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KINETIC AND STRUCTURAL ADAPTATIONS OF CYTOPLASMIC MALATE DEHYDROGENASES OF EASTERN PACIFIC ABALONE (GENUS *HALIOTIS*) FROM DIFFERENT THERMAL HABITATS: BIOCHEMICAL CORRELATES OF BIOGEOGRAPHICAL PATTERNING

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Summary

We measured the effects of temperature on cytosolic malate dehydrogenases (cMDHs) from the shell muscle of five species of eastern Pacific abalone, genus *Haliotis*, found at different latitudes and/or tidal heights. The apparent Michaelis–Menten constant (K_m) of coenzyme (nicotinamide adenine dinucleotide, NADH) was conserved within a narrow range (11–21 $\mu mol\,l^{-1}$) at physiological temperatures for all species. However, elevated temperatures perturbed the K_m of NADH for cMDHs of the two species living at higher latitudes and/or lower tidal heights [*Haliotis rufesens* (red) and *H. kamtschatkana kamtschatkana* (pinto)] to a much greater extent than for cMDHs of congeners from lower latitudes and/or higher tidal heights [*H. fulgens* (green), *H. corregata* (pink) and *H. cracherodii* (black)]. The apparent Arrhenius activation energies for the cMDHs of these five species showed a similar interspecific divergence. Furthermore, green, pink and black abalone have cMDHs that are more resistant to thermal denaturation than are cMDHs of red and pinto abalone. Native gel electrophoresis showed that cMDHs of red and pinto abalone had identical mobilities, whereas cMDHs of green, pink and black abalone are distinct both from each other and from that of the two cold-adapted species. These data suggest that cMDHs from the abalone species living in warm habitats are adapted to function optimally at higher temperatures than are the cMDHs of the two species living in cooler habitats. The relationships suggested by these criteria are in agreement with other studies that used morphological and molecular indices to predict abalone phylogeny. These results therefore provide further evidence that interspecific variation in protein structure and function may be driven by natural selection based on only small (i.e. several degrees Celsius) differences in average body temperature, and that such selection is an important element of the mechanisms of species formation and the maintenance of biogeographic patterning.

Introduction

Habitat temperature is probably the most important physical factor that affects the distribution and abundance of marine organisms. Temperature affects all aspects of an

Key words: temperature, malate dehydrogenase, abalone, biogeography, *Haliotis* sp.

ectothermic organism's biology, from its ability to feed and reproduce to the structural and functional integrity of its biochemical machinery (Vernberg, 1962; Cossins and Bowler, 1987; Prosser and Heath, 1991). It is therefore not surprising that there are numerous examples of species replacements over latitudinal and other thermal gradients in the marine environment (Hubbs, 1948; Vernberg, 1962). Studies of fish living at different temperatures have demonstrated the strong correlation between habitat (adaptation) temperature and temperature effects on kinetic and structural properties of proteins (Place and Powers, 1984; Hochachka and Somero, 1984; Graves and Somero, 1982; Coppes and Somero, 1990). For enzymes from fish, relatively small differences in average habitat temperature were found to correlate with adaptive changes in proteins. For example, Graves and Somero (1982) showed that temperature differences of only 3–8˚C were sufficient to select for compensatory differences in kinetic properties (in the apparent Michaelis–Menten constant, K_m, and in the catalytic rate constant) of M_4-lactate dehydrogenases from four species of barracudas (genus *Sphyraena*) living at different temperatures.

Although these and other studies have expanded our understanding of the role of protein adaptations in the evolution of marine fish, there has been no parallel work of this nature with congeneric species of marine invertebrates. Previous studies of thermally adaptive differences in protein structure or function of invertebrates have focused either on allozymic variations within populations living along thermal gradients (Walsh, 1981*a*,*b*; Okumura *et al.* 1981) or on confamilial species that live in very different habitats (Walsh, 1981*b*; Dahlhoff and Somero, 1991). The examination of congeneric species of marine invertebrates living in relatively similar habitats is merited for several reasons. These studies will reveal whether small differences in average body temperature found to be adequate to favour evolutionary change in enzymes of fish apply to invertebrates as well. If proteins of congeneric species of marine invertebrates exhibit a similar sensitivity to temperature, then the biogeographic patterning of invertebrates may be influenced by temperature-adaptive differences in protein structure and function. The discovery of similar temperature-adaptive patterns in the homologous enzymes of fish and invertebrates also provides the opportunity for determining whether common types of amino acid substitutions characterize adaptive changes in phylogenetically distant species.

