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PRESSURE AND TEMPERATURE ADAPTATION OF CYTOSOLIC MALATE DEHYDROGENASES OF SHALLOW- AND DEEP-LIVING MARINE INVERTEBRATES: EVIDENCE FOR HIGH BODY TEMPERATURES IN HYDROTHERMAL VENT ANIMALS

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Summary

Effects of temperature and hydrostatic pressure were measured on cytosolic malate dehydrogenases (cMDHs) from muscle tissue of a variety of shallow- and deep-living benthic marine invertebrates, including seven species endemic to the deep-sea hydrothermal vents. The apparent Michaelis–Menten constant (K_m) of coenzyme (nicotinamide adenine dinucleotide, NADH), used to index temperature and pressure effects, was conserved within a narrow range (approximately 15–25 μmol l^{-1}) at physiological temperatures and pressures for all species. However, at elevated pressures, the K_m of NADH rose sharply for cMDHs of shallow species (depths of occurrence <approximately 500 m), but not for the cMDHs of deep-sea species. Cytosolic MDHs of invertebrates from the deep-sea hydrothermal vents generally were not perturbed by elevated temperatures (15–25 °C) at *in situ* pressures, but cMDHs of cold-adapted deep-sea species were. At a single measurement temperature, the K_m of NADH for cMDHs from invertebrates from habitats with well-characterized temperatures was inversely related to maximal sustained body temperature. This correlation was used to predict the maximal sustained body temperatures of vent invertebrates for which maximal habitat and body temperatures are difficult to estimate. Species occurring on the 'smoker chimneys', which emit waters with temperatures up to 380 °C, are predicted to have sustained body temperatures that are approximately 20–25 °C higher than vent species living in cooler vent microhabitats. We conclude that, just as adaptation of enzymes to elevated pressures is important in establishing species' depth distribution patterns, adaptation of pressure-adapted enzymes to temperature is critical in enabling certain vent species to exploit warm-water microhabitats in the vent environment.

Introduction

The deep sea is characterized by high hydrostatic pressures and, in most locations, temperatures that do not exceed 4 °C (Sverdrup *et al.* 1942). Among the

Key words: malate dehydrogenase, hydrostatic pressure, hydrothermal vents.

factors allowing deep-sea organisms to thrive at high pressure are biochemical systems – membranes, structural proteins, and enzymes – that function well at high pressures and low temperatures, conditions that perturb the functions and structures of homologous biochemical systems from shallow-living species (Gibbs and Somero, 1989, 1990; Siebenaller and Somero, 1989; Somero, 1990). In addition to high pressure, organisms that inhabit deep-sea hydrothermal vent sites may encounter temperatures well above those of the ambient cold water (Fustec *et al.* 1987; Hessler and Smithey, 1984; Johnson *et al.* 1988). Therefore, success in the warmer microhabitats within the hydrothermal vent ecosytem, where sulphide-rich waters support abundant life based on chemosynthesis (Childress *et al.* 1987; Somero *et al.* 1989), may require adaptations to elevated temperatures as well as the ability to tolerate and/or exploit hydrogen sulphide (Dahlhoff *et al.* 1990, 1991).

To investigate whether enzymes of hydrothermal vent species are adapted to higher and more variable temperatures than those encountered by species from typical cold and thermally stable deep-sea environments, we studied the influences of temperature and pressure on the kinetics of malate dehydrogenases (MDH; EC 1.1.1.37; malate:NAD^+ oxidoreductase) from 15 species of marine invertebrates, including species of molluscs, crustaceans, polychaetes and a vestimentiferan (Table 1), from shallow water and deep-sea habitats. Dehydrogenases, particularly MDHs and lactate dehydrogenases (LDHs), have been studied extensively in shallow- and deep-living teleost fishes (Siebenaller and Somero, 1978; Siebenaller, 1984, 1987). These studies have identified aspects of enzyme function that are strongly perturbed by pressure and that, in deep-sea species, exhibit adaptations to high pressure. LDHs and MDHs from adult fishes living at depths greater than 500–1000 m and at typical, cold deep-sea temperatures have kinetic properties much less affected by pressure than the homologous enzymes of cold-adapted, shallow-living fishes. For example, effects of pressure (at 5 °C) on the apparent Michaelis–Menten constant (apparent K_m) of cofactor (NADH) and substrate are small or non-existent for LDHs and MDHs of deep-sea fishes, but large in the case of the enzymes from shallow-living fishes (reviewed in Siebenaller and Somero, 1989). At physiological pressures and temperatures, K_m values are strongly conserved among species, even though they are strongly affected by both environmental variables (Hochachka and Somero, 1984).

