to isozyme techniques. Academic, New York

Bridgman P W (1914) The coagulation of albumen by pressure. J Biol Chem 19:511-512.

Bridgman P W, Conant J B (1929) Irreversible transformations of organic compounds under high pressures. Proc Natl Acad Sci U.S. 15:680-683.

Buehner M, Ford G C, Moras D, Olsen K W, Rossmann M G (1974) Three-dimensional structure of D-glyceraldehyde-3-phosphate dehydrogenase. J Mol Biol 90:25-49.

Childress J J, Somero G N (1979) Depth-related enzymic activities in muscle, brain and heart of deep-living pelagic marine teleosts. Mar Biol 52:273-283.

Cossins A R, Macdonald A G (1984) Homeoviscous theory under pressure. II. the molecular order of membranes from deep-sea fishes. Biochim Biophys Acta 776:144-150.

Daniel R M, Cowan D A, Morgan H W, Curran M P (1982) A correlation between protein thermostability and resistance to proteolysis. Biochem J 207:641-644.

Everse J, Kaplan N O (1973) Lactate dehydrogenase: Structure and function. Adv Enzymol 37:61-133.

Giddings N J, Allard H A, Hite B H (1929) Inactivation of the tobacco-mosaic virus by high pressure. Phytopathology 19:749-750.

Goldberg A L, Dice J F (1974) Intracellular protein degradation in mammalian and bacterial cells. Ann Rev Biochem 43:835-869.

Harrington W F, Karr G M (1965) Subunit structure of glyceraldehyde-3-phosphate dehydrogenase. J Mol Biol 13:885-893.

Harris H, Hopkinson D A (1976) Handbook of enzyme electrophoresis in human genetics. North-Holland, Amsterdam.

Harris J I, Perham R N (1965) Glyceraldehyde-3-phosphate dehydrogenases. 1. Protein chains in glyceraldehyde-3-phosphate dehydrogenase from pig muscle. J Mol Biol 13:876-884.

Harris J I, Waters M (1975) Glyceraldehyde-3-phosphate dehydrogenases. The Enzymes 11:1-49.

Harris J I, Roos P (1959) Studies on pituitary polypeptide hormones. 2. The action of pepsin and subtilisin on β-melanocyte-stimulating hormone. Biochem J 71:445-450.

Hennessey J P Jr, Siebenaller J F (1985) Pressure inactivation of tetrameric lactate dehydrogenase homologues of confamilial deep-living fishes. J Comp Physiol B 155:647-652.

Heremans K A H (1974) The self-association of biological macromolecules: high pressure light scattering studies of glutamate dehydrogenase. In: Timmerhaus K D, Barker, M S (ed) Proc 4th Int Conf High Pressure, Kyoto, pp 627-630.

Heremans K (1982) High pressure effects on proteins and other biomolecules. Ann Rev Biophys Bioeng 11:1-21.

Hessler R R (1974) The structure of deep benthic communities from central oceanic waters. In The Biology of the Oceanic Pacific, C B Miller, ed. Oregon State University Press, Corvallis, pp. 79-93.

Hessler R R, Sanders H L (1967) Faunal diversity in the deep sea. Deep-Sea Res 14:65-78.

Hochachka P W, Storey K B, Baldwin J (1975) Gill citrate synthase from an abyssal fish. Comp Biochem Physiol B 52:43-50.

Hochachka P W, Somero G N (1984) Biochemical adaptation. Princeton University Press, Princeton, New Jersey

Holbrook J J, Liljas A, Steindel S J, Rossmann M G (1975) Lactate Dehydrogenases. The Enzymes 11:191-292.

Izui K (1973) Effects of high-pressure on stability and activity of allosteric phosphoenolphosphate carboxylase from Escherichia coli. J Biochem Tokyo 73:505-513.

Jaenicke R (1981) Enzymes under extremes of physical conditions. Ann Rev Biophys Bioeng 10:1-67.

Jaenicke R (1983) Biophysical processes under high hydrostatic pressure. Naturwissenschaften 70:332-341.

Jaenicke R, Rudolph R (1983) What NAD-dependent dehydrogenases teach us about the folding and association of oligomeric proteins. In: Sund H, Ulrich V (ed) Biological oxidations. Springer-Verlag, New York, pp 62-90.

Jeckel D, Anders R, Pfeiderer G (1975) Proteolytische spaltung nativer lactat-dehydrogenase aus schweineherz, I. Untersuchungen über die möglichkeit, durch einwirkung von trypsin enzymatisch aktive spaltprodukte zu erhalten. (Proteolytic cleavage of native dehydrogenase from pig heart, I. Studies on the possibility of obtaining enzymatically active cleavage products by the action of trypsin.) Hoppe-Seyler's Z Physiol Chem 354:737-748.

Johnson F H, Erying H (1970) The kinetic basis of pressure effects in biology and chemistry. In High Pressure Effects on Cellular Processes, AM Zimmerman, ed. Academic Press, New York, pp. 1-44.

Johnson F H, Eyring H, Stover B J (1974) The theory of rate processes in biology and medicine. Wiley and Sons, New York

Kasper C B (1975) Fragmentation of proteins for sequence studies and separation of peptide mixtures. In: Protein Sequence Determination. S B Needleman, ed. Springer-Verlag, New York. pp. 114-161.

Kauzmann W, Bodanszky A, Rasper J (1962) Volume changes in protein reactions. II. Comparison of ionization reactions in proteins and small molecules. J Amer Chem Soc 84:1777-1788.

Kiltz H H, Keil W, Griesbach M, Petry K, Meyer H (1977) The primary structure of porcine lactate dehydrogenase: Isozymes M_4 and H_4. Hoppe-Seyler's S Physiol Chem 358:123-127.

Laemmli U K (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680-685.

Laidler K J (1951) The influence of pressure on the rates of biological reactions. Arch Biochem 30:226-236.

