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Temperature adaptation of cytosolic malate dehydrogenases of limpets (genus *Lottia*): differences in stability and function due to minor changes in sequence correlate with biogeographic and vertical distributions

Yunwei Dong* and George N. Somero†

Hopkins Marine Station, Department of Biology, Stanford University, Pacific Grove, CA 93950, USA

*Current address: The Key Laboratory of Mariculture, Ministry of Education, Fisheries College, Ocean University of China, Qingdao, People's Republic of China 266003

†Author for correspondence (e-mail: somero@stanford.edu)

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SUMMARY

We characterized functional and structural properties of cytoplasmic malate dehydrogenases (cMDHs) from six limpets of the genus *Lottia* that have different vertical and latitudinal distributions. Particular attention was given to the cryptic species pair *Lottia digitalis* (northern occurring) and *L. austrodigitalis* (southern occurring) because of recent contraction in the southern range of *L. digitalis* and a northward range extension of *L. austrodigitalis*. As an index of adaptation of function, we measured the effects of temperature on the apparent Michaelis–Menten constant (K_m) of the cofactor NADH (K_m^{NADH}). K_m^{NADH} values of cMDHs from the mid- to high-intertidal, low-latitude species *L. scabra* and *L. gigantea* were less sensitive to high temperature than those of cMDHs from the low- and mid-intertidal, high-latitude species *L. scutum* and *L. pelta*. cMDH of *L. digitalis* was more sensitive to high temperatures than the cMDH ortholog of *L. austrodigitalis*. Thermal stability (rate of loss of activity at 42.5°C) showed a similar pattern of interspecific variation. Comparison of the deduced amino acid sequences showed that interspecific differences ranged from one to as many as 17 residues. Differences in K_m^{NADH} and thermal stability between orthologs of *L. digitalis* and *L. austrodigitalis* result from a single amino acid substitution. At position 291, the glycine residue in cMDH of *L. digitalis* is replaced by a serine in cMDH of *L. austrodigitalis*, a change that favors additional hydrogen bonding and reduced conformational entropy. This difference between closely related congeners demonstrates the role of minor alterations in protein sequence in temperature adaptation and suggests that such variation is important in governing shifts in biogeographic range in response to climate change.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/2/169/DC1

Key words: thermal adaptation, climate change, cytosolic malate dehydrogenase, intertidal, limpet, *Lottiidae*.

INTRODUCTION

The molecular-level adaptations that contribute to the establishment and maintenance of biogeographic patterning are a long-standing interest of environmental physiologists. Temperature has been of principal interest in this context because of its pervasive effects on the functional and structural traits of organisms and the observation that biogeographic ranges often reflect discontinuities or sharp gradients in temperature (Hochachka and Somero, 2002). Recent analyses of biogeographic patterning in a wide variety of terrestrial and aquatic ecosystems have demonstrated that the biogeographic ranges of many species have shifted upwards in elevation or towards higher latitudes in response to warming (Hughes, 2000; Beaugrand et al., 2002; Helmuth et al., 2005; Parmesan, 2006). With this recognition of how strongly global warming is affecting species and communities, it has become increasingly imperative to elucidate mechanisms of temperature adaptation, in part to provide insight into the amount of adaptive genetic change that is needed to alter thermal optima and tolerance limits. This information may prove valuable in predicting how rapidly organisms will be able to adapt to rising temperatures.

Rocky intertidal ecosystems have provided especially good examples of climate change-related shifts in species composition. In these habitats, many species alternate between aquatic and terrestrial conditions and thereby experience wide variations in body temperature that depend on local conditions of weather, timing of the tides and location on the substrate (vertical position and orientation to solar radiation) (Helmuth et al., 2002; Tomanek and Helmuth, 2002; Gilman, 2006a). Along the Pacific Coast of North America, the interaction between climate and the timing of low tides creates a complex mosaic of thermal environments (Helmuth et al., 2006a). Intertidal invertebrates, especially congeneric species, occurring along such north–south running coastlines provide excellent study systems for analyzing the roles that adaptation to temperature, desiccation and other physical stresses play in setting biogeographic range boundaries (Gilman, 2006a; Gilman et al., 2006). In central California, sampling of intertidal invertebrates over a six decade period (1930s to 1990s) revealed an increase in abundance of southern species and a decrease of northern species, possibly due to the increase in the sea-surface temperature that occurred during this period (Barry et al., 1995). In southwest Britain and the western English Channel, similar shifts in the distribution of intertidal organisms were found, based on a 70 year observation period (Southward et al., 1995). Some intertidal species currently live close to their thermal tolerance limits and thus seem especially vulnerable to further increases in temperature (Stillman and Somero, 2000; Somero, 2002; Tomanek and Helmuth, 2002; Helmuth et al.,

Table 1. The distribution of six species of *Lottia* limpets along the west coast of North America

	Northern boundary	Southern boundary	References
1978			
L. digitalis	Aleutian Islands (52°N–55°N)	Point Conception (34°45′N)	Murphy, 1978; Morris et al., 1980
L. austrodigitalis	Monterey Peninsula (36°28′N)	Baja California Sur (22°N–28°N)	
1998			
L. digitalis	Aleutian Islands (52°N–55°N)	San Simeon (35°24′N)	Curmmett and Eernisse, 2007
L. austrodigitalis	Bodega Bay (38°19′N)	Baja California Sur (22°N–28°N)	
L. gigantea	Neah Bay (38°19′N)	Bahia de Tortuga (27°38′N)	Morris et al., 1980
L. scabra	Cape Arago (43°18′N)	Baja California Sur (22°N–28°N)	
L. pelta	Aleutian Islands (52°N–55°N)	Bahia del Rosario (29°45′N)	
L. scutum	Aleutian Islands (52°N–55°N)	Point Conception (34°45′N)	

2006b). Studies of the potential causes of these changes in distribution have centered on a number of physiological systems, including cardiac function, membrane composition and protein biochemistry (reviewed by Somero, 2002).

