

## A COMPARATIVE ANALYSIS OF THE EVOLUTIONARY PATTERNING AND MECHANISTIC BASES OF LACTATE DEHYDROGENASE THERMAL STABILITY IN PORCELAIN CRABS, GENUS *PETROLISTHES*

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### Summary

The kinetic properties of orthologous homologs (orthologs) of enzymes are typically correlated with environmental temperatures in species adapted to different thermal regimes, but correlations between adaptation temperature and enzyme thermal stability are less clear. Although the thermal stability of a protein is related chiefly to its primary structure (including post-translational modification), thermal stability can also be altered by extrinsic factors present in the intracellular milieu. Here, we present a comparative analysis of the thermal stability of lactate dehydrogenase (LDH) orthologs from 22 congeneric species of porcelain crab (genera *Petrolisthes* and *Allopetrolisthes*) from a broad range of thermal habitats. Interspecific diversity of LDH stability is high: temperatures required for a 50% loss of activity in 10 min ranged from 65 to 75.5 °C, corresponding to half-lives of less than 1 min to more than 3 h at 70 °C. Although stability is positively correlated with maximal habitat temperature in some sister taxa, phylogenetic comparative analysis incorporating all 22 species does not indicate that the

interspecific diversity of LDH stability represents an adaptive response to current thermal habitats. Examination of the mechanistic bases of LDH stabilization indicates that differences in stability are related both to properties of the LDH molecule itself (intrinsic stability) and to the effects of extrinsic protein(s). Intrinsic differences were shown by the unfolding of structure during heating, as measured by circular dichroism spectroscopy. Stabilizing effects of extrinsic proteins are implied by the results of cellular fractionation experiments that removed low-molecular-mass solutes and proteins from the muscle homogenates. We conclude that the overall structural stability and functional properties of proteins can evolve independently and that *in vivo* protein–protein interactions can provide another means to regulate protein stability selectively.

Key words: lactate dehydrogenase, porcelain crab, *Petrolisthes*, protein stability, temperature.

### Introduction

Temperature is a dominant factor in governing the distribution patterns of ectothermic marine organisms (Fields et al., 1993; Barry et al., 1995; Hughes, 2000). Whole-organism thermal tolerance limits closely reflect differences in habitat temperature that result from different latitudinal and vertical distribution patterns (Vernberg and Tashian, 1959; Edney, 1961; Stillman and Somero, 1996; Stillman and Somero, 2000; Tomanek and Somero, 2000). Thermal tolerance limits of organisms are established by a combination of morphological, physiological and biochemical traits, including thermal sensitivities of enzymatic and structural proteins (Alexandrov, 1977; Hochachka and Somero, 1984; Cossins and Bowler, 1987; Somero, 1995; Somero, 1997). The need for proteins to undergo conformational changes during function, e.g. during rate-limiting steps in catalysis (Dunn et al., 1991), requires that proteins evolve a high degree of structural flexibility. Thus, net stabilization free energies of

proteins are typically of the order of the energy contained in a few non-covalent bonds, which render them highly sensitive to thermal perturbation (Jaenicke, 1991).

Studies of orthologous homologs (orthologs) of proteins have shown that functional properties, e.g. Michaelis–Menten constants ( $K_m$ =binding ability) and catalytic rate constants ( $k_{cat}$ =rate of function), are conserved within a narrow range at normal body temperatures in differently adapted species (for reviews, see Somero, 1995; Somero, 1997). Because the values of these kinetic properties can be set by energy barriers to catalytic conformational changes (Dunn et al., 1991), it has been conjectured that temperature adaptation of protein function will involve adjustments in protein stability (Arpigny et al., 1994; Somero, 1995). Cold adaptation is predicted to lead to more flexible proteins, i.e. to lower energy barriers to conformational changes, whereas adaptation to high temperatures is predicted to lead to more rigid proteins that

retain the correct three-dimensional geometry even at the high body temperatures of these organisms. Although several studies using orthologous proteins from highly divergent taxa adapted to widely different temperatures have shown correlations between adaptation temperature and protein thermal stability (McFall-Ngai and Horwitz, 1990; Jaenicke, 1991; Somero, 1995), studies of closely related, but differently adapted, species have shown that adaptive differences in kinetic properties are not invariably linked to differences in temperatures of heat denaturation (Place and Powers, 1984; Fields and Somero, 1997; Fields and Somero, 1998; Holland et al., 1997). Thus, there remains uncertainty about the general importance of adjustments in protein thermal stability during adaptation to temperature. A shortcoming of most previous studies of this issue has been the lack of a broad comparative analysis of a large number of species that, while phylogenetically closely related, are adapted to a broad range of temperatures.

A further variable in the establishment of protein stability is the role of extrinsic factors. Although the intrinsic stability of a protein, as established by its amino acid sequence and post-translational modification, may play the dominant role in setting thermal stability, the composition of the surrounding milieu can potentially have a large effect on stability; low-molecular-mass organic osmolytes may have stabilizing effects on proteins (Jaenicke, 1991; Somero and Yancey, 1997; Singer and Lindquist, 1998), as may the concentration of protein *per se* because of molecular crowding effects (Garner and Burg, 1994). *In vitro* studies of the thermal stability of purified proteins performed in simple buffers may fail, therefore, to provide an accurate estimate of a protein's stability *in vivo*.