Larson W P, Hartzell T B, Diehl H S (1918) The effect of high pressures on bacteria. J Infect Diseases 22:271-279.

Low P S, Somero G N (1975) Pressure effects on enzyme structure and function in vitro and under simulated in vivo conditions. Comp Biochem Physiol 52B:67-74.

Marquis R E, Fenn M O (1969) Dilatometric study of streptococcal growth and metabolism. Can J Microbiol 15: 933-940.

McLendon G, Radnay E (1978) Is protein turnover thermodynamically controlled? J Biol Chem 253:6335-6337.

Miller D J, Lea R N (1972) Guide to the coastal marine fishes of California. Calif Dept Fish Game, Fish Bull No 157, 235 pp. (1976 addendum, pp. 236-249).

Moon T W (1975) Effects of hydrostatic pressure on gill Na-K-ATPase in an abyssal and a surfacing-dwelling teleost. Comp Biochem Physiol B 52:59-66.

Moras D, Olsen K W, Sabesan M N, Buehner M, Ford G C, Rossmann M G (1975) Studies of asymmetry in the three-dimensional structure of lobster D-glyceraldehyde-3-phosphate dehydrogenase. J Biol Chem 250:9137-9162.

Morild E (1977) Pressure variation of enzymatic-reaction rates of yeast and liver alcohol dehydrogenase. Biophys Chem 6:351-362.

Morild E (1981) The theory of pressure effects on enzymes. Advan Protein Chem 34: 93-166.

Morita R Y, Mathemeier P F (1964) Temperature-hydrostatic pressure studies on partially purified inorganic pyrophosphatase activity. J Bacteriol 88:1667-1671.

Müller K, Lüdemann H D, Jaenicke R (1981) Reconstitution of lactic dehydrogenase from pig heart after reversible high-pressure dissociation. Biochemistry 20:5411-5416.

Müller K, Lüdemann H D, Jaenicke R (1982) Thermodynamics and mechanism of high-pressure deactivation and dissociation of porcine lactic dehydrogenase. Biophys Chem 16:1-7.

Neuman R C, Kauzmann W, Zipp A (1973) Pressure dependence of weak acid ionizations in aqueous buffers. J Phys Chem 77:2687-2691.

Ottesen M, Svendsen I (1970) The subtilisins. Methods Enzymol 19:199-215.

Paladini A A, Weber G (1981) Pressure-induced reversible dissociation of enolase. Biochemistry 20:2587-2593.

Penniston J T (1971) High hydrostatic pressure and enzymic activity: Inhibition of multimeric enzymes by dissociation. Arch Biochem Biophys 142:322-332.

Perrin D D, Dempsey B (1974) Buffers for pH and metal ion control. New York, Wiley.

Peterson G L (1983) Determination of total protein. Methods Enzymol 91:95-119.

Place A R, Powers D A (1984) Purification and characterization of the lactate dehydrogenase (LDH-B_4) allozymes of Fundulus heteroclitus. J Biol Chem 259:1299-1308.

Poulik M D (1957) Starch gel electrophoresis in a discontinuous system of buffers. Nature 1980:1477-1479.

Rahn H, Reeves R B, Howell B J (1975) Hydrogen ion regulation, temperature, and evolution. Amer Rev Resp Dis 112:165-172.

Regnard P (1891) "Recherches expérimentales sur les conditions physiques de la vie dans les eaux." Librairie de l'Academie de Médecine, Paris.

Rossmann M G (1983) Structure-function relationships of NAD-dependent dehydrogenases. In Biological Oxidations, H Sund and V Ullrich, eds. Springer-Verlag, New York, pp. 33-54.

Rossmann M G, Argos P (1978) The taxonomy of binding sites in proteins. Mol Cell Biochem 21:161-182.

Rossmann M G, Liljas A, Brändén C-I, Banaszak L J (1975) Evolutionary and structural relationships among dehydrogenases. The Enzymes 11:61-102.

Schade B C, Rudolph R, Lüdemann H D, Jaenicke R (1980) Reversible high-pressure dissociation of lactic dehydrogenase from pig muscle. Biochemistry 19:1121-1126.

Schmid G, Lüdemann H D, Jaenicke R (1978) Oxidation of sulfhydryl groups in lactate dehydrogenase under high hydrostatic pressure. Eur J Biochem 86:219-224.

Schmid G, Lüdemann H D, Jaenicke R (1979) Dissociation and aggregation of lactic dehydrogenase by high hydrostatic pressure. Eur J Biochem 97:407-413.

Siebenaller J F (1978) Genetic variability in deep-sea fishes of the genus Sebastolobus (Scorpaenidae). In: Battaglia B, Beardmore J (ed) Marine organisms: genetics, ecology and evolution. Plenum, New York, pp 95-122.

Siebenaller J F (1983) The pH-dependence of the effects of hydrostatic pressure on the M_4-lactate dehydrogenase homologs of scorpaenid fishes. Mar Biol Letters 4:233-243.

Siebenaller J F (1984a) Pressure-adaptive differences in the NAD-dependent dehydrogenases of congeneric marine fishes living at different depths. J Comp Physiol 154:443-448.

Siebenaller J F (1984b) Analysis of the biochemical consequences of ontogenetic vertical migration in a deep-living teleost fish. Physiol Zool 57:598-608.

Siebenaller J F (1984c) Structural comparison of lactate dehydrogenase homologs differing in sensitivity to hydrostatic pressure. Biochim Biophys Acta 786:161-169.

Siebenaller J F, Somero G N (1978) Pressure-adaptive differences in lactate dehydrogenases of congeneric fishes living at different depths. Science 201:255-257.

Siebenaller J F, Somero G N (1979) Pressure-adaptive differences in binding and catalytic properties of muscle-type (M_4) lactate dehydrogenases of shallow- and deep-living marine fishes. J Comp Physiol 129:295-300.