Here, we report on studies conducted with six species of intertidal limpets of the genus *Lottia*, a widespread and ecologically important group of intertidal invertebrates that has received relatively little physiological and biochemical study. Limpets are common and familiar inhabitants of rocky intertidal communities from tropical to polar regions (Brêthes et al., 1994; Williams and Morritt, 1995; Nakano and Ozawa, 2007) and occupy different intertidal zones and microhabitats (Shotwell, 1950; Haven, 1970; Wolcott, 1973). The limpets in the genus *Lottia* of the Pacific coast of North America provide an opportunity to study physiological adaptation to temperature among a group of closely related species that have different latitudinal ranges and, at a given site, distinct vertical ranges, from the low- to the high-intertidal zones (Table 1) (Morris et al., 1980; Gilman, 2006b; Sagarin et al., 2007). For example, *L. scabra* (Gould 1846), *L. gigantea* (Sowerby 1834), *L. austrodigitalis* (Murphy, 1978) and *L. digitalis* (Rathke 1833) inhabit the high-intertidal zone, whereas *L. pelta* (Rathke 1833) and *L. scutum* (Rathke 1833) are restricted to the low- to mid-intertidal zones. The different vertical distributions of these congeners expose them to different intensities of heat stress, which has led to adaptive variation in thermal tolerance. Wolcott (Wolcott, 1973) found that high-intertidal species (*L. scabra* and *L. digitalis*) could tolerate higher temperatures than low- and mid-intertidal species (*L. pelta* and *L. scutum*).

In the context of adaptations that influence latitudinal biogeographic ranges, the congeners *L. digitalis* and *L. austrodigitalis*, cryptic sibling species that are difficult to distinguish on morphological characters alone (Murphy, 1978), are of particular interest. *L. digitalis* was formerly thought to occur over a wide latitudinal range in the eastern Pacific, from the Aleutian Islands, Alaska, to Baja California Sur, Mexico (McLean, 1969; Morris et al., 1980). However, based on a geographic survey of protein polymorphism, Murphy (Murphy, 1978) found that the southern and northern populations of '*L. digitalis*' were in fact cryptic sibling species, and he named the southern population *Collisella austrodigitalis* (now *Lottia austrodigitalis*). In Murphy's survey done in the 1970s, *L. digitalis* ranged from the Aleutian Islands, Alaska, to Point Conception, California, and *L. austrodigitalis* ranged from Baja California Sur, Mexico to the Monterey Peninsula in Central California. In a 1998–1999 re-sampling of *L. digitalis* and *L. austrodigitalis* along the coast of California, a clear northern range expansion for *L. austrodigitalis* at the expense of *L. digitalis* was found (Crummett and Eernisse, 2007). There were no *L. austrodigitalis* present at two Monterey Bay sites, Santa Cruz and Pigeon Point, in 1977 (Murphy, 1978). In 1998–1999, relatively high percentages of *L. austrodigitalis* were found at these two sites (Crummett and Eernisse, 2007). Because the sea surface temperature in Monterey Bay increased significantly (yearly average increased by 0.79°C; summer maximum increased by 2.2°C) during the period 1920–1995 (Fields et al., 1993; Barry et al., 1995; Sagarin et al., 1999), the observed northward shift in range of *L. austrodigitalis* and contraction of the southern range limit of *L. digitalis* could reflect differences in thermal optima or tolerance limits between the two species.

To investigate temperature-adaptive differences among these six congeners of *Lottia* that might contribute to their vertical patterning and latitudinal distributions we focused on the enzyme cytosolic malate dehydrogenase (cMDH; EC 1.1.1.37, L-malate: NAD^+ oxidoreductase), which has previously been shown to exhibit adaptive variation related to temperature in different taxa of marine invertebrates (Dahlhoff and Somero, 1991; Dahlhoff and Somero, 1993; Fields et al., 2006). cMDH is widely distributed among organisms, and plays crucial roles in many metabolic pathways, including the tricarboxylic acid cycle, amino acid synthesis, gluconeogenesis, maintenance of oxidation/reduction balance and exchange of metabolites between the cytoplasm and subcellular organelles (Goward and Nicholls, 1994). The amino acid sequences, crystal structure and conformational changes occurring during catalysis of MDH have been studied extensively (Birktoft et al., 1982; Birktoft et al., 1989; Hall et al., 1992), which affords the opportunity to link substitutions in primary structure with adaptive variation in stability and function. We used the apparent Michaelis–Menten constant of the cofactor NADH (K_m^{NADH}) as an index of function, and resistance to denaturation at 42.5°C as an index of structural stability to explore adaptive variation among the six cMDH orthologs. We hypothesized that, for both traits, interspecific differences related to vertical and latitudinal distributions would be found. Furthermore, because of the close evolutionary relationships among certain of the congeners, deduced amino acid sequences would reveal how amino acid substitutions cause adaptive variation in function and stability. We discovered a pattern of adaptation in K_m^{NADH} and stability that reflects the congeners' distributions and show that in the case of the cMDH orthologs of *L. digitalis* and *L. austrodigitalis*, a single amino acid substitution is adequate to modify these two traits in an adaptive manner.

MATERIALS AND METHODS

Specimen collection

Lottia austrodigitalis, *L. scabra*, *L. scutum* and *L. pelta* were collected at Hopkins Marine Station (HMS), Pacific Grove, CA, USA (36°37′N, 121°54′W). *Lottia digitalis* was collected from

Bodega Marine Laboratory, Bodega Bay, CA, USA (38°09′N, 123°04′W). All limpets were acclimated to ~14°C for at least 14 days. During acclimation, limpets were immersed twice daily in ambient seawater (~14°C) for 6 h to simulate the natural high tide. Emersion (air) temperatures were 7–17°C during the April 2008 acclimation period. After acclimation, limpets were frozen in liquid nitrogen and stored at –70°C until used for enzyme studies.