That adaptations to temperature might also play a role in adaptation to the deep sea is suggested by the finding that LDH of a cold-adapted, deep-sea fish, the cosmopolitan rattail *Coryphaenoides armatus*, was strongly perturbed under pressure at temperatures above approximately 10 °C, whereas the LDH of the endemic hydrothermal vent fish *Thermarces andersoni* was not (Dahlhoff *et al.* 1990). These interspecific differences in the effects of pressure and temperature on the K_m of cofactor and substrates of enzymes are hypothesized to play important roles in establishing the vertical distribution patterns of marine teleosts and, in the hydrothermal vent environment, the fine-scale distributions of fishes in different thermal microhabitats.

Table 1. *Species studied and their habitat depths and temperature ranges*

Species	Habitat temperature (°C)	Maximal habitat depth (m)
Deep-sea hydrothermal vents		
Alvinella pompejana	2–40[b]	2600
Alvinella caudata	2–40[b]	2600
Riftia pachyptila	2–15[a]	2600
Bythograea thermydron	2–35[b]	2600
Alvinoconcha hessleri	2–25[g]	3400
Calyptogena magnifica	2–7[c]	2600
Bathymodiolus thermophilus	2–14[i]	2600
Cold deep sea		
Calyptogena phaseoliformes	2–4[f]	3300
Florida escarpment mussel	2–4[f]	3300
Shallow warm habitats		
Mytilus galloprovincialis	12–20[d]	10
Chaetopterous variopedatus	14–20[d]	10
Cancer antennarius	14–17[d]	10
Isognomen incisum	23–27[j]	10
Shallow cold habitats		
Calyptogena elongata	5–8[e]	700
Louisiana seep mussel	6–8[h]	500

Temperature data for all but the hydrothermal vent species are assumed to reflect the range of body temperatures encountered by the organisms in their habitats. For all but the hydrothermal vent species, we assume that the maximal habitat temperature given approximates the maximal sustained body temperature of the species. For hydrothermal vent species, maximal sustained body temperatures based on ambient water temperatures are reliable only for *Alvinoconcha hessleri*, *Calyptogena magnifica* and *Bathymodiolus thermophilus*. Maximal sustainable body temperatures for the other vent species are predicted (see Fig. 3 and Discussion).

[a] Johnson *et al.* (1988); [b] D. Desbruyères (IFREMER-Centre de Brest, personal communication); [c] Hessler *et al.* (1985); [d] Ricketts *et al.* (1968); [e] J. O'Brien (Scripps Institution of Oceanography, personal communication); [f] no thermal anomalies have been reported at these sites; [g] Hessler *et al.* (1988*a*); [h] Childress *et al.* (1986); [i] Fisher *et al.* (1988); [j] Armstrong (1983).

To date, the effects of pressure and temperature on enzymes from deep-living marine invertebrates have not been examined. There are several reasons why the investigation of invertebrate systems is potentially interesting. Examination of invertebrate enzymes may give an indication of the pervasiveness of protein adaptations to temperature and pressure and, therefore, of how critical protein modifications are to the colonization of warm and cold deep-sea habitats. The large number of invertebrate phyla present in the deep sea affords an excellent opportunity for studying convergent evolutionary processes related to pressure adaptation. Invertebrates provide especially good material for examining the interactions of pressure and temperature on protein function and evolution. Some species of hydrothermal vent invertebrates are likely to be exposed to elevated temperatures for extended periods (Fustec *et al.* 1987; Hekinian *et al.* 1983), and

different species are frequently restricted to microhabitats within the hydrothermal vent field that differ in temperature (Hessler and Smithey, 1984; Hessler *et al.* 1985).

We selected MDH for these studies of marine invertebrates because MDH is known to be pressure-sensitive and to exhibit adaptations to pressure in fishes and, unlike LDH, MDH is generally present at high activities in most tissues of invertebrates. In invertebrates only two gene loci typically encode MDH. One locus codes for a mitochondrial isoform (mDH) and a second for a cytosolic form (cMDH) (Basaglia, 1989). The mitochondrial isoform is primarily a Krebs citric acid cycle enzyme, and is present at high activities in aerobic tissues, but only at low activities in tissues with a high capacity for anaerobic glycolysis, e.g. the adductor muscle of bivalves and foot muscle of gastropods. Conversely, cMDH, the isoform examined in this study, is critical for maintaining redox balance in the cytosol as well as for shuttling reducing equivalents between the cytosol and the mitochondria. cMDH is present at high concentrations in most invertebrate muscles (Lazou *et al.* 1987).