Siebenaller J F, Somero G N (1982) The maintenance of different enzyme activity levels in congeneric fishes living at different depths. Physiol Zool 55:171-179.

Siebenaller J F, Somero G N, Haedrich R L (1982) Biochemical characteristics of macrourid fishes differing in their depths of distribution. Biol Bull 163:240-249.

Somero G N, Siebenaller J F (1979) Inefficient lactate dehydrogenases of deep-sea fishes. Nature 282:100-102.

Somero G N, Siebenaller J F, Hochachka P W (1983) Biochemical and physiological adaptations of deep-sea animals. In: Rowe G T (ed) The sea, vol 8. Wiley, New York, pp 261-330.

Somero G N, Siebenaller J F, Swezey R R (1985) Pressure effects on enzymatic assembly: mechanisms and adaptations. Fed Proc (in press).

Spielmann H, Erickson R P, Epstein C J (1973) The separation of lactate dehydrogenase X from other lactate dehydrogenase isozymes of mouse testes by affinity chromatography. FEBS Letters 35:19-23.

Sullivan K M, Somero G N (1980) Enzyme activities of fish skelletal muscle and brain as influenced by depth of occurrence and habits of feeding and locomotion. Mar Biol 60:91-99.

Swezey R R, Somero G N (1982) Polymerization thermodynamics and structural stabilities of skeletal muscle actins from vertebrates adapted to different temperatures and hydrostatic pressures. Biochem 21:4496-4503.

Swezey R R, Somero G N (1985) Pressure effects on actin self-assembly: interspecific differences in the equilibrium and kinetics of the G to F transformation. Biochem 24:852-860.

Torgerson P M, Drickamer H G, Weber G (1979) Inclusion complexes of poly-β-cyclodextrin: a model for pressure effects upon ligand-protein complexes. Biochem 18:3079-3083.

Torgersen P M, Drickamer H G, Weber G (1980) Effect of hydrostatic pressure upon ethidium bromide association with transfer ribonucleic acid. Biochem 19:3957-3960.

Weber G (1980) The effects of high-pressure upon proteins and other biomolecules. In New Horizons in Biological Chemistry, M Koike, ed. Tokyo:JSSP press, pp. 237-245.

Weber G, Drickamer H G (1983) The effect of high pressure upon proteins and other biomolecules. Quart Rev Biophys 16:89-112.

Wheatley D N (1984) Intracellular protein degradation: basis of a self-regulating mechanism for the proteolysis of endogenous proteins. J Theor Biol 107:127-149.

Wilson R R Jr, Waples R S (1983) Distribution, morphology and biochemical genetics of Coryphaenoides armatus and C. yaquinae (Pisces: Macrouridae) in the central and eastern North Pacific. Deep-Sea Res 30:1127-1145.

Wilson R R Jr, Waples R S (1984) Electrophoretic and biometric variability in the abyssal grenadier Coryphaenoides armatus of the western North Atlantic, eastern South Pacific and eastern North Pacific Oceans/ Mar Biol 80:227-237.

Yancey P H, Somero G N (1978a) Temperature dependence of intracellular pH: Its role in the conservation of pyruvate apparent K_m values of vertebrate lactate dehydrogenases. J Comp Physiol 125:129-134.

Yancey P H, Somero G N (1978b) Urea-requiring lactate dehydrogenases of marine elasmobranch fishes. J Comp Physiol 125:135-141.

Yancey P H, Clark M E, Hand S C, Bowlus R D, Somero G N (1982) Living with water stress: evolution of osmolyte systems. Science 217:1214-1222.

ZoBell C E, Kim J (1972) Effects of deep-sea pressures on microbial enzyme systems. Symp Soc Exp Biol 26:125-146.

ZoBell C E, Oppenheimer C H (1950) Some effects of hydrostatic pressure on the multiplication and morphology of marine bacteria. J Bacteriol 60:771-778.

APPENDIX

Table A.1. The specific activities of NAD-dependent dehydrogenases of shallow- and deep-living fishes and the apparent volume changes (ΔV) associated with pressure-inactivation of the M_4-LDH homologues. Specific activity was calculated as the activity of the enzyme at $10^{\circ}C$ at optimal substrate and coenzyme concentration per mole enzyme in solution. Enzyme activities were determined as described in Chapter IV. Protein concentration was determined using the Bradford (1976) Coomassie Blue dye binding assay as given by Peterson (1983). Bovine serum albumin was used as the standard. The ΔV values were determined at $4^{\circ}C$ as described in Chapter II.

Estimation of specific activities of the dehydrogenase homologues was necessary to determine 1) whether M_4-LDH homologues of deep-living fishes used in these studies showed the same decrease in catalytic efficiency, relative to homologues of shallow-living fishes, as observed for M_4-LDH homologues of other deep-living fishes (Somero and Siebenaller 1979), 2) whether MDH and GAPDH, which appear to show the same pressure-adaptive differences between homologues of shallow- and deep-living fishes for pressure-sensitivity of the K_m of coenzyme (Siebenaller 1984a), show a similar decrease in catalytic efficiency for homologues of deep-living fishes as seen in M_4-LDH, and 3) approximate concentrations of dehydrogenases in the dilute solutions (1 to 5 μg/ml) used in these studies.

The catalytic efficiency of LDH homologues of deep-living fishes is significantly less than that of shallow-occurring homologues (two-tailed Mann-Whitney test, $P < 0.02$). The mean value for catalytic efficiency of deep-occurring homologues is 73% of that for shallow-

occurring homologues. This is consistent with the results of Somero and Siebenaller (1979). There is no significant difference in catalytic efficiency between shallow- and deep-living homologues of MDH and GAPDH (two-tailed Mann-Whitney test $P > 0.24$, MDH; $P > 0.15$, GAPDH).