To investigate adaptive differences in protein stability among orthologous enzymes from differently adapted species, and to extend our understanding of the roles of both intrinsic and extrinsic factors in setting thermal stability, we conducted a comparative analysis of the thermal stability of lactate dehydrogenases (LDH, EC. 1.1.1.27: NAD<sup>+</sup>:lactate oxidoreductase) from 22 species of congeneric porcelain crabs. Current taxonomic nomenclature groups these species into two genera, *Petrolisthes* (20 species) and *Allopetrolisthes* (two species), but molecular taxonomy suggests that all 22 species belong to the genus *Petrolisthes* (Stillman, 1998; Stillman and Reeb, 2001). These crabs are widely distributed throughout the coastal regions of the Pacific Ocean, with species in the eastern Pacific distributed widely along both latitudinal and vertical intertidal gradients (Stillman and Somero, 2000). Maximal body temperatures of individual species range from approximately 15 °C to over 40 °C (Stillman and Somero, 2000). We examined the stabilities of LDH using (i) unfractionated supernatants of claw muscle homogenates, (ii) fractionated supernatant preparations from which classes of potentially stabilizing molecules had been removed, and (iii) purified enzymes to discern the roles of intrinsic and extrinsic stabilizing factors in setting LDH stability. We used phylogenetically based comparative analysis (Felsenstein, 1985) to determine whether the stability of LDH was

evolutionarily related to contemporary habitat temperature conditions across all 22 species.

## Materials and methods

### *Specimen collection and storage*

Crabs were collected and held submerged in flowing sea water at local water temperatures for 24–48 h before freezing and storage at –70 °C. The sites of collection were: *Petrolisthes manimaculis*, Monterey Bay, California (36°36'N, 121°53'W); *P. cabrilloi*, La Jolla, California (32°51'N, 117°16'W); *P. eriomerus*, Cape Arago, Oregon (43°21'N; 124°19'W); *P. cinctipes*, Cape Arago, Oregon, and Monterey Bay, California; *P. armatus*, *P. gracilis*, *P. sanfelipensis*, *P. hirtipes*, *P. crenulatus*, Pelican Point, Puerto Peñasco, Sonora, Mexico (31°39'N, 113°15'W); *P. granulatus*, *P. laevigatus*, *P. violaceus*, *P. tuberculatus*, *P. tuberculosus*, *Allopetrolisthes punctatus*, *A. angulosus*, Las Cruces, Chile (33°33'S, 71°36'W); *P. cf. tridentatus*, *P. armatus*, *P. galathinus*, *P. edwardsii*, *P. agassizii*, *P. lewisi austrinus*, *P. haigae*, Naos Island, Pacific Panama (8°50'N, 7°8'W).

### *Supernatant preparation*

Whole claws (merus, carpus and manus) were removed from frozen specimens and thawed on ice. The number of claws used for one supernatant preparation depended on body size; in small-bodied species, as many as 20 claws were needed to obtain enough tissue for a single preparation. Muscle tissue was removed, weighed to the nearest 0.0001 g, and homogenized on ice in 6 volumes (w/v) of 50 mmol l<sup>-1</sup> potassium phosphate buffer, pH 6.8 (homogenization buffer), in Kontes-Duall ground-glass tissue homogenizers. Crude homogenates were centrifuged at 16 000 g for 35 min at 4 °C, and supernatants were removed to fresh tubes and stored on ice until further used. Total protein concentrations of the supernatants ranged from approximately 6 to 8 mg ml<sup>-1</sup>.

### *Stabilizer identification experiment*

For identification of the molecular factors responsible for protein stabilization, the supernatant (generated as above) was divided into four fractions and prepared as illustrated in Fig. 1. One fraction was unaltered, representing the supernatant condition (code: supernatant); a second fraction was dialyzed to remove small molecules (code: –small molecules); a third was subjected to (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitation [at saturated levels of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>] to remove non-protein macromolecules (code: protein only); and the fourth fraction was used to purify the LDH (code: LDH only) (Fig. 1). The 'protein only' sample was prepared from the (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitate by centrifugation at 16 600 g for 45 min, followed by resuspension of the pellet in homogenization buffer and dialysis. LDH purification was performed following the procedure of Yancey and Somero (1978) using oxamate–Sephacryl affinity chromatography. Briefly, oxamate–Sephacryl was pre-equilibrated with 50 mmol l<sup>-1</sup> potassium phosphate, 500 mmol l<sup>-1</sup> potassium chloride and 0.2 mmol l<sup>-1</sup> NADH, pH 6.0. Samples of