The effects of temperature and pressure on the apparent Michaelis–Menten constant of cofactor, NADH, were determined for homologous cMDHs from mobile and sessile invertebrates living in environments of different temperature and pressure combinations: warm–deep, cold–deep, warm–shallow and cold–shallow (Table 1). One group of species, mussels, is represented at all four habitat types. The vesicomyid clams (*Calyptogena* spp.) are found over a wide range of depths (500–3300 m), but only in cold water (<7 °C). The worms (a vestimentiferan and three polychaetes) and brachyuran crab studied are found mostly at warmer temperatures (>15 °C) over a wide depth range (10–2600 m). This phyletically and environmentally diverse group of invertebrates enabled us to address the following questions. (i) Do cMDHs of marine invertebrates exhibit pressure sensitivities and adaptive differences similar to those described for dehydrogenase enzymes of marine fishes? (ii) Are adaptations to different temperatures evident in comparisons of cMDHs from cold-adapted deep-sea species and hydrothermal vent species? (iii) Do adaptations to different temperatures distinguish enzymes of invertebrates found in different microhabitats within the hydrothermal vent environment? (iv) Can the kinetic properties of enzymes be used to predict maximal sustained body temperatures of species for which such data are difficult to obtain, as is currently the case for some hydrothermal vent species because of the difficulty in determining the length of time these species are exposed to maximal habitat temperatures?

Materials and methods

Specimen collection

Species studied and the properties of their habitats are listed in Table 1. Vent and seep animals were collected during expeditions to eastern tropical Pacific hydrothermal vents, to vents at the Marianas Trough in the western Pacific, and to

cold hydrocarbon and sulphide seeps in the Monterey Canyon, Florida escarpment and Gulf of Mexico. Animals were collected by submersible at depths between 500 and 3300 m, and were transported from the collection site to the surface in an insulated chest. Whole specimens were frozen immediately, either in liquid N_2 or at −80°C. *Calyptogena elongata* were collected by otter trawl from the Santa Barbara Basin using the RV *R.G. Sproul* (July 1990) and were maintained in mud from the site in a dark aquarium at 5°C until killed. *Cancer antennarius* were collected by baited trap near San Diego, California, or purchased live from local seafood suppliers. *Mytilus galloprovincialis* were collected by skin diving from the Scripps Institution of Oceanography pier. *Chaetopterous variopedatus* were collected by SCUBA diving near San Diego. The coastal crabs, mussels and worms were maintained in ambient temperature (approximately 15°C) seawater aquaria until killed. The oyster *Isognomen incisum* was collected intertidally near Honolulu, Hawaii.

Enzyme isolation

Malate dehydrogenase was isolated from different muscle tissues of the various species: posterior adductor muscle of bivalves, claw muscle of crabs, ventral body wall of alvinellid polychaetes, vestimentum of *Riftia pachyptila* and cephalothorax of *C. variopedatus*. Single individuals were used whenever possible, but tissue from several individuals was pooled for some species (*M. galloprovincialis*, *I. incisum*, *Bathymodiolus thermophilus*, *C. variopedatus*, *C. elongata*, *Bythograea thermydron* and *Alvinella* spp.) because adequate amounts of enzyme could not be extracted from a single individual. Muscle was dissected from freshly killed animals or partially thawed frozen material, and homogenized in 8 vols of ice-cold 50 mmol l^{-1} potassium phosphate buffer (pH 6.8 at 5°C), using a Polytron mechanical homogenizer. Samples were held on ice throughout the homogenization. Tissue from *C. variopedatus* was homogenized in 15 vols of buffer because of the high mucous content of muscle of this species.

After centrifugation (23 500 ***g***, 20 min, 4°C) the supernatant was poured through glass wool to remove lipids and solid ammonium sulphate was added to a final saturation of 50 % (0.313 g ml^{-1}). The suspension was allowed to stand for 30 min and was then centrifuged (24 000 ***g***, 20 min). The supernatant, containing essentially all of the MDH activity, was brought to 75 % ammonium sulphate saturation (0.176 g ml^{-1}). The pH was maintained between 7.0 and 7.4 by addition of 0.1 mol l^{-1} NH_4OH. The suspension was allowed to stand at least 2 h, and was often stored (4°C) at this stage. MDH stored in 75 % ammonium sulphate was stable for at least 4–6 months, as has been found in other work on dehydrogenases (Lazou *et al.* 1987).

For kinetic studies, the precipitate containing the MDH was collected by centrifugation (25 000 ***g***, 20 min) and dialysed overnight at 4°C to remove all ammonium sulphate (dialysis buffer: 20 mmol l^{-1} Tris–HCl, pH 8.2 at 5°C; 0.01 mmol l^{-1} 2-mercaptoethanol, 0.01 mmol l^{-1} EDTA, 0.10 μmol l^{-1} sodium azide and 0.10 μmol l^{-1} phenylmethylsulphonyl chloride, a protease inhibitor).