Because it is difficult to distinguish between the cryptic congeners *L. austrodigitalis* and *L. digitalis* on the basis of morphological traits, a genetic method was used to distinguish the two species. Partial sequences of 16S mtDNA were amplified using specific primers 16sAr and 16sBr (Palumbi, 1996). The products were digested with the restriction enzyme *Hae* II (New England Biolabs, Ipswich, MA, USA). *Lottia digitalis* yields only the original uncut band (690 bp), and *L. austrodigitalis* yields two bands (171 and 520 bp).

Determination of kinetics of cMDH

Approximately 1 g of foot muscle was dissected from 1–10 individuals, depending on the body sizes of the limpets, and pooled. Tissue was homogenized in ice-cold potassium phosphate buffer (50 mmol l^{-1}, pH 6.8 at 4°C) as described by Fields and colleagues (Fields et al., 2006). The homogenate was centrifuged at 3000 ***g*** for 1 h and the supernatant was removed and heated at 51.0°C for 3 min. The mitochondrial isoform of MDH (mMDH) is more thermally labile than cMDH and could be fully eliminated by this heat treatment (supplementary material Fig. S1). The heated supernatant was brought to 40% saturation with solid ammonium sulfate. The sample was stirred at 4°C for 15 min, put on ice for 30 min, and then centrifuged at 18,000 ***g*** for 30 min. The supernatant was decanted and brought to 80% saturation with ammonium sulfate. The sample was stirred for a further 15 min, and after 30 min on ice it was centrifuged at 18,000 ***g*** for 30 min. The pellet was resuspended in 50 mmol l^{-1} potassium phosphate buffer (pH 6.8) and dialyzed against this buffer overnight at 4°C.

Apparent Michaelis–Menten constants of NADH (K_m^{NADH}) were determined at four temperatures (25°C, 30°C, 35°C and 40°C) for *L. scabra*, *L. gigantea*, *L. scutum* and *L. pelta*, and six temperatures (15°C, 20°C, 25°C, 30°C, 35°C and 40°C) for *L. digitalis* and *L. austrodigitalis*. Activity was determined using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) equipped with a temperature-controlled cell attached to a Lauda RM6 recirculating water bath (Brinkmann, Westbury, NY, USA). The temperature of the cuvette was maintained to within ±0.2°C. An imidazole chloride buffer (200 mmol l^{-1}, pH 7.0 at 20°C) was used to ensure that the pH levels in the experimental system corresponded to intracellular values over the range of measurement temperatures (Yancey and Somero, 1978; Hochachka and Somero, 2002). For each K_m^{NADH} determination, seven concentrations of NADH were used: 10, 15, 20, 30, 40, 60 and 75 μmol l^{-1}. The co-substrate oxaloacetic acid (OAA) was present at a starting concentration of 200 μmol l^{-1}. K_m^{NADH} values were calculated from initial velocity measurements at different [NADH] with Prism 5.0 software (Graphpad Software, San Diego, CA, USA) using a non-linear least-squares fit to the Michaelis–Menten equation.

Determination of thermal stabilities of cMDH

Thermal stabilities of cMDH were determined using enzyme prepared as described above. Enzyme denaturation and activity assays were carried out as described by Fields and colleagues (Fields et al., 2006). After overnight dialysis, enzymes of the six species were diluted to equivalent activity and incubated at 42.5°C. Samples of each enzyme were transferred to ice at t=0, 5, 10, 15, 20, 30, 45 and 60 min and activity at 25°C was determined in triplicate using a reaction mixture containing 200 mmol l^{-1} imidazole-HCl (pH 7.0 at 20°C), 150 μmol l^{-1} NADH and 200 μmol l^{-1} OAA. Residual activity was defined as the ratio between the mean activity at time t and the mean activity at time 0.

Sequencing of cMDH cDNA

Total RNA was purified from foot muscle of each of the six species using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase (RT) reactions were performed using Affinity Script^{TM} multiple temperature reverse transcriptase (Stratagene, La Jolla, CA, USA). PCR (94°C for 2 min, followed by 35 cycles of 94°C 30 s, 54°C 1 min, with a final 10 min extension at 72°C) was used to amplify partial sequences using a pair of primers (MDF1 and MDR1) based on the *cmdh* sequences of *Mytilus galloprovincialis* (DQ149970), *Crassostrea virginica* (CV089210) and *Nucella lapillus* (AF218065). The full-length cDNAs of *L. digitalis* and *L. scabra* were obtained using the rapid amplification of cDNA ends (RACE) protocol (Generacer, Invitrogen). 5′ and 3′ gene-specific primers (Br and Sf) were designed based on the partial sequences amplified above. PCR (94°C for 2 min, followed by 35 cycles of 94°C 30 s, 65–72°C 1 min, with a final 10 min extension at 72°C) was used to amplify the 5′ and 3′ ends of the cDNA. The sequences of primers used in this study are given in supplementary material Table S1.

Based on the 5′ and 3′ untranslated regions (UTRs) of *L. digitalis* and *L. scabra*, a pair of primers (0404MF1 and 0404MR2) were designed to amplify the full-length *cmdh* coding region. The full-length *cmdh* sequences for the six species were amplified using PCR (94°C for 3 min, followed by 35 cycles of 94°C 45 s, 50°C 45 s, 72°C 90 s, with a final 10 min extension at 72°C).

The PCR products were cleaned using exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, OH, USA), and then sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Products of the sequencing reaction were analyzed with an ABI 3100 DNA analyzer (Applied Biosystems).

The *cmdh* sequences were assembled using Sequencher software (GeneCodes, Ann Arbor, MI, USA). The deduced amino acid sequences were aligned using the CLUSTAL X algorithm (Thompson et al., 1997).