Marine archaeogastropod molluscs of the genus *Haliotis* (abalone) are an ideal group of organisms in which to examine questions of temperature-induced protein adaptations of marine invertebrates. The biogeographic distribution of abalone suggests that habitat temperature is an extremely important factor in determining a given species' range and abundance (Fig. 1). For example, the two most southern-occurring species, *Haliotis fulgens* (green) and *H. corregata* (pink), experience habitat temperatures (Fig. 1) that are much greater than those experienced by the most northern-occurring species,

Fig. 1. The distribution of five species of abalone along the west coast of North America. Species distribution data from Cox (1962) and Lindberg (1991). Habitat temperature data from Ricketts *et al.* (1985), Leighton (1974; green, pink and red abalone), Hines *et al.* (1980; black abalone) and Paul and Paul (1980; pinto abalone).

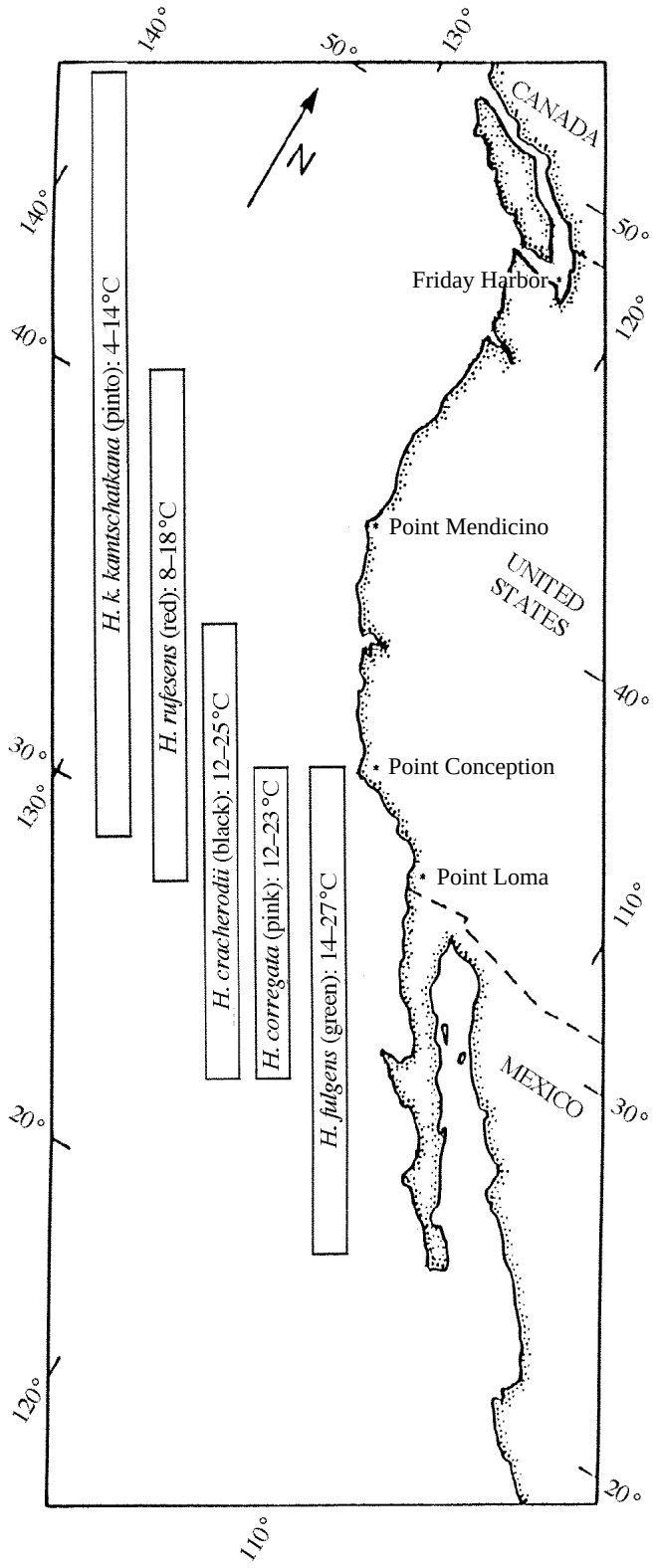
140°
50°
130°
CANADA
N
140°
50°
Friday Harbor
40°
120°
H. k. kamtschatkana (pinto): 4–14 °C
H. rufescens (red): 8–18 °C
H. cracherodii (black): 12–25 °C
H. corregata (pink): 12–23 °C
H. fulgens (green): 14–27 °C
Point Mendicino
UNITED STATES
30°
130°
40°
Point Conception
Point Loma
110°
MEXICO
20°
30°
120°
20°
110°

Fig.1

H. k. kamtschatkana (pinto). Abalone distribution varies with tidal height as well. For example, green and black (*H. cracherodii*) abalone are found in the mid to low intertidal region in southern California, whereas the pink and red (*H. rufesens*) abalone are found subtidally. These different latitudinal and depth distributions suggest that green, pink and black abalone experience much higher habitat temperatures than do red or pinto abalone.