The ΔV of inactivation of M_4-LDH homologues was calculated to determined whether the ΔV of inactivation is less for homologues of deep-living species than it is for homologues of shallow-living species (see also Table II.1). There is no difference in the ΔV of inactivation for M_4-LDH homologues of shallow- and deep-living fishes (two-tailed Mann-Whitney test, $P > 0.81$) and the correlation between the pressure at which an LDH homologue is half-inactivated (see Table II.1 and III.1) and the ΔV of inactivation is not significantly different than zero (Pearson's correlation coefficient, $P > 0.43$).

Table A.1

SPECIES	DEPTH OF ABUNCANCE (m)	SPECIFIC ACTIVITY (moles substrate converted to product/min/mole enzyme)			ΔV (ml/mol)
		MDH	LDH	GAPDH	LDH
Shallow-living species					
Sebastes melanops	0- 100	10,100	163,800	13,500	-307 ±31
Sebastes pinniger	0- 300	---	140,300	---	-248 ± 6
Platichthys stellatus	0- 300	---	131,000	---	-653 ±58
Parophrys vetulus	20- 330	6,600	110,100	14,100	-365 ±28
Microstomus pacificus	30-1000	---	118,900	---	-240 ±21
Deep-living species					
Anoplopoma fimbria	200-1670	---	122,700	---	---a
Antimora microlepis	400-3300	---	102,800	---	-337 ±23
Antimora rostrata	825-2500	---	98,000	---	-176 ±19
Coryphaenoides rupestris	550-1960	9,000	99,800	12,200	-219 ±30
Coryphaenoides acrolepis	475-2825	19,100	101,000	13,500	-439 ±45
Coryphaenoides leptolepis	2288-4639	13,900	60,200	3,900	-308 ±20

[a]. No detectable loss of activity after 1 h at 1750 atm and 4°C.

Table A.2. The rates of thermal inactivation of NAD-dependent dehydrogenases of shallow- and deep-living fishes. Samples were incubated in 20 mM potassium phosphate, pH 7.0, 5 mM 2-mercaptoethanol, 1 mM EDTA and 100 µg/ml chicken egg albumin. Enzyme concentration was approximately 1 µg/ml. Aliquots of 1.0 ml in 1.5 ml polypropylene centrifuge tubes with caps were placed in a heating block to maintain incubation temperatures within $\pm$ 0.5°C. LDH homologues were incubated at 50°C, MDH homologues at 45°C and GAPDH homologues at 60°C. Thermal inactivation was monitored over the course of 2 h by removing 100 µl aliquots from the heated sample and assaying for enzyme activity. Enzyme activity was determined as described in Chapter IV. Thermal inactivation rates were calculated as the slope of the regression of log percent activity remaining versus time. Six to eight time points were used for each regression.

These experiments were performed to determine the relationship between susceptibility to thermal inactivation, susceptibility to pressure-inactivation and susceptibility to proteolytic inactivation of homologous enzymes. Homologues of MDH are, in general, more susceptible to thermal inactivation than are LDH homologues. LDH homologues are more susceptible to thermal inactivation than GAPDH homologues. The rates of thermal inactivation of GAPDH homologues is related to species depth of abundance (two-tailed Mann-Whitney test, $P < 0.01$); homologues from deeper-occurring species are more resistant to thermal inactivation. In contrast, LDH homologues (at least those from shallow-living fishes) are more susceptible to inactivation by hydrostatic pressure and proteolysis than are MDH and GAPDH

homologues. MDH homologues of marine fishes are similar in susceptibility to inactivation by pressure and proteolysis as LDH homologues of deep-occurring fishes. GAPDH, however, is very resistant to inactivation by either hydrostatic pressure or proteolysis (see Chapter IV). Thus, while fish GAPDH homologues are least susceptible to any of the three inactivating agents, the differences in susceptibility of LDH and MDH homologues to inactivation by pressure (LDH $\geq$ MDH), temperature (MDH > LDH), and proteolysis (LDH $\geq$ MDH) prevents any reliable conclusions about relationships between susceptibility to inactivation by these agents based on these data.

Table A.2

SPECIES	DEPTH OF ABUNDANCE (m)	RATE OF INACTIVATION (h^{-1}) LDH	MDH	GAPDH
Shallow-living species				
Sebastes melanops	0- 100	383 ±36	>1600	>1170
Parophrys vetulus	20- 330	9 ± 1	121 ± 4	729 ±78
Deep-living species				
Coryphaenoides rupestris	550-1960	22 ± 2	178 ± 8	429 ±12
Coryphaenoides acrolepis	475-2825	38 ± 3	125 ± 4	197 ± 8
Coryphaenoides leptolepis	2288-4639	170 ±11	136 ± 2	53 ± 3

Table A.3. The effect of coenzyme on the susceptibility of NAD-dependent dehydrogenases from marine fishes to pressure-inactivation and proteolysis. LDH and MDH homologues were incubated at 10°C in the presence and absence of 600 μM NADH. GAPDH was incubated at 30°C in the presence and absence of 2 mM NAD. Pressure-inactivation of LDH was at 1000 atm pressure; MDH and GAPDH at 1750 atm pressure. See Chapter IV for details of the incubation buffer and activity determinations.

Bound coenzyme appears to decrease the susceptibility M_4-LDH homologues to inactivation by pressure (Schade et al. 1980; Müller et al. 1982) and by proteases (Jeckel et al. 1973; Place and Powers 1984). These experiments were performed to determine the effect of coenzyme binding on the stability of NAD-dependent dehydrogenases of marine fishes. With the exception of M_4-LDH from Sebastes melanops, all of the NAD-dependent dehydrogenases used in this study showed decreased susceptibility to inactivation by hydrostatic pressure upon binding of coenzyme. MDH and GAPDH homologues appear to be less susceptibility to inactivation by proteases in the presence of coenzyme. However, fish LDH homologues appear to be more susceptible to inactivation by proteases in the presence of coenzyme than in the absence of coenzyme. Thus the MDH and GAPDH homologues examined appear to be stabilized upon coenzyme binding. LDH homologues, with the exception of the S. melanops homologue, are more stable to pressure-inactivation in the presence of coenzyme. These findings are consistent with other studies (Schade et al. 1980; Müller et al. 1982; Jeckel et al. 1973; Place and Powers 1984). However, the increased

susceptibility of the LDH homologues to inactivation by proteases is contrary to other reports (Jeckel et al. 1973; Place and Powers 1984).