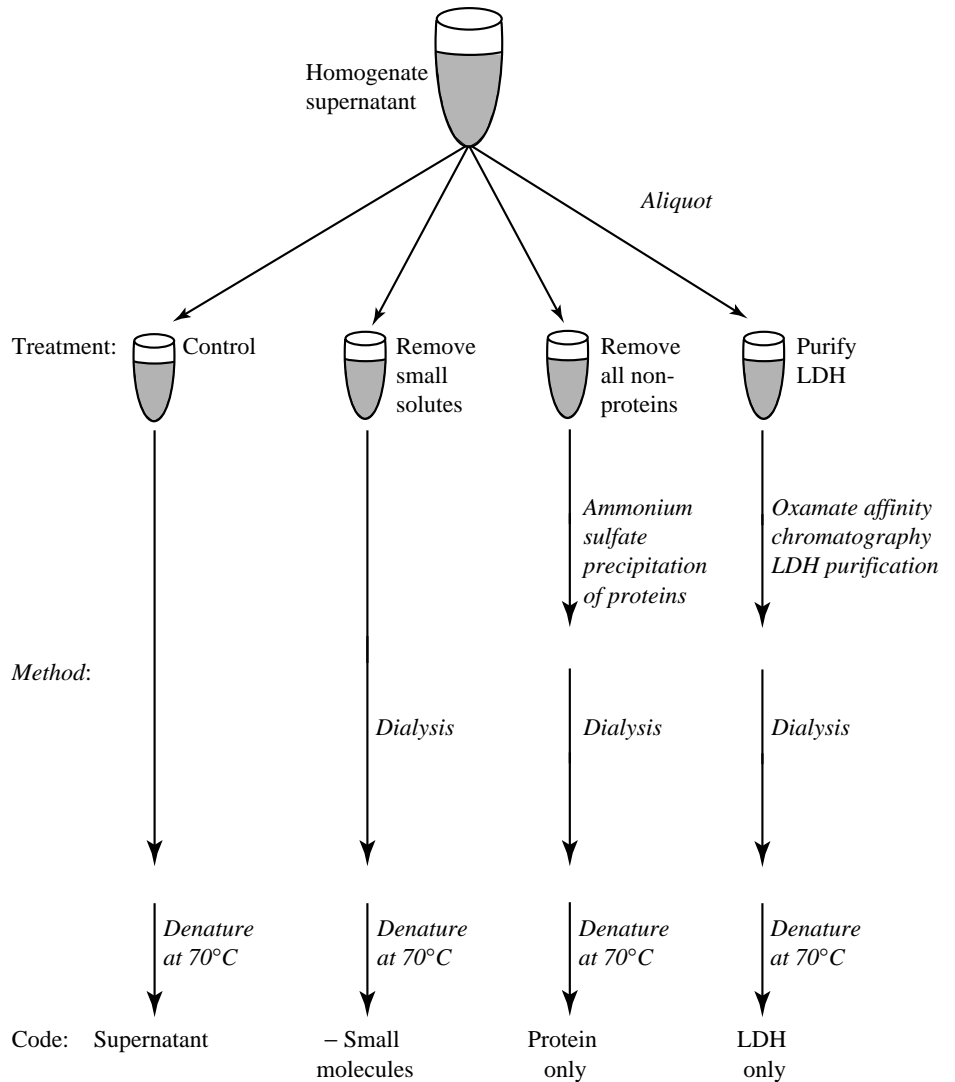


Fig. 1. Preparation of samples for identification of macromolecular species involved in lactate dehydrogenase (LDH) stabilization. Diagram of the experimental procedure used in the stabilizer identification experiments. Sample identification is in normal type, and methods are in italicized type. 'LDH only' samples also contained bovine serum albumin at a concentration of  $1 \text{ mg ml}^{-1}$ . See text for further details.

supernatant were made up to  $500 \text{ mmol l}^{-1}$  potassium chloride and  $0.2 \text{ mmol l}^{-1}$  NADH by addition of the appropriate amounts of dry chemicals; pH was not adjusted. This solution was passed over the column and was followed by a wash of approximately 25 bed volumes of column buffer. LDH was eluted with  $50 \text{ mmol l}^{-1}$  potassium phosphate,  $500 \text{ mmol l}^{-1}$  potassium chloride and  $10 \text{ mmol l}^{-1}$  pyruvate, pH 6.0. Fractions containing significant levels of LDH activity were pooled, concentrated and dialyzed. In all cases, dialysis was performed in Slide-A-Lyzers with a 10 kDa molecular mass cut-off (Pierce, Rockford, IL, USA) against 4 l of homogenization buffer for 4 h followed by 4 l of fresh homogenization buffer for at least 12 h. Bovine serum albumin (BSA) was added to the 'LDH only' fraction at a concentration of  $1 \text{ mg ml}^{-1}$  during thermal denaturations to mimic the higher protein concentrations of the other three fractions.

#### *Thermal denaturations and LDH assay*

Aliquots of each sample (25–100  $\mu\text{l}$ ) were placed into 200  $\mu\text{l}$  thin-walled thermal cyclers tubes, and denaturation was

performed in an MJ-Research thermal cycler (model PTC-100), either as a function of time at one temperature, generally  $70^\circ\text{C}$  (half-life), or as a function of temperature with incubation times set at 10 min ( $T_{50}$ ). After thermal incubation, the tubes were immediately cooled on ice and then centrifuged for 2 min at  $16\,600 \text{ g}$  to pellet any precipitate that formed during the thermal incubation. The supernatants were then used directly in activity assays. LDH activity was measured spectrophotometrically by following the change in absorbance at 340 nm, which corresponds to the enzymatic oxidation of cofactor NADH to  $\text{NAD}^+$ . Reaction conditions were:  $80 \text{ mmol l}^{-1}$  imidazole-Cl,  $150 \mu\text{mol l}^{-1}$  NADH,  $5 \text{ mmol l}^{-1}$  pyruvate, pH 6.9, in a volume of 2 ml at  $20^\circ\text{C}$  (following Yancey and Somero, 1978) and 10  $\mu\text{l}$  of the sample. pH 6.9 was found to give the highest enzymatic activity under these reaction conditions (data not shown). The initial LDH activities of unheated supernatants varied approximately threefold among samples.

#### *Data analysis*

To determine the time at one temperature required for the

loss of 50% of activity (the half-life), the data were analyzed as follows. Replicate activity measurements at each time point were averaged. Percentage residual activity was calculated on the basis of the activity of the unheated sample, which never varied during the time that it took to measure the activity of all of the samples. These percentages were log-transformed to make them linear with respect to time, and regression analysis was employed to calculate the slope of the correlation line between activity and time. The slope was then used to calculate the half-life, or time at which 50% (log-transformed=1.699) of the activity remained.

To determine the temperature required for 50% loss of activity after a 10 min incubation ( $T_{50}$ ), the data were analyzed as follows. Replicate activity measurements at each temperature were averaged. The average activity at each time point was expressed as a percentage of the residual activity of the sample incubated at a temperature just below the level required for loss of activity to be measurable. This sample was used as the 100% activity, rather than the unheated sample, because activity increased slightly following incubation at elevated, but non-denaturing, temperatures. Percentages were transformed by taking the arcsine of the square root of percentage residual activity, which made the activities linear with respect to incubation temperature over most of the denaturation temperature range. Regression analysis was performed over this linear range, and the slope of the correlation line was used to calculate the  $T_{50}$ , or temperature at which 50% (arcsine square root=0.7853) of the activity remained.  $r^2$  values were generally greater than 0.95 for both half-life and  $T_{50}$  determinations.

Half-life and  $T_{50}$  are log-linear when plotted against one another ( $r^2=0.98$ ), and the relationship between these two measures of thermal stability was used to generate half-life values from  $T_{50}$  values to compare species whose half-lives at 70°C were too short to measure accurately. Half-lives were calculated for each  $T_{50}$  value using the equation  $T_{50}=65.5135+4.2075x$ , where  $x$  is the  $\log_{10}$ (half-life) at 70°C (half-life in min).