Studies of growth and survival of abalone at elevated temperatures indicate that green, pink and black abalone are less sensitive to high temperatures than are red or pinto abalone. For example, the optimal growth temperature for juvenile red abalone is 18˚C, whereas it is 22˚C for pink abalone and 24˚C for green abalone (Leighton, 1974). Pinto abalone die if they are held at 16˚C for longer than a day, whereas green and black abalone can be held at 25˚C indefinitely (Paul and Paul, 1980; Hines *et al.* 1980; Leighton, 1974). Differential responses to elevated temperatures have also been observed in the field. For example, red abalone in a southern Californian kelp bed suffered severely reduced recruitment during warming caused by the 1982–1983 El Niño Southern Oscillation, whereas pink abalone showed enhanced recruitment during this same period (Tegner and Dayton, 1987). Although this differential recruitment may have been caused by shifting current patterns, as the authors suggested, elevated water temperatures could have been a factor as well. These studies provide further evidence that green, pink and black abalone are adapted to life at higher temperatures than are red and pinto abalone. However, none of the biochemical mechanisms underlying these differences has been investigated.

To elucidate these mechanisms, we examined the effects of temperature on the structure and function of cytosolic malate dehydrogenases (cMDHs) purified from the shell muscle of these five species of abalone. Kinetic function was indexed by the effects of temperature on the Michaelis–Menten constant, K_m, and interspecific differences in the apparent Arrhenius activation energy (E_a). Both of these kinetic variables have been shown to reflect adaptation temperature in previous studies of proteins from marine and terrestrial vertebrates (Hochachka and Somero, 1984; Cossins and Bowler, 1987).

Structural differences between homologous cMDHs from the five species of abalone were determined by measuring both thermal stability and mobility on non-denaturing polyacrylamide gels. Thermal stability was examined because it is well established that proteins from species with high body temperatures are more resistant to thermal denaturation than homologous proteins from species with low body temperatures (Johnston and Walesby, 1977; Swezey and Somero, 1982; Low and Somero, 1976). Although the thermal denaturation of proteins usually occurs well above the upper lethal temperature of the organism, the strong correlation between adaptation temperature and protein thermal stability suggests that thermal stability, like kinetic function, is an excellent indicator of adaptation temperature. To date, thermal stability differences for proteins from marine invertebrates have not been examined.

Materials and methods

Animal collections

Abalone were obtained using snorkel or SCUBA except for *Haliotis fulgens*

specimens, which were obtained from Dr David Leighton at San Diego, CA, USA. *H. corregata* were collected at Point Loma, CA. *H. cracherodii* were collected at Diablo Canyon, CA. *H. rufesens* were collected from both Point Loma and Point Mendicino, CA, and *H. k. kamtschatkana* were collected near Friday Harbor, WA. Individuals were maintained in controlled-temperature aquaria at their capture or culture temperature until being killed (*H. fulgens*, 20˚C; *H. corregata*, 20˚C; *H. cracherodii*, 18˚C; *H. rufesens*, 12˚C; *H. k. kamtschatkana*, 9˚C).