For some species, lactate dehydrogenase (LDH) was removed from the MDH preparation. LDH can interfere with measurement of MDH activity if present at high concentrations. For most species of invertebrates used in this study, LDH activity was extremely low and did not interfere with MDH analysis (Hand and Somero, 1982; E. Dahlhoff and G. N. Somero, unpublished observations). However, mobile crustaceans like crabs have high LDH activity in locomotory muscles, and it was necessary to remove LDH from the MDH preparations of *B. thermydron* and *C. antennarius*. The dialysed MDH preparation was applied to an oxamate–aminohexyl–Sepharose 4B column, following the procedures of Yancey and Somero (1978). Over 90% of the LDH bound to the column, and MDH was recovered in the void volume. The fractions containing MDH were pooled and precipitated with ammonium sulphate (75% saturation).

The isozyme composition of the MDH preparations was analyzed using native acrylamide gels and an MDH activity stain (Wheat *et al.* 1972). For all species studied, essentially all (estimated as greater than 90%) of the MDH activity was due to the cytosolic isoform. We did not further separate the cytosolic and mitochondrial isoforms.

Enzyme kinetic studies

Apparent Michaelis–Menten constants of the cofactor NADH were determined kinetically by measuring the relationship between initial reaction velocity and NADH concentration. The assay mixture contained 80 mmol l^{-1} imidazole chloride buffer (pH 7.2 at 15°C), 100 mmol l^{-1} KCl, 0.20 mmol l^{-1} oxaloacetate and various concentrations of NADH. This concentration of oxaloacetate gave optimal activity and was approximately an order of magnitude greater than K_m (oxaloacetate) for the species so examined (data not shown). An imidazole buffer was used because the pK of imidazole varies with temperature in parallel with the pH of body fluids (Reeves, 1977) and is essentially pressure-independent (Marquis and Fenn, 1969). K_m values were determined at 0.1, 6.9, 20.6, 34.3 and 48.1 MPa. MDH activity was adjusted by dilution into 80 mmol l^{-1} imidazole buffer (pH 7.2 at 15°C) at the beginning of each K_m determination to yield an initial activity (using 25 μl of enzyme in an assay containing 150 μmol l^{-1} NADH) of 0.050±0.005 absorbance units min^{-1}. Decrease in absorbance at 340 nm was followed, using either a 3 ml cuvette (0.1 MPa assays) or a high-pressure optical cell (Mustafa *et al.* 1971) mounted in a Perkin-Elmer lambda 3B spectrophotometer. Measurement temperatures were 5, 15 and 25°C, the latter being the highest temperature at which high-pressure assays could be conducted reliably with our instruments. Assay temperature was regulated to within ±0.1°C. Duplicate (or more) measurements of initial rate were made at each of seven NADH concentrations, and the average rate at each [NADH] was used in statistical analysis. K_m values were determined from Lineweaver–Burk plots using Wilman software (Brooks and Suelter, 1986) based on the weighted linear regression analysis of Wilkinson (1961). Confidence limits were based on errors of triplicate (*Mytilus galloprovincialis*, *Bathymodiolus thermophilus*, *Calyptogena magnifica* and *Calyptogena*

elongata) or duplicate (all other species) K_m determinations made using all averaged rates at the seven concentrations of NADH.

Results

Conservation of K_m *at physiological pressures and temperatures*

The effects of increasing pressure (at 5 °C) on the K_m of NADH of cMDHs from deep- and shallow-living marine invertebrates (Fig. 1) indicate that cMDHs from species occurring at depths greater than 500–1000 m are much less sensitive to pressure than cMDHs from species found at lesser depths. For cMDHs from mussels, an increase in measurement pressure to 6.9 MPa increased the K_m of