#### Circular dichroism (CD) spectroscopy

To determine whether interspecific differences in thermal stabilities, as determined by assays of loss of activity, are related to large-scale structural unfolding of the protein, we used circular dichroism to monitor the thermal transition of protein secondary structural elements ( $\alpha$ -helix and  $\beta$ -sheet) from folded to unfolded states with a JASCO J-710 spectropolarimeter. Temperature was controlled with a Peltier-driven thermal jacket (model PTC-348W, JASCO). CD temperature scans of purified LDHs at concentrations of  $8\ \mu\text{mol l}^{-1}$  were performed at 220 nm by increasing the temperature in 0.1°C increments from 20°C to 95°C at a rate of 1°C min<sup>-1</sup>. 220 nm was chosen because, at this wavelength, a large difference exists between the ellipticity of the native and denatured states (spectral data not shown). Apparent  $T_m$  values (the temperature at the midpoint of the transition from

the folded to the unfolded state) were determined by fitting the data to a thermodynamic equilibrium model (Johnson et al., 1995; Kasimova et al., 1998).

#### Independent contrasts analyses

Molecular sequence data of the 16S rDNA mitochondrial gene (approximately 450 base pairs) were obtained by polymerase chain reaction (PCR) amplification from whole genomic extracts using the universal primers 16SAR and 16SBR (Palumbi et al., 1991) and cycle sequencing as described elsewhere (Stillman, 1998; Stillman and Reeb, 2001). Phylogenetic analysis was performed by neighbor-joining of bootstrapped distance matrices generated by the PHYLIP software package (Felsenstein, 1989) as described elsewhere (Stillman, 1998; Stillman and Reeb, 2001). Phylogenetically independent contrasts (Felsenstein, 1985) of  $T_{50}$  and maximal habitat temperatures were generated using the CAIC software package (Purvis and Rambaut, 1995) and the results of PHYLIP analyses. These phylogenetically independent contrasts were used in linear regression analyses in which the regression was forced through the origin, as is required for analyses of independent contrasts (Purvis and Rambaut, 1995).

## Results

#### Comparative analysis of LDH thermal stability

The thermal stability of LDH, as indexed by half-life, varied by over two orders of magnitude among species of eastern Pacific *Petrolisthes* and *Allopetrolisthes* (Fig. 2). Average half-lives at 70°C ranged from 0.8 min ( $T_{50}=65^\circ\text{C}$ ) for *P. lewisi austrinus* (18) and *P. crenulatus* (17) to 240 min ( $T_{50}=75.5^\circ\text{C}$ ) for *P. edwardsii* (9) (numbers in parentheses refer to Fig. 2). The thermal stability of LDH differs on average between the two main clades of *Petrolisthes* (Fig. 3): *P. armatus*, *P. agassizii*, *P. edwardsii*, *P. haigae* and *P. galathinus* have LDHs that are generally more stable than those of any of the species in the other clade, with the exception of *P. sanfelipensis* (Figs 2, 3).

In two groups of sister taxa living in different vertical zones, LDH thermal stability was positively correlated with maximal habitat temperatures, as an adaptational hypothesis would predict: (i) in a group of four north-temperate sister species (Fig. 3), the two intertidal species, *P. cinctipes* (13) and *P. cabrilloi* (14), have LDHs that were significantly more stable than the LDHs of the two subtidal species, *P. eriomerus* (2) and *P. manimaculis* (4); and (ii) in two south-temperate species, the intertidal species *A. angulosus* (7) has an LDH that is more thermally stable than that of the low intertidal and subtidal species *A. punctatus* (3). However, sister species that occur in similar thermal habitats can have LDHs with different stabilities, e.g. *P. edwardsii* (9) versus *P. agassizii* (10) and *P. tuberculosis* (1) versus *P. tuberculatus* (5), indicating that LDH thermal stability is not necessarily correlated with habitat temperature in sister species. Despite the two positive correlations between LDH



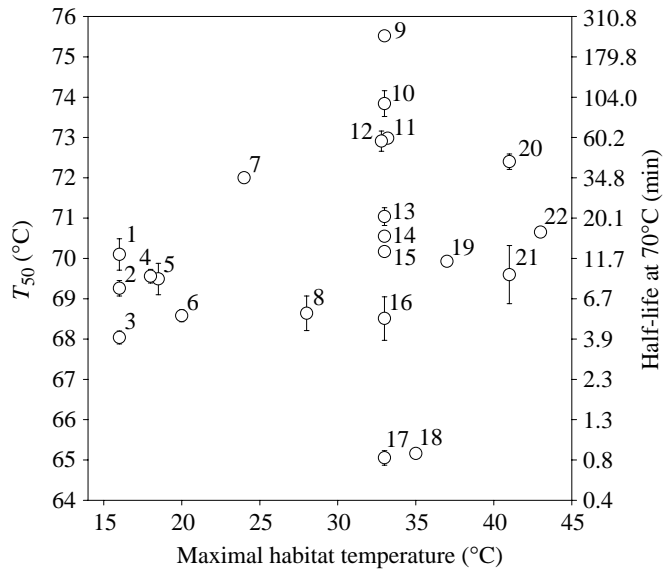


Fig. 2. Thermal stability of lactate dehydrogenases (LDHs) in muscle homogenate supernatants from 22 species of eastern Pacific porcelain crabs, genera *Petrolisthes* and *Allopetrolisthes*, plotted as a function of maximal microhabitat temperature. Data plotted are the temperatures required for 50% loss of activity after a 10 min incubation ( $T_{50}$ ) values; calculated half-lives have been added to the right-hand y-axis for reference. Maximal microhabitat temperature data are from Stillman (Stillman, 1998) and Stillman and Somero (Stillman and Somero, 1996; Stillman and Somero, 2000). Each point represents the mean  $\pm$  1 s.d. ( $N=5$ ) for a single species. Species are numbered as follows: 1, *P. tuberculatus*; 2, *P. eriomerus*; 3, *A. punctatus*; 4, *P. manimaculis*; 5, *P. tuberculatus*; 6, *P. violaceus*; 7, *A. angulosus*; 8, *P. laevigatus*; 9, *P. edwardsii*; 10, *P. agassizii*; 11, *P. haigae*; 12, *P. galathinus*; 13, *P. cinctipes*; 14, *P. cabrilloi*; 15, *P. sanfelipensis*; 16, *P. granulatus*; 17, *P. crenulatus*; 18, *P. lewisi austrinus*; 19, *P. hirtipes*; 20, *P. armatus*; 21, *P. gracilis*; 22, *P. cf tridentatus*.