Table A.3

	PRESSURE-INACTIVATION		PROTEOLYTIC INACTIVATION	
	% ACTIVITY LOST		RATE WITH COENZYME / RATE WITHOUT COENZYME	
	NO COENZYME	COENZYME	TRYPSIN	SUBTILISIN
LDH				
Sebastes melanops	13	23	1.45	1.78
Parophrys vetulus	6	2	1.10	----
Coryphaenoides rupestris	58	24	8.78	2.57
Coryphaenoides acrolepis	34	25	1.69	----
Coryphaenoides leptolepis	14	12	1.85	----
MDH				
Sebastes melanops	10	5	0.27	0.16
Coryphaenoides rupestris	10	0	0.21	0.37
GAPDH				
Sebastes melanops	49	0	0.08[a]	0.58[a]
Coryphaenoides rupestris	11	0	----[a]	----[a]

[a] No activity lost after 2 h in the presence of coenzyme.

Figure A.1 The effect of incubation temperature on (a) the apparent volume change (ΔV) of dissociation and (b) the pressure at which half-inactivation occurs ($P_{1/2}$) for M_4-LDH homologues of six macrourid fishes. Experiments procedures and enzyme activity determinations were as described in Hennessey and Siebenaller (1985). The pH of the incubation medium was allowed to vary with temperature. Incubation conditions were as described in Chapter II. Nezumia bairdii: ○; Coryphaenoides rupestris: ■; C. acrolepis: △; C. carapinus: ●; C. armatus: □; C. leptolepis: ▲. Standard errors are shown.

These experiments show the dependence of pressure-inactivation of these LDH homologues on incubation temperature. Increased temperature decreases the magnitude of the ΔV. The free volume at the interfaces of the subunits increases but trapping of solvent at the interfaces results in a net decrease in the ΔV with increasing temperature (Heremans 1982). This rationale can be used to explain why temperature increases also result in decreased susceptibility of the LDH homologues to pressure-inactivation i.e. increases in the $P_{1/2}$. Increased temperature increases the free volume at the subunit interfaces whereas pressure decreases these volumes. Thus temperature can counteract some pressure effects on subunit interactions.