Molecular modeling

The three-dimensional structure of the cMDH monomer was constructed using the ternary complex of pig cMDH [PDB, 5mdhA (Chapman et al., 1999)] as a template with Swiss-Model software (Schwede et al., 2003). The sequence identity between 5mdhA and cMDH of *L. digitalis* is 69%. Hydrogen bonds and distances between atoms were computed with Swiss-PDB Viewer, and the three-dimensional structure was also visualized with Swiss-PDB Viewer (Guex and Pietsch, 1997). The verifications of protein structure were performed using WHATCHECK (Hooft et al., 1996) and PROCHECK (Laskowski et al., 1993) procedures. These two mathematical evaluations of the deduced three-dimensional structures yielded values included in the predicted range for homology-based models.

Statistics

Data were analyzed using an SPSS for Windows (version 11) statistical package (Chicago, IL, USA). Differences in K_m^{NADH} among species were analyzed using one-way ANOVA followed by a Duncan *post-hoc* multiple range test. The difference in K_m^{NADH}

between *L. digitalis* and *L. austrodigitalis* was analyzed using independent samples *t*-tests. To test for significant differences in the relationship of K_m^{NADH} *versus* temperature and temporal changes of residual enzymatic activity following incubation at 42.5°C among different species, the slopes of regressions were analyzed using an analysis of covariance (ANCOVA) followed by a least significant difference (LSD) multiple comparisons test (α=0.05).

RESULTS

Kinetics of cMDH orthologs

K_m^{NADH} values of cMDH orthologs for all congeners increased with increasing measurement temperature (Figs 1 and 2), but at the upper end of the range of measurement temperatures the orthologs of the more warm-adapted species showed lower rates of increase in K_m^{NADH} than those of more cold-adapted species. Thus, when temperature increased from 25°C to 40°C, the K_m^{NADH} values of cMDHs of *L. pelta* and *L. scutum*, two low- to mid-intertidal species whose biogeographic ranges extend to ~55°N latitude (Table 1), increased from 20.63 µmol l^{-1} to 30.69 µmol l^{-1} and from 18.37 µmol l^{-1} to 30.97 µmol l^{-1}, respectively. At 40°C, the K_m^{NADH} values of these two species were statistically higher than those of *L. gigantea* (27.86 µmol l^{-1}) and *L. scabra* (27.33 µmol l^{-1}) two high-intertidal species whose latitudinal ranges do not extend beyond ~48°N and ~43°N, respectively (one-way ANOVA followed by a Duncan *post-hoc* multiple range test: $F_{3,11}$=5.270, P=0.027; Fig. 1). The average slope of the relationship of K_m^{NADH} to temperature is shown by the Arrhenius plots given as insets in Fig. 1. The ANCOVA result shows that the slope of K_m^{NADH} *versus* temperature for the *L. pelta* ortholog is significantly higher than the slope for the *L. scabra* ortholog ($F_{4,16}$=36.863, P<0.001).

Relative to the ortholog of its cryptic sister species *L. digitalis*, the cMDH of *L. austrodigitalis* has a significantly smaller increase in K_m^{NADH} with increasing measurement temperature (independent samples *t*-tests: 35°C, t=0.024; 40°C, t=0.002). The slope of the Arrhenius plot (Fig. 2, inset) is significantly lower for the ortholog of *L. austrodigitalis* (ANCOVA, $F_{2,12}$=44.912, P<0.001).

Thermal stability of cMDH

Differences in thermal stability generally mirrored the pattern observed among the orthologs in the temperature sensitivity of K_m^{NADH} (Fig. 3). After 60 min incubation, orthologs of the two mid- to high-intertidal species from lower latitudes, *L. gigantea* and *L. scabra*, retained 71.0% and 44.8% of their original activities, respectively, whereas the residual activities of orthologs of the two low- to mid-intertidal species with biogeographic ranges extending into the Arctic, *L. pelta* and *L. scutum*, were only 29.1% and 19.8%, respectively (Fig. 3A). The slopes of the regressions of the two low- and mid-intertidal species' orthologs are significantly greater than those of the two mid- and high-intertidal species (ANCOVA, $F_{4,32}$=62.480, P<0.001).

L. austrodigitalis cMDH had a higher resistance to heat denaturation than the *L. digitalis* ortholog (Fig. 3B). After 60 min incubation, *L. austrodigitalis* possessed 54.7% of its original activity, compared with 33.7% for *L. digitalis*. The slope of the regression of residual activity *versus* temperature of cMDH from *L. digitalis* is significantly steeper than that of *L. austrodigitalis* (ANCOVA, $F_{2,16}$=99.337, P<0.001).

cmdh cDNA sequence and deduced amino acid sequence

An alignment of the 999 bp coding regions of *cmdh* cDNAs from *L. digitalis* (GenBank accession no. EU863452), *L. pelta* (EU863453), *L. austrodigitalis* (EU863454), *L. gigantea* (EU863455), *L. scutum* (EU863456) and *L. scabra* (EU863457) is presented in supplementary material Fig. S2. The sequences of the