Enzyme isolation and purification

Cytosolic malate dehydrogenase (cMDH; EC 1.1.1.37; malate: NAD^+ oxidoreductase) was isolated from the adductor muscle (also called shell muscle) of each species of abalone according to the method of Dahlhoff and Somero (1991). Muscle was dissected from frozen samples and homogenized in 8 vols of ice-cold 50mmol l^{-1} potassium phosphate buffer (pH6.8 at 5˚C) using a Waring blender. After centrifugation (23500 ***g*** for 20min at 4˚C), the supernatant was precipitated by the addition of solid ammonium sulphate to give a final ammonium sulphate concentration of 45% saturation (0.258 g l^{-1}). The suspension was allowed to stand for 30min and was then centrifuged (24000 ***g*** for 20min). The supernatant, containing essentially all of the MDH activity, was brought to 80% ammonium sulphate saturation (0.226 g l^{-1}). The precipitate, containing 95% of the MDH activity, was collected by centrifugation (25000 ***g*** for 20min) and then dialysed overnight against TEB at 4˚C to remove ammonium sulphate (TEB: 20mmol l^{-1} TrisCl, pH8.2 at 5˚C, 0.01mmol l^{-1} 2-mercaptoethanol and 0.01mmol l^{-1} EDTA). The desalted MDH preparation in TEB was applied to a Matrix red A affinity column (Amicon Co.). The column was washed sequentially in 20mmol l^{-1} KCl, 20mmol l^{-1} malate and 0.2mmol l^{-1} β-nicotinamide adenine dinucleotide (oxidized; NAD^+). All wash buffers were in TEB, and the column was washed exhaustively with TEB between each step. The cMDH was eluted from the column using a continuous gradient (buffer A: TEB only; buffer B: 40mmol l^{-1} malate and 0.4mmol l^{-1} NAD^+ in TEB). In all species, at least 80% of the cMDH loaded onto the column was eluted at approximately 20mmol l^{-1} malate and 0.2mmol l^{-1} NAD^+. The exact concentration of NAD/malate necessary to elute the MDH from the column varied among species. Elution was monitored by measuring cMDH activity as described below [assay buffer: 80mmol l^{-1} imidazole, pH7.2 at 15˚C, 100mmol l^{-1} KCl, 0.15mmol l^{-1} nicotinamide adenine dinucleotide (reduced; NADH) and 0.2mmol l^{-1} oxaloacetic acid]. Active fractions were pooled and then filtered and concentrated using a Centricon-30 microconcentrator (Amicon).

Gel electrophoresis

The purity of the cMDH from each species was confirmed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) gels stained with Coomassie Blue. The cMDHs were approximately 92–95% pure, based on the detection limits of the Coomassie stain. Non-denaturing gel electrophoresis was performed according to the methods of Wheat *et al.* (1972) as modified by Dahlhoff and Somero (1991).

Enzyme kinetics

Apparent Michaelis–Menten constants (K_m) of the cofactor NADH of cytosolic malate dehydrogenase were determined kinetically according to the method of Dahlhoff and Somero (1991). K_m values were determined at 5, 15, 25, 35 and 45°C. The assay medium contained 80mmol l^{-1} imidazole chloride buffer (pH7.2 at 15°C), 100mmol l^{-1} KCl, 0.20mmol l^{-1} oxaloacetate and NADH at concentrations ranging from 7.5 to 75 μmol l^{-1}. Imidazole was selected as the buffer system because previous work has shown that imidazole mimics changes of *in vivo* pH caused by body temperature variation (Yancey and Somero, 1978; Cossins and Bowler, 1987). Buffer pH was selected to mimic intracellular pH, as discussed in previous studies of cMDHs from fish and invertebrates (Yancey and Siebenaller, 1987; Dahlhoff and Somero, 1991). The reaction was initiated by the addition of 10 μl of purified cMDH in TEB (enzyme stocks: 100 μg ml^{-1} for 5°C, 50 μg ml^{-1} for 15°C, 25 μg ml^{-1} for 25°C, and 12.5 μg ml^{-1} for 35 and 45°C). The decrease in absorbance of NADH was followed at 340nm using a Perkin–Elmer lambda 3B spectrophotometer. Assay temperature was regulated to within ±0.2°C by a water-jacketed cell holder. Duplicate (or better) measurements were made at each of the six NADH concentrations used. K_m (and maximal velocity, V_{max}) values were determined from Lineweaver–Burk plots using the weighted linear regression analysis of Wilkinson (1961), as calculated using Wilman4 software (Brooks and Suelter, 1986).

Apparent activation energies were calculated as follows. The natural logarithm of V_{max} of the enzyme was plotted against $1/T$ (where T is the assay temperature in degrees Kelvin). The slope of this (Arrhenius) plot is $-E_a/\boldsymbol{R}$, where E_a ($=\Delta H^{\ddagger}+\boldsymbol{R}T$) is the apparent Arrhenius activation energy of the process, $\Delta H^{\ddagger}$ is the activation enthalpy, and $\boldsymbol{R}$ is the gas constant.