stability and habitat temperature mentioned above, there was no overall correlation between LDH stability and maximal habitat temperature across all 22 species (Fig. 2). Phylogenetically independent contrast analysis, based on the phylogenetic tree shown in Fig. 3, also lends no support for the 'adaptive' hypothesis that LDH thermal stability is positively correlated with microhabitat temperature across all species because there was no significant correlation between contrast of  $T_{50}$  and contrast of maximal habitat temperature (Fig. 4; regression line not shown).

#### Mechanistic analysis

##### Factors affecting the stability of LDH

To investigate the mechanisms of stabilization in porcelain crab LDHs, we selected five species (*Petrolisthes eriomerus*, *P. cinctipes*, *P. armatus*, *P. agassizii* and *P. edwardsii*) whose LDHs had stabilities representing a wide range of the observed variation within the genus (the LDH half-lives in muscle homogenate supernatants of these species ranged from 8 to 240 min at 70 °C; Fig. 2). The protein pools in the 'supernatant',

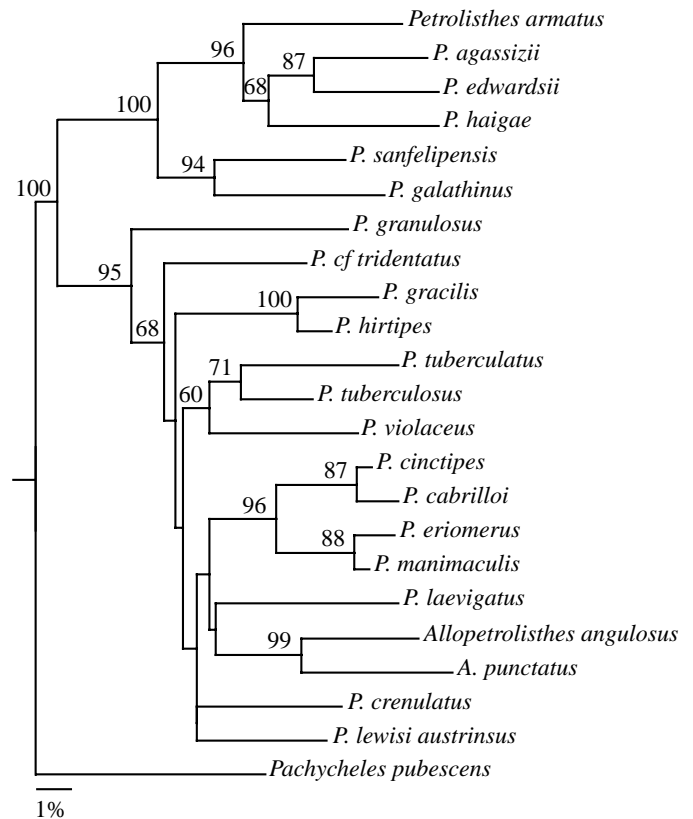


Fig. 3. Phylogenetic tree of porcelain crabs used in this study. Tree construction was based on the 16S RNA gene. Phylogenetically independent contrasts were generated using this tree. The tree was generated using distance analysis, and numbers next to nodes are bootstrap values out of 100 trees. Bootstrap values less than 60 have not been added to the tree. This tree was rooted with *Pachycheles cf pubescens*, although this terminal node was not used to generate an independent contrast.

'small molecules' and 'protein only' fractions were similar (Fig. 5). 'LDH only' fractions analyzed by silver-stained SDS-PAGE typically contained only one band, indicating that the LDH was at least 95% pure (Fig. 5). Interspecific differences in thermal stability were consistent regardless of what classes of intracellular constituent (low-molecular-mass or proteins) were present (Fig. 6). The half-lives of LDH in the 'small molecules' and 'protein only' fractions were consistently slightly higher than the half-lives of the supernatant samples ( $P<0.5$ ; one-tailed  $t$ -test) (Fig. 6). Decreased thermal stability was only observed in the 'LDH only' fractions, in which the half-lives were reduced in all species compared with the 'protein only' fractions, although the degree of reduction in stability differed among species. Half-lives were reduced in each case ( $P$ -values are from one-tailed  $t$ -tests): *P. eriomerus* by 35% ( $P=0.067$ ), *P. cinctipes* by 20% ( $P=0.036$ ), *P. armatus* by 56% ( $P=0.016$ ), *P. agassizii* by 66% ( $P=0.008$ ) and *P. edwardsii* by 68% ( $P=0.020$ ) (Fig. 6). The results of these homogenate fractionation studies suggest that there are differences among the LDHs of these species that are intrinsic to the LDH molecules (as suggested