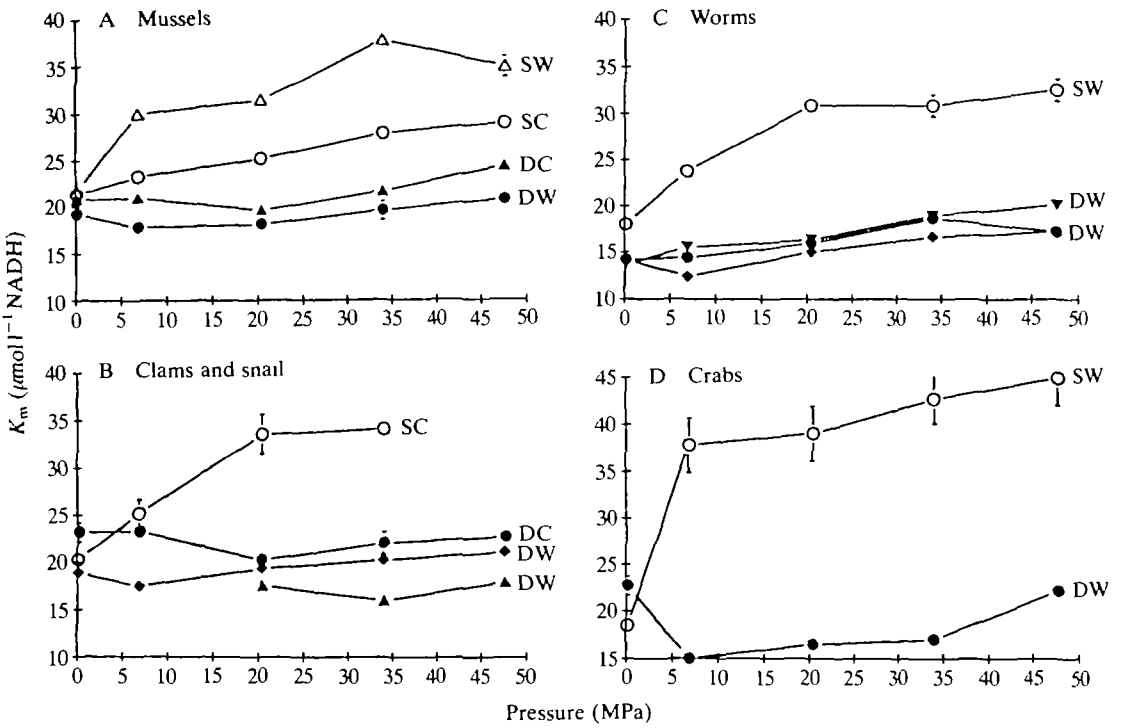

Fig. 1. The effect of pressure on the K_m of NADH of cytosolic malate dehydrogenases (cMDHs) from several shallow- and deep-living marine invertebrates (see Table 1 for habitat data). (A) Mussels: *Bathymodiolus thermophilus* (●), Florida escarpment mussel (▲), *Mytilus galloprovincialis* (△), Louisiana petroleum seep mussel (○). (B) Clams and snail: *Calyptogena magnifica* (◆), *Calyptogena phaseoliformes* (●), *Calyptogena elongata* (○), *Alvinoconcha hessleri* (▲). (C) Worms: *Riftia pachyptila* (▼), *Alvinella caudata* (●), *Alvinella pompejana* (◆), *Chaetopterus variopedatus* (○). (D) Crabs: *Bythograea thermydron* (●), *Cancer antennarius* (○). Triplicate (*M. galloprovincialis* and *B. thermophilus*) or duplicate (all other species) K_m determinations were made at 5 °C. 95 % confidence intervals are given (when no error bars are evident it is because the 95 % confidence intervals are hidden by the symbols). SW, shallow warm; DW, deep warm; SC, shallow cold; DC, deep cold conditions.

NADH for the cMDH of the coastal mussel *Mytilus galloprovincialis* to approximately 150% of the value at 0.1 MPa, and by 34.3 MPa, the K_m was almost doubled (Fig. 1A). The cMDH of the unnamed mussel from the Louisiana seep environment was perturbed by non-physiological pressures, but not as strongly as the enzyme of *M. galloprovincialis*. For cMDHs of the two deep-sea mussels, *Bathymodiolus thermophilus* from the hydrothermal vents and the unnamed mussel from the Florida escarpment site, K_m of NADH was not significantly affected by pressures within the physiological range (26.3 and 33.3 MPa, respectively).

Similar differences were found in comparisons of shallow- and deep-living representatives of other invertebrate groups. For vesicomyid clams, the K_m of NADH for the cMDH of the shallow-living clam *Calyptogena elongata* increased by approximately 75% when the measurement pressure was increased from 0.1 to 20.6 and 34.3 MPa (Fig. 1B). The cMDHs of the deep-sea clams *Calyptogena magnifica* and *Calyptogena phaseoliformes* and the deep-sea gastropod mollusc *Alvinoconcha hessleri* exhibited no significant increase between 0.1 and 48.1 MPa (Fig. 1B). For polychaete (*Alvinella pompejana* and *Alvinella caudata*) and vestimentiferan (*Riftia pachyptila*) worms, the cMDHs of deep-sea species were consistently less sensitive to pressure at 5°C than the cMDH of the shallow-living species, *Chaetopterous variopedatus* (Fig. 1C). Similarly, the cMDH of the hydrothermal vent crab *Bythograea thermydron* did not exhibit the sharp rise in K_m of NADH at high pressure noted for cMDH of the shallow-living crab *Cancer antennarius* (Fig. 1D). Thus, shallow- and deep-living representatives of eight families within four invertebrate phyla show similar adaptive differences to the effects of pressure on cMDH function.