Thermal stability

The thermal stability of purified cMDHs from the five species of abalone was determined using a method modified from Place and Powers (1984). Purified cMDH of each species was brought to a final concentration of 25 μg ml^{-1} in a buffer containing 10mmol l^{-1} potassium phosphate (pH7.0 at 20°C), 150mmol l^{-1} KCl and 0.1% bovine serum albumin. Tubes containing 70 μl of this stock were incubated at 40°C for 0 (no heat), 5, 10, 20, 40 or 60min and placed on ice immediately after heat treatment. After cooling for at least 15min, samples were assayed at 20°C for residual activity (assay buffer: 80mmol l^{-1} imidazole, pH7.2 at 15°C, 100mmol l^{-1} KCl, 0.15mmol l^{-1} NADH and 0.2mmol l^{-1} oxaloacetic acid). MDHs did not lose activity when kept on ice for a day.

Protein concentrations

Protein concentrations were determined by the bicinchoninic acid (BCA) method (Pierce Co., Rockford, IL).

Statistical determinations

Statistical analyses were performed using Systat 5 for the Macintosh (Version 5.1; copyright 1991, Systat Inc.).

Results

Enzyme kinetics

The effects of increasing temperature on the K_m of NADH of cMDHs purified from the five species of abalone (Fig. 2) indicate that cMDHs from species that experience elevated habitat temperatures, due either to latitudinal or to tidal distribution, are much less sensitive to thermal perturbation than cMDHs from species found in cooler habitats. The K_m values of NADH are conserved within a fairly narrow range (11–21 μmol l^{-1} NADH) for cMDHs of all five species when measured at the approximate habitat temperatures of the species (Fig. 2). However, the K_m values of NADH for cMDHs from red and pinto abalone are significantly elevated above this range at even moderate (25 °C) assay temperatures, whereas the K_m values of cMDH homologues from the green, pink and black congeners show much less perturbation at higher assay temperatures.

Interspecific differences in the apparent Arrhenius activation energies of the five cMDHs also reflected a division between warm- and cold-adapted species. The E_a values of cMDHs from the three species living at warmer temperatures (green, 52.7±2.5 kJmol^{-1}; pink, 44.4±2.1 kJmol^{-1}; black, 47.3±2.4 kJmol^{-1}) are significantly higher than the E_a values of cMDHs from the two cool-adapted species (red, 40.2±2.0 kJmol^{-1}; pinto, 40.6±2.0 kJmol^{-1}: Wilcoxon signed-ranks test; P=0.021).

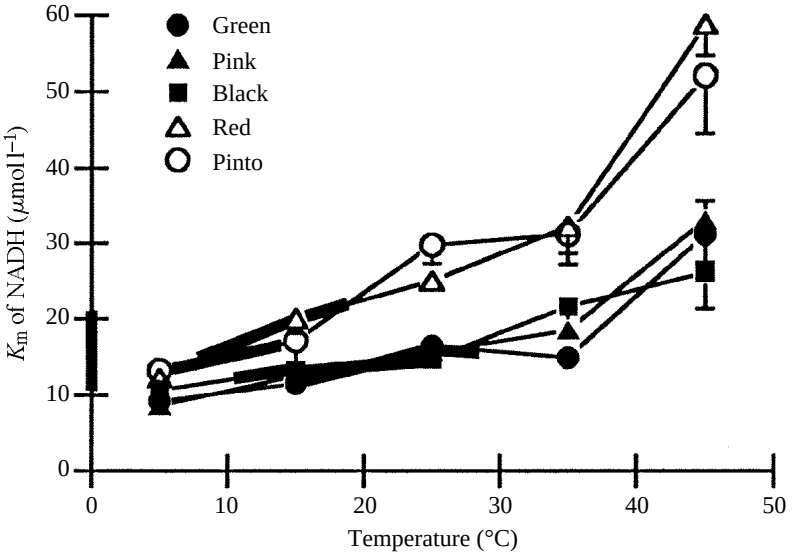

Fig. 2. The effect of temperature on the K_m of NADH of cMDHs purified from abalone living in warm [*Haliotis fulgens* (green), *H. corregata* (pink) and *H. cracherodii* (black)] and cool [(*H. rufesens* (red) and *H. k. kamtschatkana* (pinto)] habitats. N=2 for all species except for green and pinto abalone (N=3). Values are means ± 1 S.E.M. Thicker lines indicate the range of K_m values measured at each species' habitat temperature.