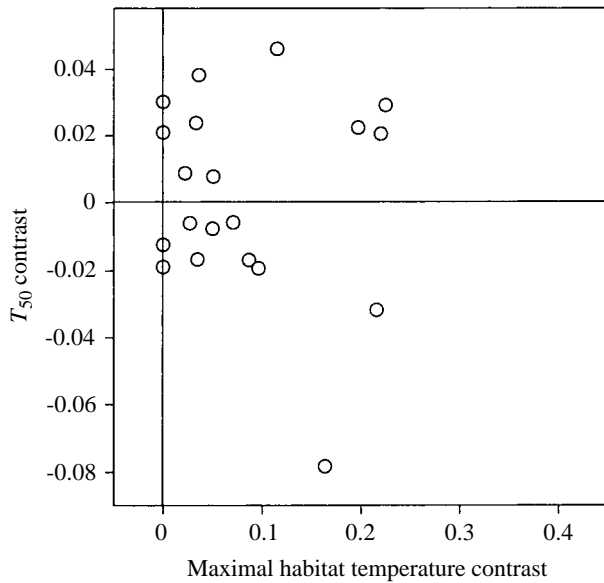


Fig. 4.  $T_{50}$  phylogenetically independent contrasts plotted against contrasts of maximal habitat temperature. No significant correlation exists between these two contrasts.  $T_{50}$  is the temperatures required for 50% loss of activity after a 10 min incubation.

by the consistent ranking of half-lives among species) and that extrinsic protein stabilizers also influence LDH stability, although to differing degrees.

*Structural differences among LDHs*

Interspecific differences in structure among LDH molecules are apparent from SDS-PAGE analysis of purified proteins. The LDHs of *Petrolisthes eriomerus* and *P. cincipetes* are

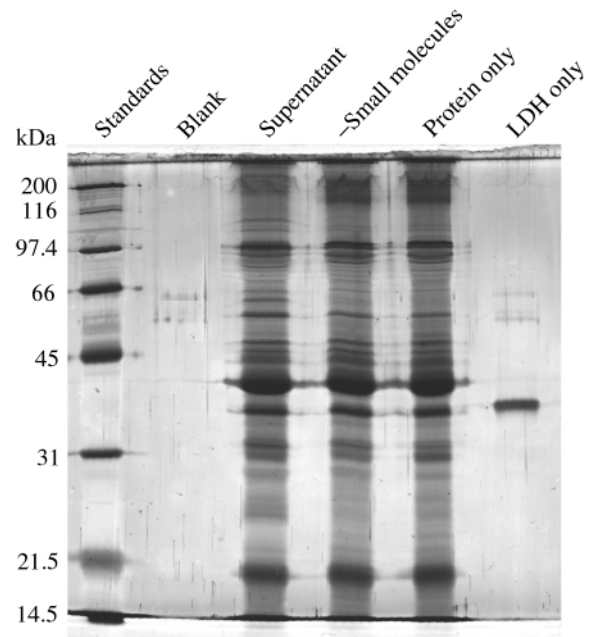


Fig. 5. Silver-stained SDS-PAGE (12%, Laemmli, 1970) analysis of *Petrolisthes cincipetes* samples prepared as illustrated in Fig. 1.

approximately 1 kDa smaller than the LDHs of *P. agassizii* and *P. edwardsii* (Fig. 7). Whether the difference in mass among these LDHs is due to differences in primary structure or to post-translational modification, such as phosphorylation or glycosylation, is not known. The contribution of these differences in mass to differences in thermal stability is not known.

Intrinsic differences in stability among LDHs observed by

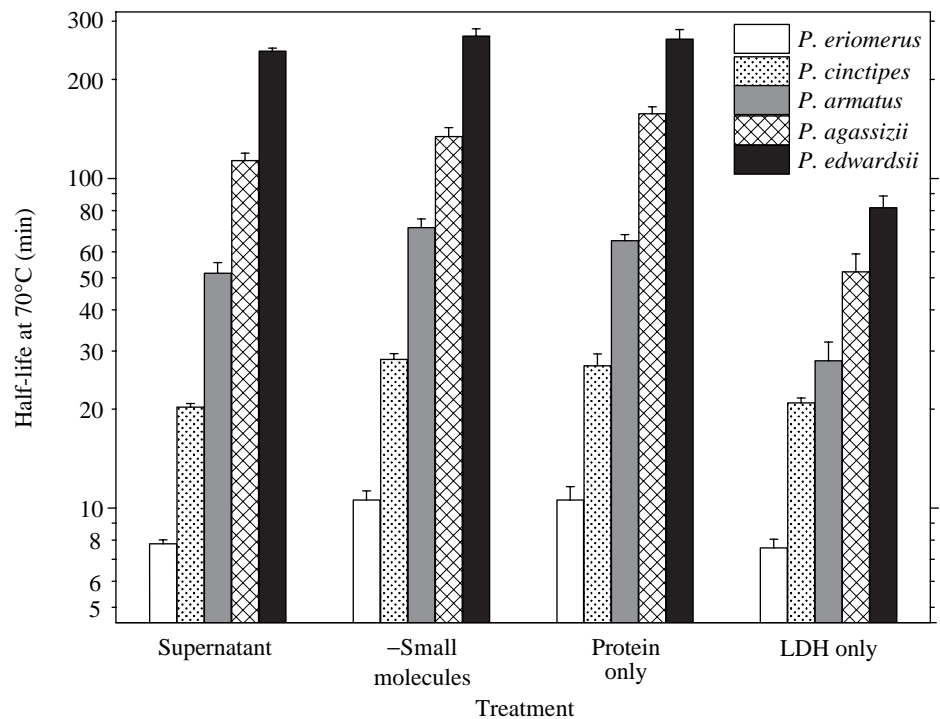


Fig. 6. Analysis of the molecular classes responsible for stabilization of *Petrolisthes* spp. lactate dehydrogenases (LDHs). Each column represents the mean  $\pm$  S.E.M. half-life at 70°C for each sample. Sample sizes are as follows: for *P. eriomerus* and *P. edwardsii*,  $N=2$ ; for *P. cincipetes*, *P. armatus* and *P. agassizii*,  $N=3$ .