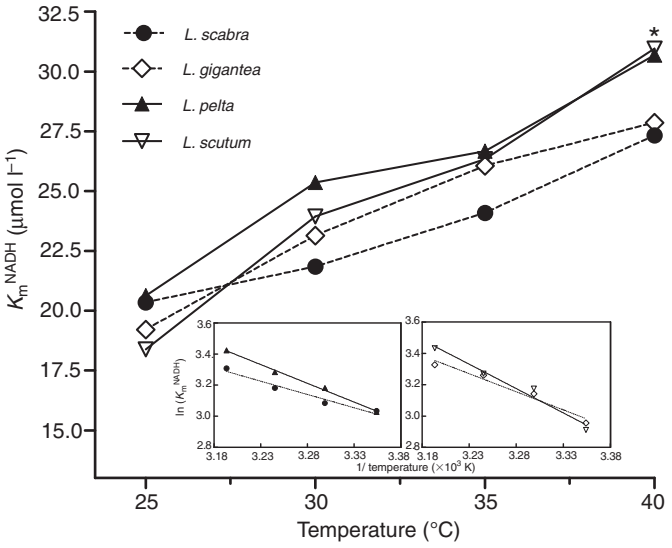


Fig. 1. Apparent Michaelis-Menten constant for the cofactor NADH (K_m^{NADH}) of cMDHs from foot muscle of *Lottia gigantea*, *L. scabra*, *L. pelta* and *L. scutum*. The asterisk indicates a significant difference in K_m^{NADH} between the two low- to mid-intertidal species (*L. pelta* and *L. scutum*) and the two mid- to high-intertidal species (*L. gigantea* and *L. scabra*; P<0.05). Inset: Arrhenius plot (ln K_m^{NADH} *versus* reciprocal temperature in Kelvins) of cMDH for *L. gigantea*, *L. scabra*, *L. pelta* and *L. scutum*. The slope of the relationship for cMDH of *L. pelta* was significantly steeper than that for *L. scabra* (P<0.001; ANCOVA). Statistics (means ± s.d.) for the K_m^{NADH} determinations are given in supplementary material Table 2.

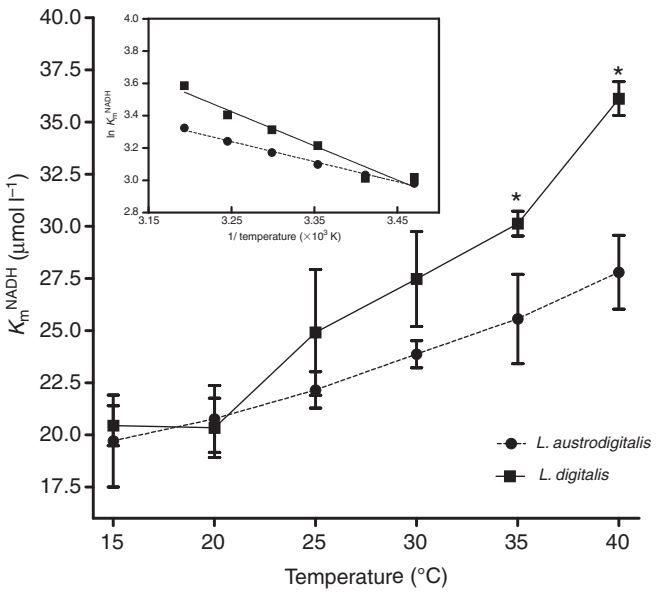


Fig. 2. Michaelis–Menten constant for cofactor NADH (K_m^{NADH}) of cMDHs from foot muscle of *L. digitalis* and *L. austrodigitalis*. Asterisks indicate the temperatures at which K_m values were significantly different between species (α=0.05). Inset: Arrhenius plot (ln K_m^{NADH} *versus* reciprocal temperature in Kelvins) of cMDHs of *L. digitalis* and *L. austrodigitalis*. The slope of the relationship for cMDH of *L. digitalis* is significantly steeper than that for *L. austrodigitalis* (P<0.0001; ANCOVA).

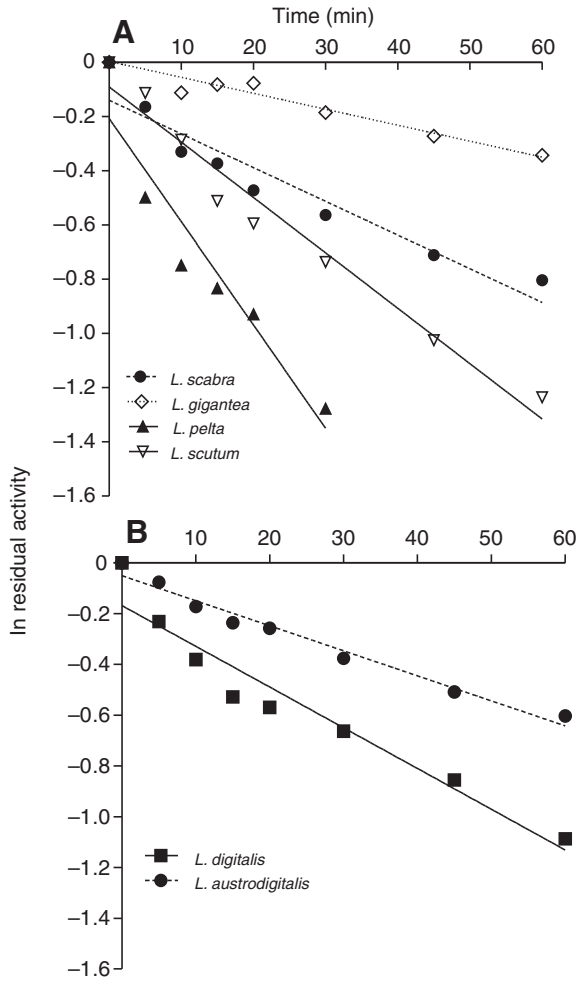


Fig. 3. Residual activities of cMDHs from *Lottia* limpets after heat treatment at 42.5°C for different times. (A) Residual activities of *L. gigantea*, *L. scabra*, *L. pelta* and *L. scutum*. The slopes of the regressions for cMDHs of *L. pelta* and *L. scutum* are significantly higher than those of the orthologs of *L. gigantea* and *L. scabra* (P<0.001). (B) Residual activities of cMDHs of *L. digitalis* and *L. austrodigitalis*. The denaturation rate of cMDH for *L. digitalis* is significantly higher than that of the *L. austrodigitalis* ortholog (P<0.001; ANCOVA).

six *Lottia cmdh*s share high identity. The coding regions of genes from the two most closely related species, *L. digitalis* and *L. austrodigitalis*, differ at only three sites, yielding an identity of 99.70%. The genes from the two most distantly related species, *L. gigantea* and *L. scabra*, differ at 107 sites, yielding an identity of 89.29% (Table 2).