Figure A.1a

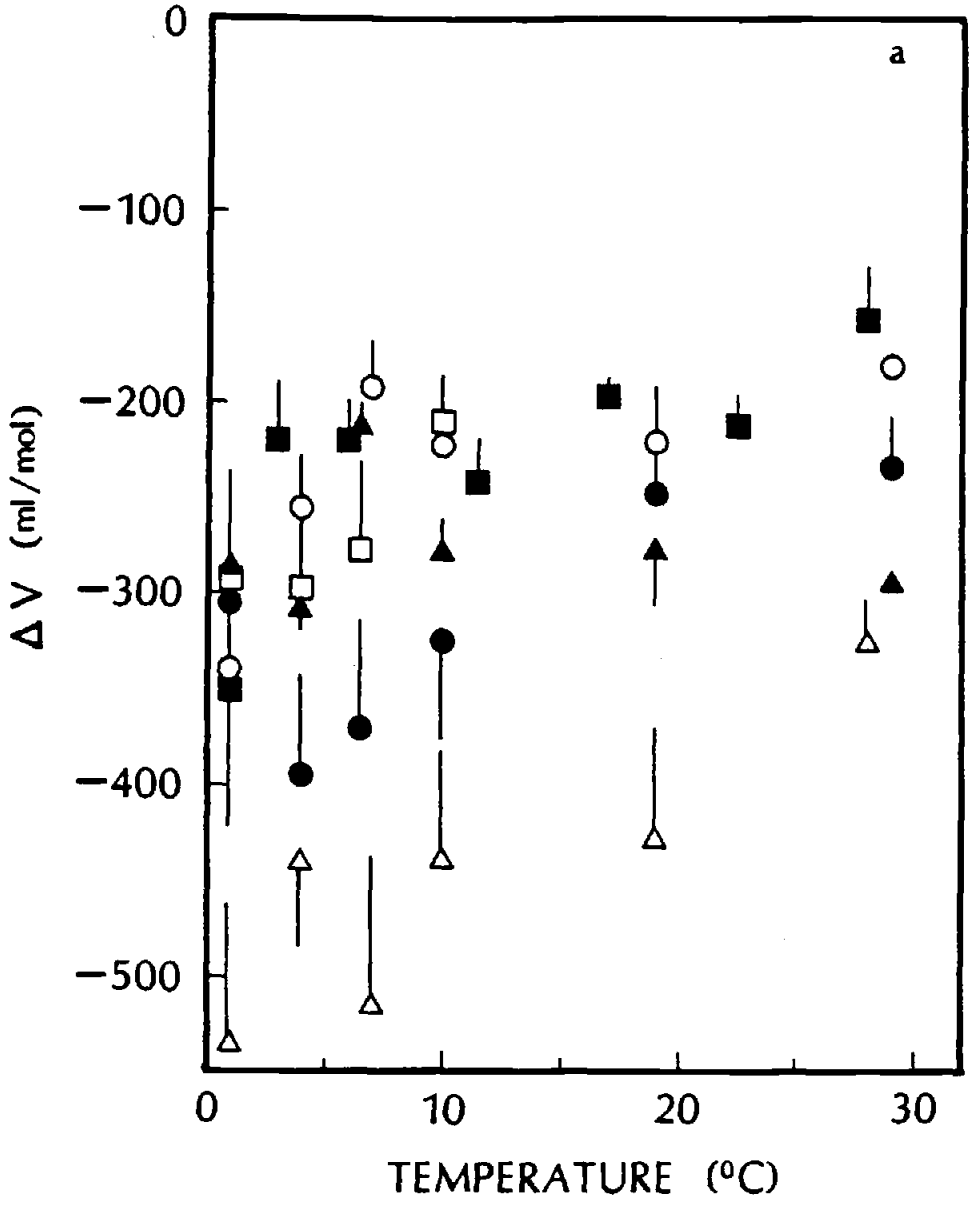


Figure A.1b

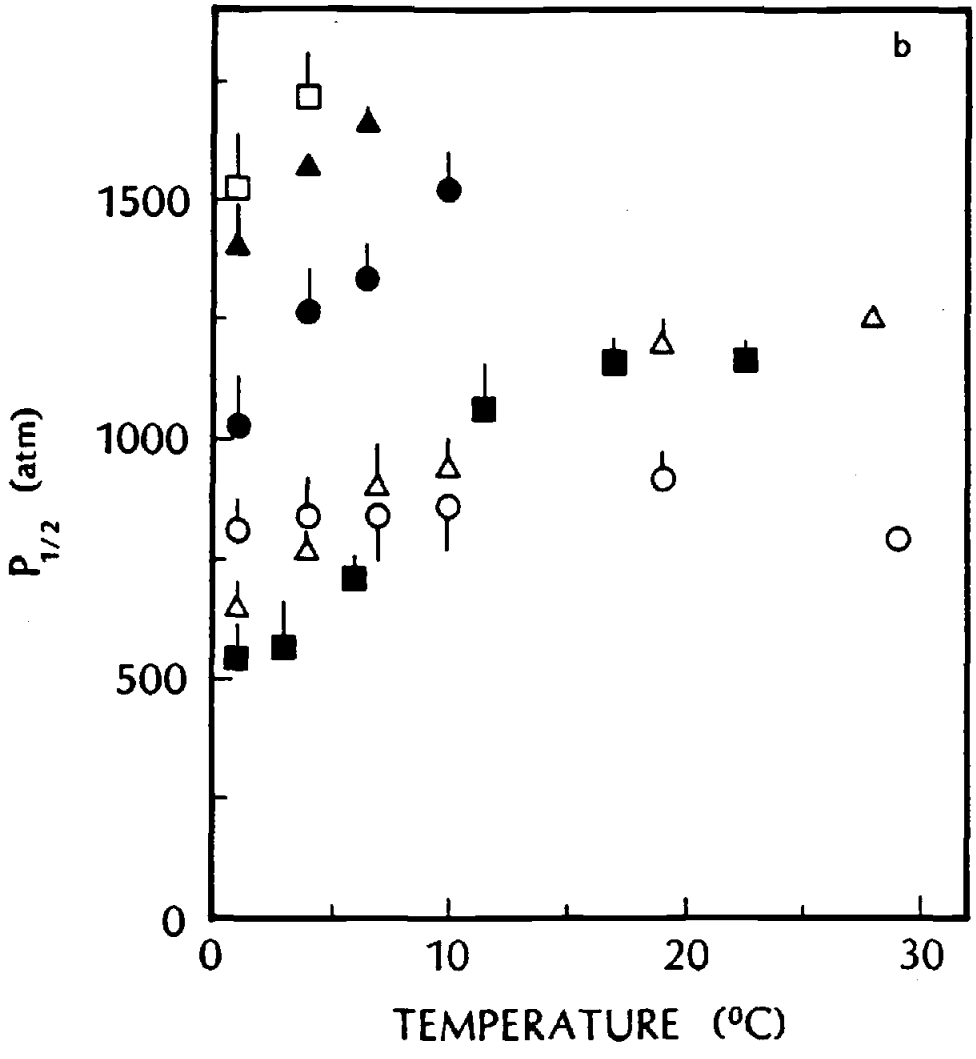


Figure A.2. The effect of incubation pH at 4°C and constant ionic strength (I = 0.054) on the pressure inactivation of M_4-LDH homologues. Samples were incubated for 1 h in 20 mM imidazole-HCl at the desired pH, 5 mM 2-mercaptoethanol and 1 mM EDTA. Experimental procedures and enzyme activity determinations were as described in Hennessey and Siebenaller (1985). Nezumia bairdii (700 atm): ● and Coryphaenoides rupestris (600 atm): ■ .

These experiments show the pH-dependence of LDH stability at high hydrostatic pressure for homologues from two marine fishes. A decrease of 1 pH unit (from approximately pH 7.6 to pH 6.6) results in a greater than three-fold increase in percent activity remaining for both homologues studied. The pH of imidazole-HCl decreases 0.02 pH units for each one degree (Celsius) increase in temperature (Perrin and Dempsey 1974), similar to that of biological fluids (Rahn et al. 1975). The strong influence of pH on susceptibility of LDH homologues to pressure-inactivation indicates that the temperature dependence of the ΔV of dissociation and the $P_{1/2}$ may emanate, at least in part, from pH effects on the LDH tetramer.