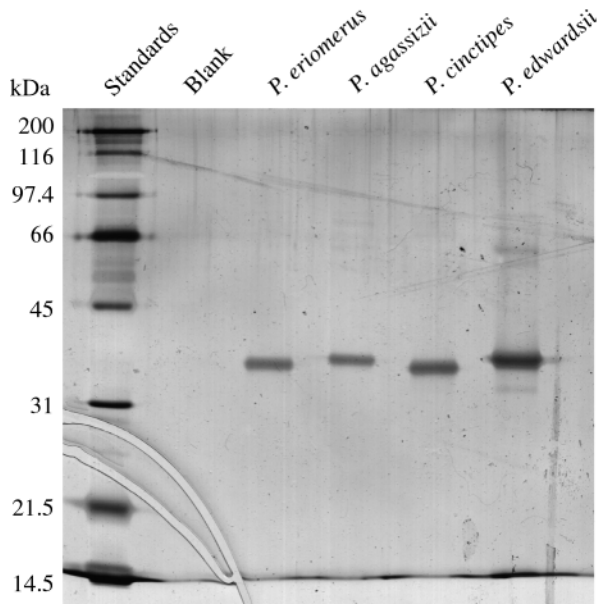


Fig. 7. SDS-PAGE analysis of purified lactate dehydrogenase (LDH) from four species of *Petrolisthes*. Electrophoresis and staining conditions were the same as in Fig. 5.

following loss of activity at elevated temperatures are probably the result of thermal unfolding of secondary and tertiary structure, not artifacts of aggregation and precipitation (see Discussion). Using CD spectroscopy to monitor the loss of  $\alpha$ -helical and  $\beta$ -sheet structures, the same trends found in loss-of-activity experiments were observed (Fig. 8). Apparent  $T_m$  values were 62 °C, 72 °C and 78 °C for *Petrolisthes violaceus*, *P. cincipies* and *P. galathinus*, respectively.

## Discussion

### Comparative analysis: thermal stabilities of porcelain crab LDHs

Porcelain crab LDHs are unusually thermally stable in comparison with previously studied vertebrate and crustacean LDHs. The temperature required to denature fully the most stable porcelain crab LDH (from *P. edwardsii*) within a 10 min period was 79 °C. In previous studies of crustacean LDH thermal stability,  $T_{50}$  values (after a 20 min incubation as opposed to the 10 min as used here) were as high as 61 °C, but were commonly lower (Gleason et al., 1971). Incubation of LDHs from all species of *Petrolisthes*, except for the two less-stable LDHs from *P. crenulatus* and *P. lewisi austrinus* (Fig. 2), for 10 min at temperatures below 65 °C resulted in almost no loss in activity. Loss of activity in *P. edwardsii* LDH did not occur at incubation temperatures below 71 °C. A direct comparison of LDH thermal stabilities between the data reported here and data from the literature is possible for species such as *P. cincipies* and *P. cabrilloi* that have half-lives of approximately 20 min at 70 °C. Those species would have  $T_{50}$  values of approximately 70 °C if thermal incubations lasted for 20 min, much higher values than

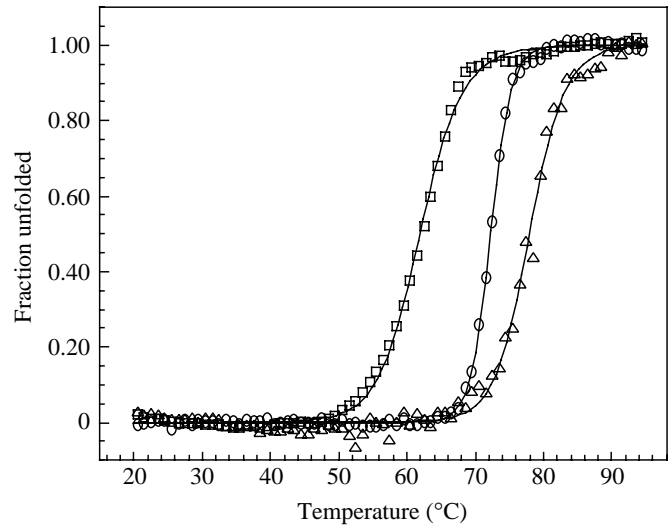


Fig. 8. Thermal melts of purified lactate dehydrogenase (LDH) monitored at 220 nm by circular dichroism spectroscopy. Squares, *Petrolisthes violaceus*; circles, *P. cincipies*; triangles, *P. galathinus*. Every tenth data point is plotted for clarity, although fits (solid curves) were performed on all the data.

previously reported from any eukaryotic organism. In fish A<sub>4</sub>-LDHs, the temperatures required for total loss of enzymatic activity within 20 min are generally well under 60 °C (Fields and Somero, 1997; Fields and Somero, 1998). Comparisons of thermal stabilities of crustacean LDHs with vertebrate A<sub>4</sub>-LDHs are potentially confounded by the fact that the homology of crustacean LDHs and vertebrate A<sub>4</sub>-LDHs is unknown (Zietara et al., 1996).

Comparative analyses of LDH thermal stability with respect to maximal habitat temperatures did not indicate that the LDHs of *Petrolisthes* congeners have evolved different thermal stabilities in response to environmental temperature (Fig. 4). Other studies of the thermal stability of orthologous proteins have also found a lack of correlation with adaptation temperature, even though adaptive differences in kinetic properties ( $K_m$  and  $k_{cat}$ ) were observed (Place and Powers, 1984; Holland et al., 1997; Fields and Somero, 1998). For example, in a comparison of A<sub>4</sub>-LDHs of Antarctic and South American notothenioid fishes,  $K_m$  and  $k_{cat}$  values were conserved within a narrow range for each group of fishes and reflected a high level of cold adaptation in the Antarctic fishes. However, the thermal stabilities of these A<sub>4</sub>-LDHs varied widely within each group of fish and showed no correlation with adaptation temperature (Fields and Somero, 1998). The lack of consistent correlations between adaptive differences in kinetic properties and thermal stability could be a consequence of the fact that changes in the flexibility of the regions of proteins that set energy barriers to catalytic conformational changes, and thereby establish kinetic properties, may not alter the overall stability of the molecule (Fields, 2001). Experimental evidence that kinetic properties and thermal stability can evolve separately is presented in recent studies that have used site-directed mutagenesis to 'evolve' protein

thermal stability and kinetic properties independently (Giver et al., 1998; Miyazaki et al., 2000).