The amino acid sequences of the six cMDH orthologs were deduced from the nucleotide sequences (Fig. 4). Amino acid sequence identity is higher in the nucleotide binding domain (approximated by the N-terminal half of the subunit) (Birktoft et al., 1989) than in the catalytic domain (approximated by the C-terminal half of the subunit); 16 of 24 variable sites in the primary structure are found in the latter region. The majority of amino acid substitutions occur in helices αC′, α1F, α2F, α1G and αH, and in some of the β-strands (βL and βM) in the catalytic domain. Several highly conserved regions were found, including 11 of the 12 β-strands (βA, βB, βC, βD, βE, βF, βG, βH, βJ, βK and βL), the catalytic loop (residues 92–112), and two helices, α2G and α3G (Fig. 4).

Percentage identities between each possible pair of orthologs are shown in Table 2. There is one amino acid substitution between *L. digitalis* and *L. austrodigitalis* (G291S), two between *L. digitalis* and *L. pelta* (A72C and I320T), eight between *L. digitalis* and *L. scutum*, 14 between *L. digitalis* and *L. gigantea*, and 17 between *L. digitalis* and *L. scabra*. Closeness in sequence identity as indexed by the number of substitutions between species is not correlated with similarities in kinetic properties (K_m^{NADH}; Figs 1 and 2) or stability (Fig. 3). For example, orthologs of *L. digitalis*, *L. austrodigitalis* and *L. pelta* have a close relationship based on primary structure (Fig. 4), yet the kinetics and stabilities of the orthologs are substantially different.

Molecular modeling

Based on structural information available for pig heart cMDH (5mdhA; ternary complex), three-dimensional models of the cMDH subunit were constructed for *L. digitalis* (Fig. 5A) and *L. austrodigitalis* (Fig. 5B). The single amino acid substitution differentiating the two orthologs occurs near the C-terminal end (residue 291) of a β-strand, βL (Figs 4 and 5). At this site, *L. austrodigitalis* has a serine and *L. digitalis* has a glycine. Based on structural analyses using Swiss-Model software, the larger side-chain of serine changes the structure of the C-terminal region of *L. austrodigitalis* cMDH relative to that of its congener's ortholog. In *L. digitalis*, βL extends from Leu-280 to Gln-289, whereas in the ortholog of *L. austrodigitalis* βL extents to Ser-291. Furthermore, in the cMDH of *L. austrodigitalis* Ser-291 can form two additional hydrogen bonds with other amino acid residues (Fig. 6). One of them is between O Ser-291 and N Arg-294, and the other is between N Ser-291 and O Val-295. These two hydrogen bonds reduce the distances separating other nearby amino acids, allowing two more hydrogen bonds to form (Table 3). One is between N Thr-261 and O Ile-290, and the other is between OD1 Asp-293 and O Arg-294. In the ortholog of *L. digitalis*, the long distances between potentially hydrogen-bonding atoms at these two locations, 5.63 and 8.49 Å, respectively, make it unlikely that they can contribute to stabilization of the structure. The same caveat applies to the potential bond between Thr-292 and Asp-293 in *L. austrodigitalis* (Table 3). Thus, in this region of the enzyme's three-dimensional structure, the ortholog of *L. austrodigitalis* is likely to have five hydrogen bonds and that of *L. digitalis* only two.

Table 2. Percentage identity of protein and cDNA sequences of cMDHs for *Lottia* limpets

	Percentage identity – protein					
Percentage identity – cDNA	1	2	3	4	5	6
(1) *L. digitalis*	–	99.70	99.40	97.60	94.89	95.80
(2) *L. austrodigitalis*	99.70	–	99.10	97.30	94.89	95.50
(3) *L. pelta*	98.60	98.50	–	97.00	94.89	95.50
(4) *L. scutum*	95.30	95.20	95.10	–	94.89	95.50
(5) *L scabra*	92.99	92.89	92.39	91.99	–	95.80
(6) *L. gigantea*	92.19	91.89	91.59	91.69	89.29	–

```
                βA          αB                 βB           αC               βC    αC′      βD
l.d.     MSEPVKVLVTGAAGQIAYSLLYSIAKGDVFGEKQPISLVLLDIEPMMAVLDGVVMELQDCALPLLADVIPTADESAAFKDIDVALLVGAQPRRQGMERKD [100]
l.a.     .................................................................................................... [100]
l.p.     .......................................................................C............................ [100]
l.scu.   ..........................................................................AV........................ [100]
l.g.     ...............................D..........................................A......................... [100]
l.sca.   .......................................................................SE.AI.................E...... [100]
                  αDE              βE         α1F            βF          α2F               βG               βH
l.d.     LLKANVKIFKSQGAALDAHAKKTVKVVVVGNPANTNALVIAQFAPSIPKENFSCLTRLDQNRAQAQVASRLGISNENVQRTIIWGNHSSTQFPDLAHAVV [200]
l.a.     .................................................................................................... [200]
l.p.     .................................................................................................... [200]
l.scu.   ....................................................................TK.............................. [200]
l.g.     ........................................SK...........................K.............................. [200]
l.sca.   ........................................SR..........................NK.............................. [200]
                βJ αG′       α1G           α2G            α3G                βK               βL          βM
l.d.     HVNGKLMPAQEAIKDDDWVKNDFIKTVQTRGGAVIQARKLSSAMSAAKAICDHVRDWWFGTGERWVSMGIISKGDYGIKEGLMYSFPVQIGTDRVVKVVP [300]
l.a.     ..........................................................................................S......... [300]
l.p.     .................................................................................................... [300]
l.scu.   ................N...DE..........................................................................Q... [300]
l.g.     ................N.............................................................QQ.............K..Q... [300]
l.sca.   ................N..................K..........................................S...........A......... [300]
                         αH
l.d.     GLTISNFAREKMDATQAELIEEKDCAFSFLEA [332]
l.a.     ................................ [332]
l.p.     ...................T..........X. [332]
l.scu.   ................................ [332]
l.g.     .....D.............V..R....L.... [332]
l.sca.   .....D.......V.....V..R......... [332]
```

Fig. 4. Deduced amino acid sequence alignment of cMDHs from *L. digitalis*, *L. austrodigitalis*, *L. pelta*, *L. scutum*, *L. gigantea* and *L. scabra*. Above each segment of sequence is the designation for secondary structure in the cMDH of *L. digitalis*, which was calculated using the Swiss-Model software based on the pig cMDH [PDB, 5mdhA (Chapman et al., 1999)]. The cofactor binding residues, the residues functioning in catalysis and the residues involved in subunit–subunit interactions are shown in blue, red and green, respectively. The single substitution between *L. digitalis* and *L. austrodigitalis* (position 291) is shown in bold.