To determine whether differences related to adaptation temperature existed among the cMDHs of deep-sea invertebrates, values of K_m of NADH were determined at temperatures of 5, 15 and 25°C at pressures of 0.1, 6.9, 20.6, 34.3 and 48.1 MPa for animals from cold and warm deep-sea habitats (Table 1). Fig. 2 presents the temperature *versus* K_m relationships for several species at their physiological pressures. All cMDHs exhibited an increase in K_m with rising temperature. However, large differences in the responses of K_m to temperature were found between species living in cold and warm environments. cMDHs from species living at low, stable temperatures, e.g. *C. phaseoliformes* and the Florida escarpment mussel, were strongly perturbed by increased temperatures under physiological pressures. For hydrothermal vent species the effect of temperature on K_m of NADH was relatively slight except in the case of cMDH of *R. pachyptila* at 25°C, a temperature that probably exceeds the worm's upper maximal sustained body temperature (see below).

When values of the K_m of NADH at normal physiological combinations of temperature and pressure were estimated for all species for which these environmental data were available (Table 1), the K_m was found to be conserved between approximately 15 and 25 μmol l^{-1} NADH (Figs 1 and 2), a narrow range relative to the values observed at non-physiological pressures and temperatures.

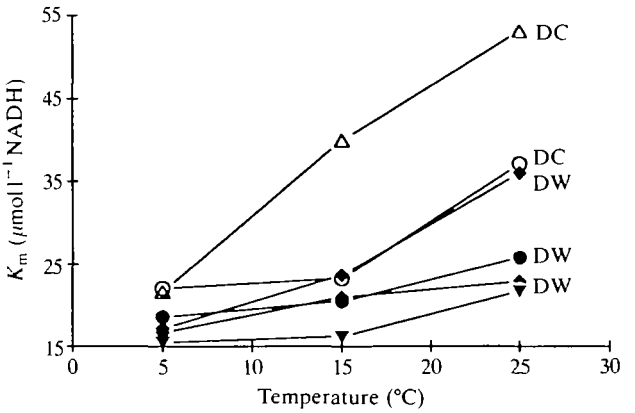

Fig. 2. The effect of temperature on the K_m of NADH of cMDHs of deep-sea invertebrates from cold habitats [*Calyptogena phaseoliformes* (○) and Florida escarpment mussel (△)] (DC) and the hydrothermal vents [*Riftia pachyptila* (◆), *Bathymodiolus thermophilus* (●), *Alvinella pompejana* (▼) and *Bythograea thermydron* (▲)] (DW). N=2 for all species except *B. thermophilus* (N=3). Data for *Calyptogena magnifica*, *Alvinella caudata* and *Alvinoconcha hessleri* cluster within the data for *B. thermophilus* and *B. thermydron*. K_m values are those at the approximate *in situ* pressures of the species (values determined by interpolation, using K_m data at 20.6 and 34.3 MPa).

Estimates of maximal sustained body temperatures of vent invertebrates

For the mobile vent species *B. thermydron*, *A. pompejana* and *A. caudata*, maximal body temperatures are difficult to estimate because the animals are apt to move through extremely large thermal gradients such as those found near the walls of smoker chimneys. Sessile species like *R. pachyptila* may also experience large fluctuations in water temperature as bursts (approximately 1 s) of warm water exit from the vents. Because times of exposure to heated vent effluents may be too short to permit thermal equilibration of the animals, it is difficult to predict, using available water temperature data, the highest sustained body temperatures of these species. To address this issue we sought to estimate maximal sustained body temperatures through the use of an enzymatic index based on the finding that the value of the K_m of NADH at any single measurement temperature was inversely related to the species' upper habitat temperatures (Figs 1 and 2). The relationship between K_m of NADH (at 5°C and *in situ* pressure) and the approximate upper temperature to which the species would be exposed for long periods (which we assume reflects the maximal sustained body temperature) for 11 invertebrates with known temperature ranges is shown by the open symbols and regression line in Fig. 3. When the K_m values for the three mobile vent species (*B. thermydron*, *A. caudata* and *A. pompejana*) and for *R. pachyptila* are placed on the regression line determined for the 11 other species, their predicted maximal sustained body temperatures are approximately 25, 25, 31 and 23°C, respectively.

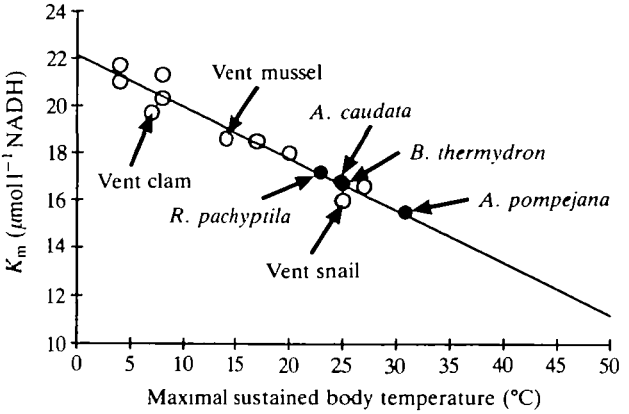

Fig. 3. The relationship between maximal sustained body temperature and K_m of NADH at 5°C and *in situ* pressure. Open symbols represent data for 11 species (two points are superimposed) from well-characterized thermal habitats. The regression line ($y=-0.219x+0.213$; $r^2=0.95$) was computed using these data. The filled symbols are for the mobile vent species, *Bythograea thermydron*, *Alvinella caudata* and *Alvinella pompejana*, and the tube worm *Riftia pachyptila*, for which habitat temperature ranges are uncertain. Maximal sustained body temperatures for these four species were estimated by placing the K_m data (5°C, *in situ* pressure) on the regression line, and determining the corresponding temperature value on the abscissa.