#### *Mechanisms of LDH thermal stability*

The results of the fractionation experiments suggest that the differences in stability among porcelain crab LDHs observed with unfractionated homogenates are due to intrinsic differences in the LDH molecules and to influences of extrinsic factors, probably intracellular proteins (Fig. 6). The slight increase in stability observed in the '–small molecules' and 'protein only' samples may have been due to the removal of destabilizing solutes (e.g.  $\text{Cl}^-$ ) and the relative increase of stabilizing  $\text{PO}_4^{2-}$  from the dialysis buffer (Timasheff, 1992). Intrinsic differences among LDHs are indicated by the consistent interspecific differences seen in all four fractions (Fig. 6). In all species, a reduction in LDH thermal stability occurred when LDH was purified from other cytosolic proteins, as demonstrated by the consistent differences between the 'LDH only' and the 'protein only' fractions. This observation, along with the observation that no decrease in LDH stability occurred when small solutes or large non-protein molecules were removed in the '–small molecules' and 'protein only' treatments, leads to the conclusion that the extrinsic stabilizer is a protein or multiple proteins. The stabilizing interactions with extrinsic proteins may involve highly specific interactions with a limited number of proteins, rather than effects due to protein concentration *per se*, because stability was not altered when bovine serum albumin at concentrations of 1–10 mg ml<sup>-1</sup> was added to samples of purified LDH (data not shown).

Several protein candidates exist that could interact with and stabilize LDH. Proteins known to have a role in the rescue and recovery of thermally damaged proteins include the family of chaperone proteins known as heat-shock proteins (Parsell and Lindquist, 1993). Many of the heat-shock proteins (e.g. hsp70) are ATP-dependent; because no ATP was present in the dialyzed samples, we can rule out these particular heat-shock proteins as candidates. However, heat-shock proteins that can function in the absence of ATP (e.g. the hsp27 family, the TF55 family and the cyclophilins; Parsell and Lindquist, 1993) are potential candidates.

Non-chaperone proteins may also interact with LDH and confer increased stabilization. Glycolytic enzymes, including LDH, have been shown to be associated with ultrastructural components of the muscle contractile apparatus (Amberson et al., 1965), such as the intracellular microtrabecular lattice (Clegg, 1984; Masters, 1984; Pagliaro, 1993). The physical arrangement of enzymes that catalyze reactions in a common metabolic pathway into a single metabolic unit may be advantageous for maximally efficient substrate–product trafficking (Weber and Bernhard, 1982). The microtrabecular lattice may provide a framework for such physical arrangements to be made. In addition, in some cases, glycolytic enzymes require binding to actin for proper function (Bronstein and Knull, 1981), suggesting that these enzymes are readily poised to bind to the cellular microtrabecular lattice or contractile

apparatus (Clegg, 1984). Further investigation of the extrinsic protein stabilization of *Petrolisthes* LDHs may reveal new types or new modes of protein–protein interaction and stabilization.

Differences intrinsic to the LDH molecules may represent differences in primary structure or may represent post-translational modification through addition of phosphate, sugar or other groups to the LDH molecules. Size analysis by SDS–PAGE suggests that there is an approximately 1 kDa difference in apparent molecular mass between, for example, the LDHs of *P. cinctipes* and *P. edwardsii* (Fig. 7). This mass difference could represent the presence of approximately nine additional amino acid residues in the primary sequence of *P. edwardsii* (and *P. agassizii*). Differences of this magnitude among vertebrate LDHs have not been recorded (Holland et al., 1997). The linkage between this 1 kDa increase in mass and elevated thermal stability is not clear.

Analysis of the unfolding of secondary structural elements by CD spectroscopy suggests a concordance between thermal stability, as indexed by the loss of activity, and thermal stability, as indexed by the loss of  $\alpha$ -helix and  $\beta$ -sheet secondary structure (Fig. 8). Because heat inactivation of enzymatic activity may result from minor unfolding followed by aggregation and/or precipitation of protein (see discussion in Fields and Somero, 1998) rather than from large-scale unfolding, it is important to corroborate loss-of-activity measurements with physical measurements, as obtained with CD spectroscopy.  $T_m$  values from the CD thermal melts follow the same ranking as the  $T_{50}$  values determined using loss-of-activity assays: 62 °C versus 68.5 °C for *P. violaceus*, 72 °C versus 71 °C for *P. cinctipes* and 78 °C versus 73 °C for *P. galathinus*. These observations support the hypothesis that differences in thermal stability among *Petrolisthes* LDHs are related to intrinsic differences that have global influences on the structural properties of the molecules.

Further analysis of the intrinsic differences in LDH stability among species is hindered in the absence of information about the primary or three-dimensional structure of crustacean LDHs. The large interspecific differences in thermal stability may be determined by only a few mutations in primary structure, as has been seen in fish LDHs (Holland et al., 1997; Fields and Somero, 1997; Fields and Somero, 1998). Knowledge of the three-dimensional structure of crab LDHs and of the locations of specific differences in primary structure may reveal new mechanisms that enzymes use to adjust thermodynamic properties. For example, given the large difference in thermal stability between crustacean LDHs and fish LDHs, it would be interesting to see whether site-specific mutations shared any common characteristics, such as has been shown in genetically engineered protein variants with hugely increased thermal stabilities (Van Den Burg et al., 1998).

In summary, this study represents the first phylogenetically based comparative analysis of enzyme thermal stability and adaptation temperature. We have shown that the diversity of LDH thermal stability in a group of marine crustaceans is not related to an evolutionary adaptive response to maximal habitat temperature. Examination of the mechanistic bases of LDH



thermal stability in these species suggests that intrinsic properties of the LDH molecules and extrinsic stabilizing proteins may act in concert *in vivo* to regulate protein stability selectively.

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