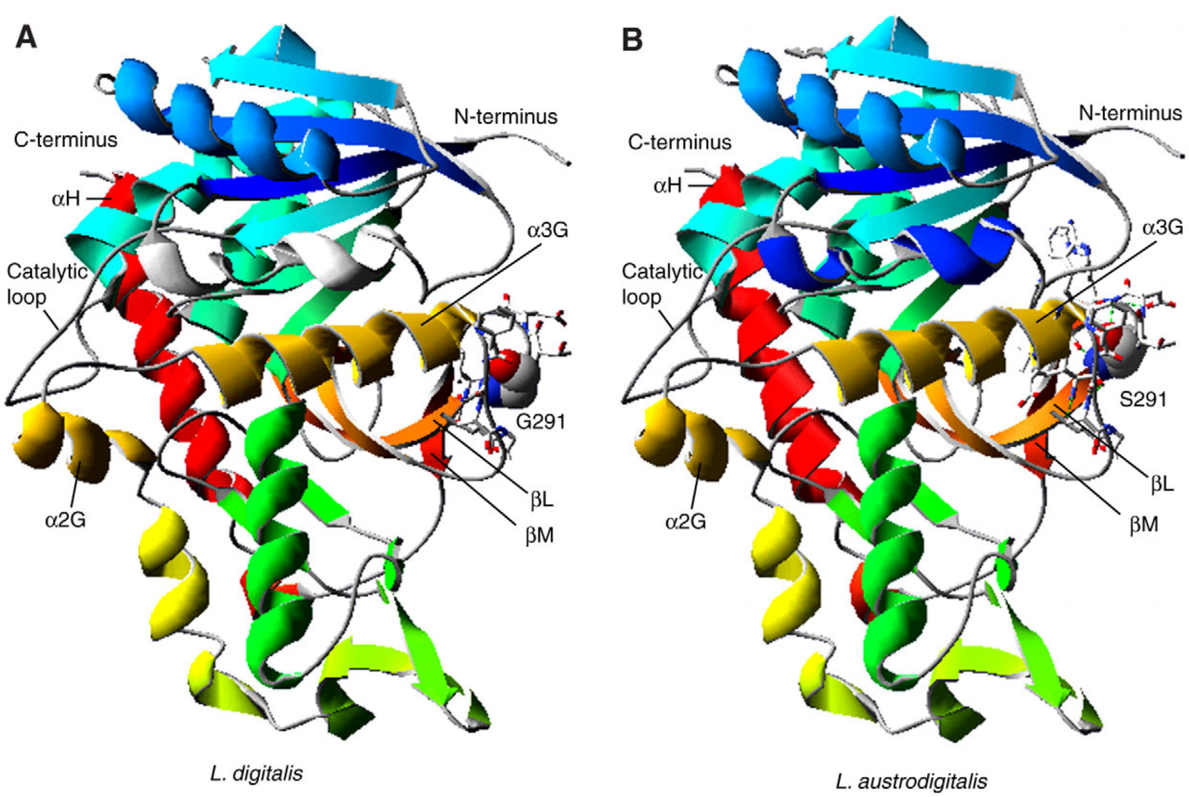
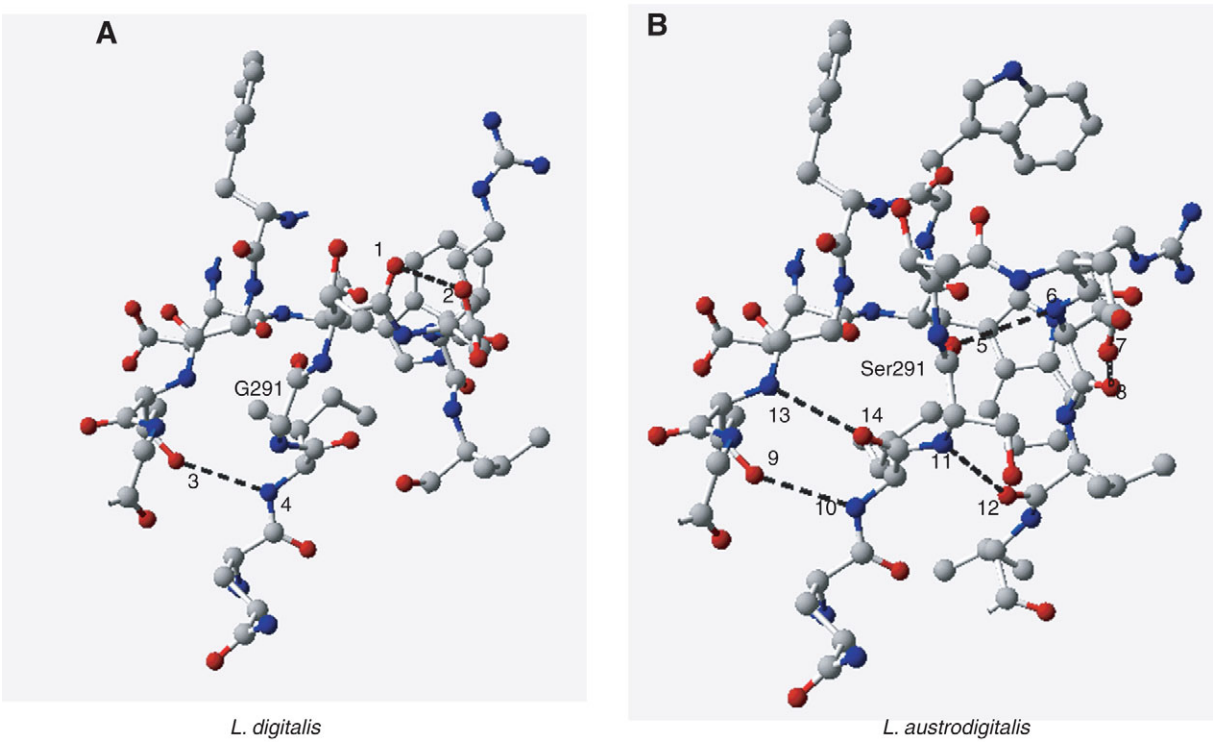