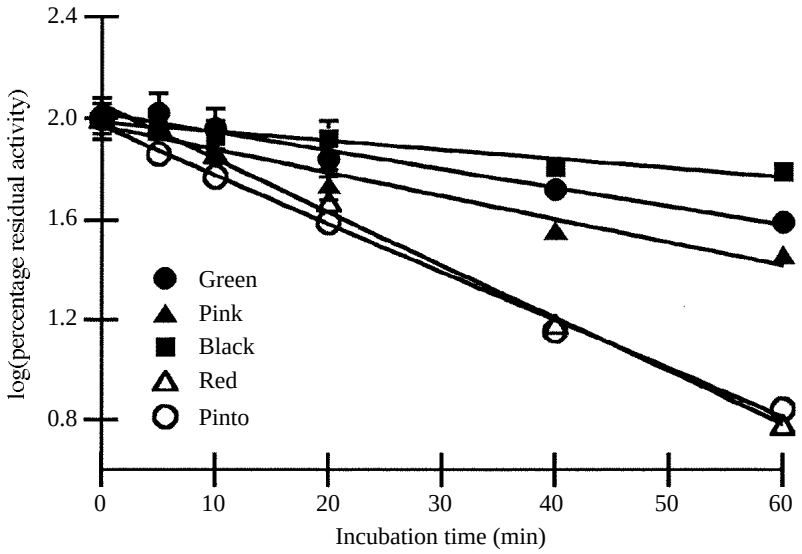

Fig. 3. The thermal stability of cMDH homologues for five species of eastern Pacific abalone. cMDHs from black, green and pink abalone were significantly more resistant to thermal denaturation at 40˚C than were cMDHs of red and pinto abalone, i.e. the slopes of the regression equations for warm- and cool-adapted species were significantly different (ANOVA, P=0.01). Values are means ± 1 S.E.M. Error bars are within limits of symbols when not visible. N=2 for all species.

Thermal stability and native gel electrophoresis

The thermal stabilities of the cMDHs also differed among species (Fig. 3). Using the slopes of thermal denaturation plots for statistical comparisons, the cMDH homologues from the black, pink and green abalone were found to be the most thermostable. When the thermal stabilities of cMDHs from the warm-adapted green, pink and black abalone are treated as a group, these enzymes are significantly more thermostable than the cMDHs from the cold-adapted red and pinto abalone (analysis of variance; P=0.01).

Non-denaturing gel electrophoresis (Fig. 4) yields two results. First, each of the cytoplasmic MDHs used in this study was free from contamination by the mitochondrial isoform (mMDH), confirming that the kinetic and thermal stability data are based solely on the cytoplasmic isozyme of MDH. Mitochondrial MDH migrates significantly more slowly than the cytosolic form on non-denaturing PAGE gels (Wheat *et al.* 1972). Second, because native gels separate on the basis of charge as well as size, differences in surface charge between proteins of similar size can be detected. These data show that the cMDHs from the two northern abalone species, the red and pinto abalone, are electrophoretically indistinguishable. Furthermore, the migration of red and pinto cMDHs is distinct from that of homologues of the green, pink and black abalone. The green, pink and black homologues migrate differently from each other, indicating that they are structurally, if not kinetically, distinct.

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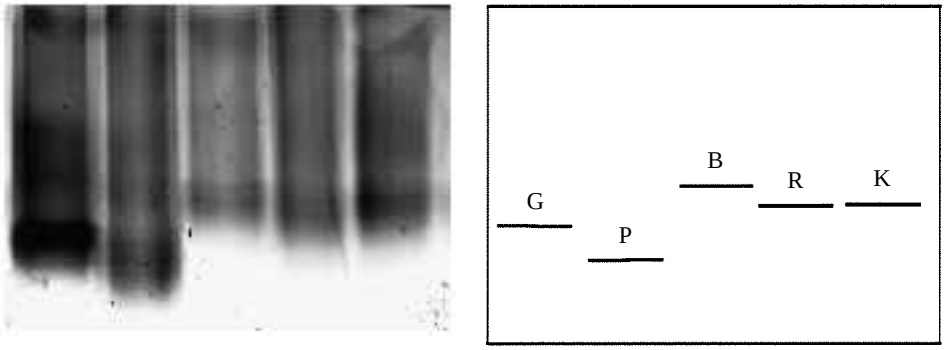

Fig. 4. Non-denaturing polyacrylamide gel for cMDHs of five species of abalone: *H. fulgens*, G; *H. corregata*, P; *H. cracherodii*, B; *H. rufesens*, R; and *H. k. kamtschatkana*, K.