Discussion

At physiological pressures and temperatures a strong degree of conservation of K_m of NADH was found for the cMDHs from the 15 species of marine invertebrates. The K_m of NADH was conserved between approximately 15 and 25 μmol l^{-1} under physiological temperature plus pressure combinations, despite large increases in K_m of NADH at non-physiologically high temperatures or pressures (Figs 1 and 2). This conservation pattern resembles that found in studies of malate, lactate and glyceraldehyde-3-phosphate dehydrogenases of marine fishes (Siebenaller, 1984, 1987).

The significantly different responses to pressure of cMDHs from shallow-living and deep-sea invertebrates belonging to four phyla suggest that adaptation of enzymes to pressure, specifically the acquisition of pressure-resistant K_m values, is a pervasive feature of colonization of the deep sea. The pressures at which perturbation of enzyme function becomes large enough to favour selection for pressure-resistant enzymes appear to be around 5–10 MPa (Table 1). Only species occurring below depths of 500–1000 m had pressure-resistant enzymes. This is the same pressure perturbation threshold discovered in studies of dehydrogenases from shallow- and deep-living teleost fishes (Siebenaller, 1987). Therefore, for a given class of enzyme, similar pressure perturbation thresholds may exist across all animal phyla.

Despite similarities in pressure perturbation threshold among cMDHs from teleost fishes and invertebrates, the degree of perturbation of K_m by elevated

pressure differs substantially between fishes and invertebrates and, to a lesser extent, among the different phyla of invertebrates. For example, the cMDH (=MDH-2) of a shallow-living fish, *Sebastolobus alascanus*, increased from approximately 20 μmol l^{-1} to approximately 90 μmol l^{-1} as pressure increased from 0.1 to 34.3 MPa (Siebenaller, 1984). Among the marine invertebrates studied, the K_m of NADH at 34.8 MPa for shallow-living species was generally no greater than 40 μmol l^{-1}, except in the case of the crab *C. antennarius*. The molecular basis of the lower pressure sensitivities of cMDHs of some invertebrates relative to the cMDHs of fishes is not known.

Although the cMDHs from all deep-sea invertebrates were resistant to pressure at 5°C, increases in measurement temperature strongly perturbed the cMDHs of species from cold deep-sea environments like the Florida escarpment site (unnamed mytilid) and the Monterey Canyon (*C. phaseoliformes*). Cytosolic MDHs from hydrothermal vent species were less temperature sensitive, except in the case of cMDH of *R. pachyptila* at 25°C (Fig. 2). These interspecific differences in sensitivity to elevated temperature under high pressure suggest that enzymes of species adapted to the cold and stable temperatures found in most of the deep sea are not pre-adapted for function in the warmer and thermally variable waters found in certain regions of the hydrothermal vent environments. A similar conclusion was reached in studies of LDHs of deep-sea fishes found either in cold deep-sea habitats or at the hydrothermal vents (Dahlhoff *et al.* 1990).

Previous studies of temperature effects on K_m of substrate and cofactor of dehydrogenases have found that, at a common temperature of measurement, the K_m is inversely related to the species' adaptation temperatures (Baldwin, 1971; Yancey and Somero, 1978; Graves and Somero, 1982; Yancey and Siebenaller, 1987; Coppes and Somero, 1990). This inverse relationship is a reflection of the underlying conservation of temperature-dependent K_m values at physiological temperatures. The K_m of NADH for the invertebrate cMDHs at 5°C and physiological pressures also differed regularly among species according to their estimated maximal sustained body temperatures (Fig. 3; Table 1).