Figure A.2

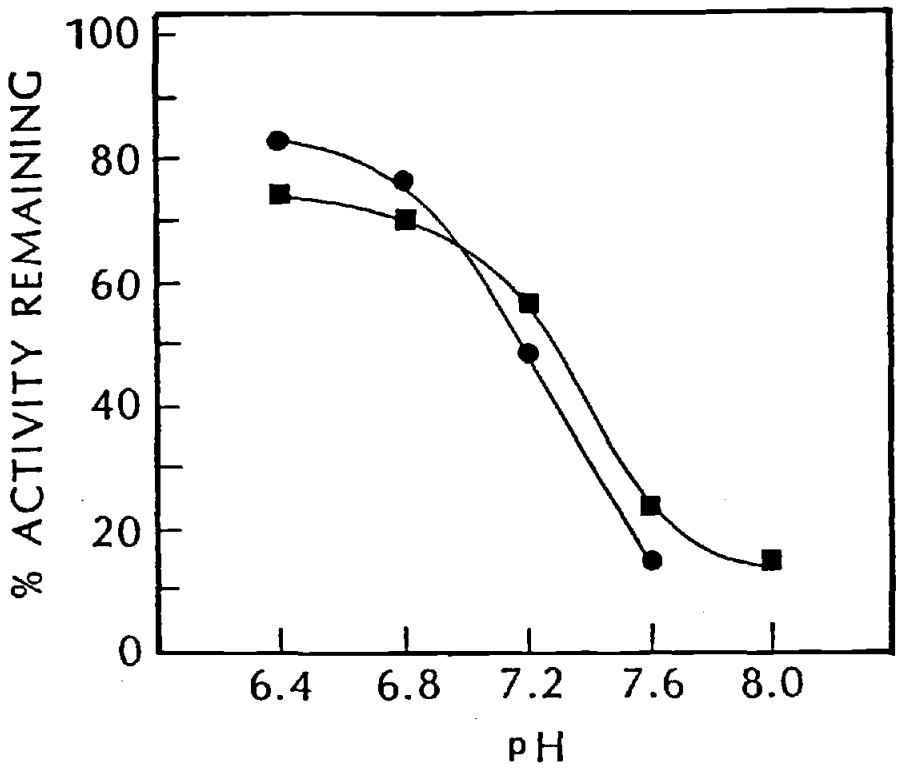


Figure A.3. The effect of pressure on ln K_{eq} of the M_4-LDH homologues of five shallow- and two deep-living fishes. Values are for 1 h incubations at 4°C. Protein concentrations (μg/ml) used are indicated in parentheses below. See Hennessey and Siebenaller (1985) for the method of calculation and assumptions involved in those calculations. Sebastes melanops (6.1): +; S. pinniger (9.6): ◇; Platichthys stellatus (10.7): △; Parophrys vetulus (8.5): ×; Microstomus pacificus (10.2): □; Antimora microlepis (7.3): ■; A. rostrata (8.9): ▲.

These data plus data from Figure II.4 were used to calculate the volume change (ΔV) of dissociation and pressure of half-inactivation ($P_{1/2}$) values reported in Table A.1, II.1 and III.1. Plots were linear over the range of pressures where inactivation was detectable (i.e. from 5 to 95% activity remaining).