Discussion

Enzyme kinetics

The effects of temperature on cytoplasmic malate dehydrogenases isolated from five species of eastern Pacific abalone indicate that cMDHs from species living at lower latitudes or higher tidal heights (green, pink and black abalone) are adapted to function at higher temperatures than are homologous enzymes from congeners living at higher latitudes or greater depths (red and pinto abalone). The K_m of NADH was conserved between 11 and 21 $\mu mol\,l^{-1}$ for cMDHs of all five species when measured at the appropriate physiological temperatures. The range within which the K_m of NADH is conserved for these five species' cMDHs is similar to that observed for the K_m values of NADH for other dehydrogenases when measured at physiological temperatures (Yancey and Siebenaller, 1987; Dahlhoff and Somero, 1991). For example, Yancey and Siebenaller (1987) found that the kinetics of M_4-lactate dehydrogenases of six species of marine teleosts living at different habitat temperatures were extremely temperature-sensitive. However, when measured over the habitat temperature range of each species, the K_m of NADH was conserved between 9 and 16 $\mu mol\,l^{-1}$. The conservation of K_m values between phylogenetically distinct groups of marine organisms suggests that the adaptive modification of protein function is a pervasive strategy of temperature adaptation in the marine environment.

Elevated temperatures perturbed the K_m of NADH for cMDHs of the two species living at higher latitudes or lower tidal heights to a much greater extent than for cMDH homologues of species from lower latitudes or higher tidal heights. At 25°C, the K_m values of cMDHs from red and pinto abalone are about 35 $\mu mol\,l^{-1}$ NADH, significantly higher than the values of 11–21 $\mu mol\,l^{-1}$ NADH measured at these two species' common habitat temperatures (5–15°C). Conversely, K_m values of cMDHs from green, pink and black abalone are maintained at approximately 11–21 $\mu mol\,l^{-1}$ NADH until very high assay temperatures (45°C). Given that an increase in the K_m corresponds to a decrease in

binding effectiveness (and loss of NADH saturation), these data suggest that cMDHs from green, pink and black abalone function more effectively at higher temperatures than do cMDHs from red and pinto abalone.

The different responses to temperature of cMDHs of abalone from different latitudes and tidal heights suggest that adaptation of enzymes to small (less than 5˚C) differences in average or maximal habitat temperature is critical in evolutionary processes. For example, the maximal habitat temperature of red abalone is approximately 18˚C, whereas that for pink abalone is approximately 23˚C. Although these two species only experience a difference in maximal habitat temperature of approximately 5˚C, the cMDH homologue from the pink abalone is much less perturbed by elevated assay temperatures than is the red cMDH homologue. This suggests that differences in habitat temperature of about 5˚C are sufficient to select for adaptive modification of enzymatic function, as was observed for lactate dehydrogenases from closely related species of barracuda experiencing differences in body temperatures of 3–8˚C (Graves and Somero, 1982). The observation that similar, small changes in habitat temperature are sufficient to induce protein modifications in dehydrogenases from both teleosts and molluscs suggests that adaptive modifications of protein function may be an important aspect of the mechanism of species formation (discussed below).

The differences observed in Arrhenius activation energy (E_a) between the cMDHs of the three warm-adapted species and the two cold-adapted species are consistent with trends observed in other studies. Comparisons of several sets of homologous enzymes from species adapted to different temperatures have shown that E_a values are lower for enzymes of species with low body temperatures (Johnston and Goldspink, 1975; Johnston and Walesby, 1977; Hochachka and Somero, 1984). Although E_a is a function of the activation enthalpy ($\Delta H^{\ddagger}$) alone (i.e. it does not take into account differences in the activation entropy, $\Delta S^{\ddagger}$), previous studies have shown that $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are positively correlated (Low *et al.* 1973). Therefore, differences in E_a indicate differences in the Gibbs free energy of activation ($\Delta G^{\ddagger}$). Differences in E_a (or $\Delta G^{\ddagger}$) have been interpreted to be an indication that the energy barriers to catalysis are lower in proteins from cold-adapted species, so as to compensate for the reduced levels of energy available to drive catalysis. The lower E_a values for enzymes of cold-adapted species may be a consequence of these proteins' more flexible structures (as indexed by thermal stability), which allow catalytic conformational changes to occur with lower energy costs.

Lower E_a values could also result from differences between species in active site structural complementarity to the ground-state and transition-state structures of the substrate. Thus, the tight substrate binding required to stabilize the enzyme–substrate complex at high temperatures in warm-adapted species may favour selection for a high degree of active site structural complementarity with the ground-state substrate. The need for strong enzyme–substrate interactions to offset thermal perturbation of binding is suggested by the observation that, at any single measurement temperature, the K_m values of enzymes from warm-adapted species are lower than those of enzymes from cold-adapted species. In cold-adapted species, enzyme–substrate complex formation is favoured by the low kinetic energy of the system, and the binding energy needed by enzymes of warm-adapted species for enzyme–substrate complex stabilization can

instead be used to stabilize the transition-state complex. Because any increase in the stabilization of the transition-state complex will lower the energy barriers to the reaction (Fersht, 1985), the enzymes of cold-adapted species may gain higher turnover numbers compared with enzymes of warm-adapted species.