We used the relationship shown in Fig. 3 to derive estimates of the maximal sustained body temperatures of vent species that encounter large and rapid fluctuations in water temperatures. Uncertainty about upper thermal limits exists for mobile invertebrates like *B. thermydron*, *A. caudata* and *A. pompejana*, as well as for the sessile tube worm *R. pachyptila*. The crab is found in warm venting areas populated with vestimentiferans and mussels, as well as on the walls of smoker chimneys that emit waters with temperatures up to approximately 380°C. The two polychaetes occur only on the walls of smoker chimneys. Only a few centimetres away from the smoker chimneys, water temperatures are only about 2°C. Although habitat temperature ranges for sessile species are becoming well characterized at particular vent sites (see Fisher *et al.* 1988, for *B. thermophilus*; Hessler *et al.* 1985, for *C. magnifica*; Table 1), maximal exposure temperatures for mobile species are inherently less readily determined and are much less meaningful physiologically. This is mainly because the time of exposure of rapidly

moving mobile species to elevated temperatures is not well characterized and short-term exposure to warm temperatures may not be accompanied by thermal equilibration of the body. For sessile species like *R. pachyptila*, bursts of warm water may last for less than 1 s (Johnson *et al.* 1988), and typical body temperatures may remain at or only slightly above the approximately 2°C ambient temperature. Although Johnson *et al.* (1988) and Hessler and Smithey (1984) report *R. pachyptila* occurring at sites with temperatures no greater than approximately 14°C, unpublished data (K. Johnson, personal communication) suggest that these worms may be exposed to water with temperatures up to approximately 28°C. However, the extremely rapid fluctuations of temperature in the warm-water vents (Johnson *et al.* 1988) may preclude thermal equilibration between the worms and the medium.

Even though the maximal sustained body temperatures for vent species are probably much lower than the highest temperatures ever encountered, available data suggest that the brachyuran crab and polychaete worms are adapted to higher temperatures than other vent species. Mickel and Childress (1982*a*,*b*) reported that the critical thermal maximum for *B. thermydron* was approximately 38°C, although the likely maximal temperature for sustained survival was closer to 25°C. Tunnicliffe and Juniper (1990) report that alvinellid polychaetes colonize newly formed hot vents and are responsible for some solidification of the sulphide chimneys. Under such conditions, these worms are almost certain to see temperatures well above the approximately 2°C of the surrounding water. Desbruyères *et al.* (1982) conjectured that alvinellid polychaetes may encounter temperatures as high as 50°C on smoker chimneys. Using the oxygen-binding characteristics of haemoglobins as an index of body temperature, Toulmond *et al.* (1990) predicted body temperatures of 10–20°C for *A. caudata* and 20–30°C for *A. pompejana*. Dahlhoff *et al.* (1991) studied the effects of temperature on mitochondrial respiration of different hydrothermal vent species and found that the mitochondria of the alvinellid polychaetes and *B. thermydron* withstood significantly higher temperatures than the mitochondria of *C. magnifica* and *B. thermophilus*.

The relationship shown in Fig. 3 yields estimates of maximal sustained body temperatures for the mobile vent species that are generally in accord with available ecological and physiological data. When the K_m values at 5°C and physiological pressure for cMDHs of *B. thermydron*, *A. caudata* and *A. pompejana* are placed on the regression line determined using data from the 11 other species, their predicted maximal sustained body temperatures are approximately 25°, 25° and 31°C, respectively (Fig. 3). For *R. pachyptila*, the estimated maximal sustained body temperature is approximately 23°C.

Although these are only approximations of the highest temperatures that these species can withstand indefinitely, the values do support conclusions reached in ecological and physiological studies that certain vent species may be able to sustain body temperatures up to 20–25°C higher than species found in typical cold deep-sea habitats. Despite the general agreement between predicted maximal sustained

body temperatures obtained using the analysis of Fig. 3 and ecological data, there would appear to be a discrepancy in the case of the mussel *B. thermophilus* and the tube worm *R. pachyptila*. These two species commonly co-occur as adults (Hessler and Smithey, 1984; Hessler *et al.* 1985), yet their predicted maximal sustained body temperatures are different by several degrees (Fig. 3). This discrepancy might be a reflection of different temperatures encountered during larval and adult stages by the species. Mussels are thought to colonize a vent site after tube worms are established and after the flow of vent water is reduced. As the mussel clumps grow, vent flow is further reduced (Hessler *et al.* 1988*b*). It is possible, then, that the apparently higher thermal tolerance of *R. pachyptila* relative to *B. thermophilus* (Fig. 3; Dahlhoff *et al.* 1991) reflects selection operating at the non-adult life history stages of the tube worm.

In conclusion, the differences in pressure sensitivity found between the cMDHs of invertebrates from shallow-water and deep-sea habitats support the hypothesis that adaptations of enzymes to pressure are important in establishing depth distribution patterns of invertebrates in the marine water column. Interspecific differences in sensitivity to temperature indicate that adaptations to elevated and variable temperatures are of importance in colonization of the hydrothermal vents. Moreover, within the vent environment, species appear to differ in thermal adaptation according to the microhabitats in which they occur. Because different thermal microhabitats are separated by distances of less than 1 m in many cases, hydrothermal vent species provide an exceptionally clear illustration of the importance of fine-scale thermal adaptations in supporting species distribution patterns within a single ecosystem.

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