Figure A.3

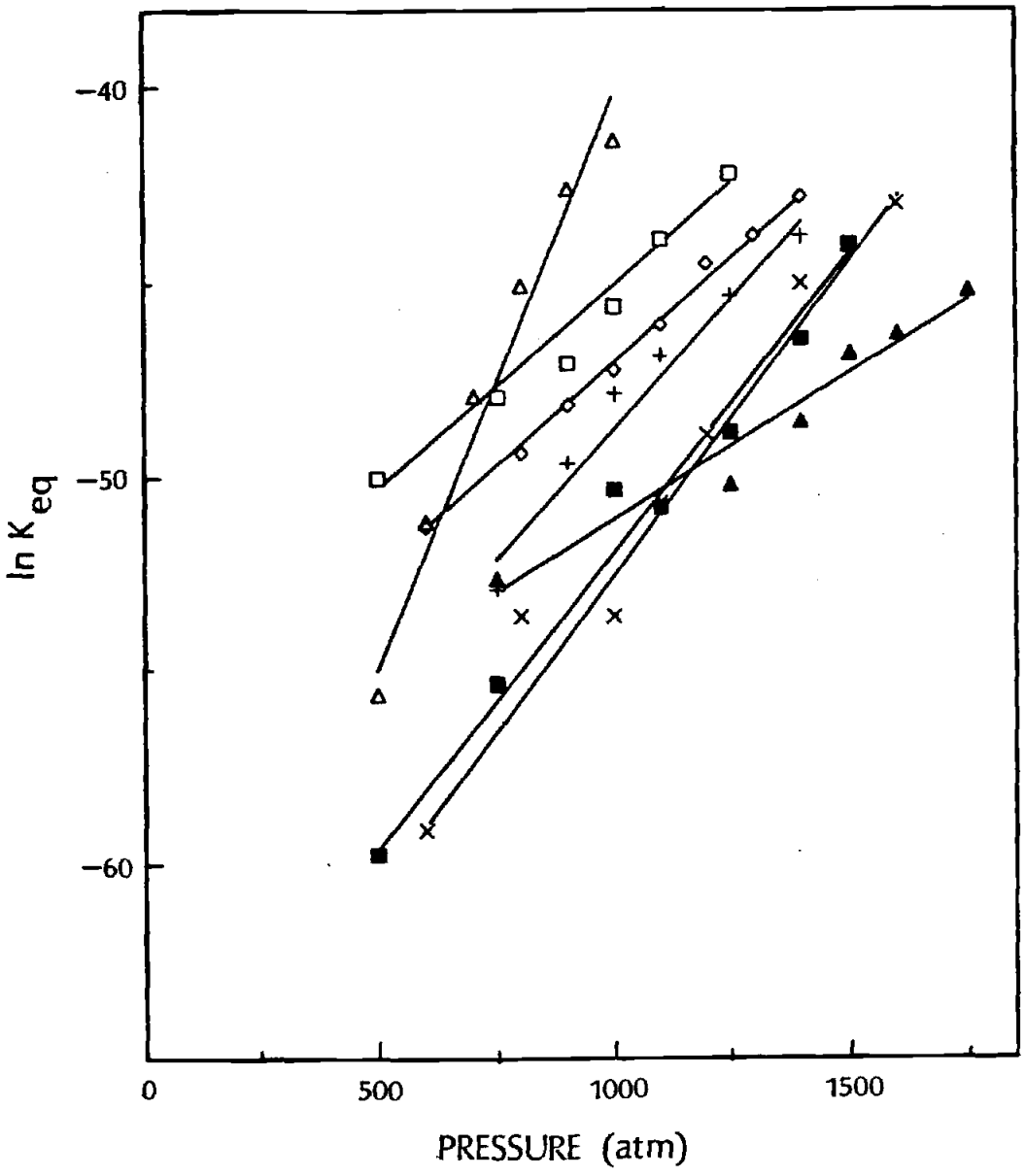
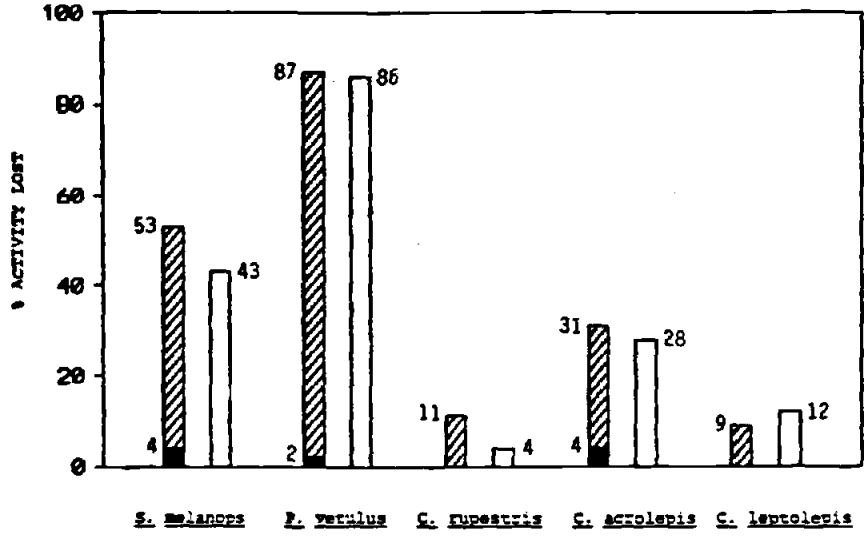
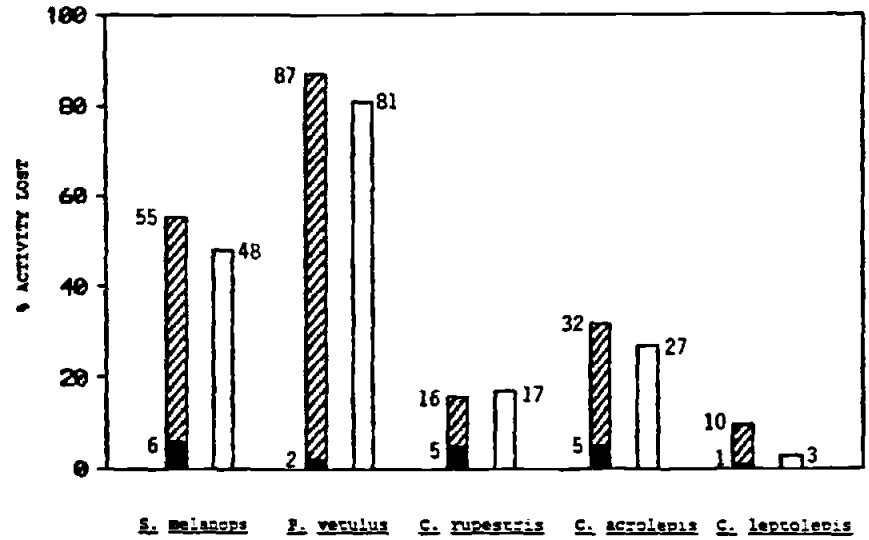


Figure A.4. Inactivation of GAPDH homologues of marine fishes by hydrostatic pressure and proteolysis. A) 1.0 mg/ml trypsin. B) 1.0 mg/ml subtilisin. GAPDH was incubated for 30 min at 30°C: (■) with protease at atmospheric pressure; (▨) with albumin at 1750 atm pressure; (□) with protease at 1750 atm pressure. Experimental procedures and enzyme activity determinations were as described in Chapter IV.

These data show the susceptibility of fish GAPDH homologues to inactivation by hydrostatic pressure and proteolysis relative to LDH and MDH homologues from the same species (see Figure III.1, III.2 and IV.1). Loss of GAPDH activity due to tryptic inactivation at 1750 atm pressure and 30°C is the same as the combined effects of inactivation by 1750 atm pressure and 30°C and inactivation by trypsin at atmospheric pressure and 30°C (t-test, $P > 0.188$). Inactivation by subtilisin at 1750 atm pressure and 30°C is less than the combined effects of inactivation by 1750 atm pressure and inactivation by subtilisin at atmospheric pressure (t-test, $P < 0.033$). Inactivation of GAPDH homologues by protease at 1750 atm pressure and 30°C was not significantly different from inactivation by 1750 atm pressure and 30°C (t-test, $P > 0.477$, trypsin; $P > 0.65$, subtilisin). The low susceptibility of GAPDH homologues to inactivation by hydrostatic pressure and proteolysis, relative to LDH and MDH homologues from the same species, is a pattern similar to that seen for thermal inactivation of these enzymes (see Table A.2). Thus, of the three NAD-dependent dehydrogenases, GAPDH appears to be the most stable to high temperatures, high hydrostatic pressures, and proteases.

Figure A.4


% ACTIVITY LOST
100
80
60
40
20
0
53
43
87
86
11
4
31
28
9
12
4
2
4
S. melanops
P. vetulus
C. rupestris
C. acrolepis
C. leptolepis


% ACTIVITY LOST
100
80
60
40
20
0
55
48
87
81
16
17
32
27
10
3
6
2
5
5
1
S. melanops
P. vetulus
C. rupestris
C. acrolepis
C. leptolepis