Protein thermal stability

Cytoplasmic MDHs from green and black abalone are more able to withstand exposure to elevated temperatures than are cMDHs from red and pinto abalone. This indicates that cMDHs of species that live in the intertidal zone and experience very high body temperatures (up to 35°C in some cases; Ricketts *et al.* 1985) for short periods are more resistant to thermal stress than are cMDHs of species that occur subtidally or at high latitudes. The intermediate thermal stability of cMDH from pink abalone may be a reflection of this species' subtidal distribution. These data suggest that the evolution of proteins that are resistant to thermal denaturation may confer some selective advantage on marine invertebrates, in agreement with studies that have demonstrated adaptive differences in thermal stabilities of proteins from marine fish and other vertebrates (Johnston *et al.* 1973; Johnston and Walesby, 1977; McFall-Ngai and Horwitz, 1990). Although it is possible that an increase in the structural stability of proteins may be directly important for intertidal species, which experience short exposures to elevated temperatures, it is more probable that these stability differences are a result of the conservation of physiologically important functional properties rather than resistance to thermal denaturation (Somero, 1991). For example, partial unfolding of an enzyme's tertiary structure may affect its ability to bind substrate, resulting in a decrease in binding effectiveness. It is interesting to note that, for the cMDH homologues examined in the present study, the two homologues that are most kinetically thermolabile (the cMDHs of the red and pinto abalone) are the least structurally thermostable. The elevation of the K_m of NADH at high temperatures for cMDHs from red and pinto abalone may be an indirect result of partial thermal unfolding of the enzyme. More detailed studies are necessary to test this hypothesis.

Evolutionary implications

The differences observed in thermal stability and sensitivities of kinetic properties to elevated temperatures of cMDHs from these five species of abalone reflect the evolutionary history of the genus. The common ancestor of all eastern Pacific abalone lived in warm temperate or tropical seas, conditions similar to modern-day southern California and Baja California (Lindberg, 1991; Talmadge, 1963). This suggests that modern abalone living in warm habitats (green, pink and black abalone) will have cMDHs that are functionally, although not necessarily structurally, similar to the ancestral form. Phylogenetic trees based on two distinct data sets suggest that red and pinto abalone were formed from more recent speciation events relative to the event(s) that led to the separation of green, pink and black abalone and therefore are more closely related to each other than to green, pink or black species (Cox, 1962; Lee and Vacquier, 1992).

The cMDHs of the red and pinto abalone are identical in their thermal stabilities,

kinetic properties and electrophoretic mobilities, and appear to be adapted to function optimally at cool temperatures. The kinetics and thermal stabilities of cMDH homologues from green, pink and black abalone are similar, yet these homologues are electrophoretically distinct. Previous studies of electrophoretic and kinetic properties of lactate dehydrogenases of pairs of teleost species separated by the Isthmus of Panama showed that kinetic and electrophoretic differences between closely related species living at different temperatures do not necessarily co-vary (Graves *et al.* 1983). This observation, along with the data reported in the present study, suggests that some of the amino acid substitutions leading to electrophoretic differences are 'neutral' with respect to temperature adaptation.

The data presented in this study, when considered in the light of the phylogenetic relationships of modern abalone species, suggest that the cMDH of red and pinto abalone was present in an ancestor that had evolved biochemical and physiological adaptations that allowed it to exploit cool habitats, whereas the cMDHs of green, pink and black species are functionally similar to that of their warm-adapted common ancestor. Although these warm-adapted cMDHs may have accumulated 'neutral' mutations since divergence, they are still adapted to function optimally at the physiological temperatures of the species from which they were isolated, suggesting that positive selection has been critical in maintaining optimal kinetic function and thermal stability.

In conclusion, differences in thermal stability and in the sensitivities of kinetic properties to temperature change between cMDHs from abalone living at different temperatures indicate that temperature adaptations are critical in establishing species distribution, both with latitude and at different tidal heights at a given latitude, for this group of marine invertebrates. The results obtained with cMDH indicate that a protein's thermal stability and kinetic sensitivity to elevated temperatures are selective traits that may co-vary with speciation events and are probably one part of the complex mechanism by which new species are formed.

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