Fig. 5. Three-dimensional model of a single cMDH monomer from (A) *L. digitalis* and (B) *L. austrodigitalis*. The positions of Gly-291/Ser-291 and amino acids within 4 Å of Gly-291/Ser-291 are indicated. Van der Waals radii are indicated for the atoms comprising Gly-291/Ser-291. The position of βL is also indicated. These models were produced using Swiss-Model software visualized with the Swiss-PDB Viewer (Guex and Peitsch, 1997).



L. digitalis *L. austrodigitalis*

Fig. 6. A magnified view of the region near residue 291 (Gly/Ser) showing amino acids within 6 Å of Gly-291 (Ser-291) of cMDHs from (A) *L. digitalis* and (B) *L. austrodigitalis*. Hydrogen bonds were calculated using Swiss-PDB Viewer (Guex and Peitsch, 1997), and are indicated as dashed lines. Atoms that form hydrogen bonds in the ortholog from *L. digitalis* are marked as: 1, Thr-292 O; 2, Asp-293 OD1; 3, Thr-261 OG1; 4, Ile-290 N. Atoms that form hydrogen bonds in the ortholog from *L. austrodigitalis* are marked as: 5, Ser-291 O; 6, Arg-294 N; 7, Asp-293 OD1; 8, Arg-294 O; 9, Thr-261 OG1; 10, Ile-290 N; 11, Ser-291 N; 12, Val-295 O; 13, Thr-261 N; and 14, Ile-290 O.

DISCUSSION

The differences observed in the values and thermal sensitivities of K_m^{NADH} and in the resistance to denaturation of the six cMDH orthologs mirror the thermal conditions encountered by the six *Lottia* congeners as a consequence of their different latitudinal and vertical distributions (Table 1). Numerous studies of the effects of measurement temperature on K_m values of orthologous enzymes have shown that orthologs of warm-adapted species have lower K_m values, corresponding to higher substrate and cofactor affinity, at a common temperature of measurement than orthologs of cold-adapted species (reviewed by Hochachka and Somero, 2002). These intrinsic differences in substrate and cofactor affinity, which appear to be based on differences in structural rigidity among orthologs (Fields and Somero, 1998), allow a high degree of conservation of K_m values at typical body temperatures. Another common pattern observed in comparative studies of thermal sensitivity of K_m is a more rapid increase in K_m with rising temperature for cold-adapted orthologs, especially at the higher end of the range of measurement temperatures where unfolding of structure may be initiated in the more thermally labile orthologs of cold-adapted species.

Table 3. Hydrogen bonds between neighboring amino acids within 6 Å of Gly-291 (Ser-291) in *Lottia digitalis* and *L. austrodigitalis*

	Distance between related amino acids (Å)	
Hydrogen bonds	*L. austrodigitalis*	*L. digitalis*
O Ser-291...N Arg-294	3.16	–
N Ser-291...O Val-295	2.92	–
N Thr-261...O Ile-290	3.27	(5.63)
OD1 Asp-293...O Arg-294	3.32	(8.49)
OG1 Thr-261...N Ile-290	3.14	3.19
O Thr-292...OD1 Asp-293	(5.31)	2.65

The hydrogen bonds and distances between atoms were calculated using Swiss-PDB Viewer (Guex and Peitsch, 1997).
Data in parentheses are for distances between related atoms that were greater than the hydrogen bond detection threshold.

By these criteria, the cMDHs of the six *Lottia* congeners reflect adaptation to the different thermal conditions they encounter as a result of their latitudinal and vertical distributions. Among the three species that occur at highest latitude (~55°N), the mid- to high-intertidal species *L. digitalis* has the highest K_m^{NADH} values at all measurement temperatures (Figs 1 and 2) and thus appears to be the most cold-adapted species. The extensive periods of emersion it encounters relative to its low- to mid-intertidal congeners, *L. pelta* and *L. scutum*, may favor selection for function at the near-freezing temperatures that would be encountered over a substantial fraction of its biogeographic range. Orthologs of the mid- to high-intertidal species that occur at lower latitudes, *L. austrodigitalis*, *L. scabra* and *L. gigantea*, have K_m^{NADH} values that are intrinsically lower and less perturbed by increasing temperature at the upper ranges than the ortholog of *L. digitalis* (Figs 1 and 2).

This pattern of interspecific variation in K_m^{NADH} related to latitude and vertical position resembles the relationships found previously (Dahlhoff and Somero, 1993) in our study of congeners of *Haliotis* (abalone). Elevated temperatures perturbed the K_m^{NADH} for cMDHs of the two abalones living at higher latitudes or lower tidal heights to a much greater extent than for cMDH homologs of species from

lower latitudes or higher tidal heights. Similarly, a comparison of cMDHs of blue mussel congeners with different latitudinal distributions revealed higher K_m^{NADH} values for the ortholog of a northern-occurring species, *M. trossulus*, relative to that of a more warm-adapted congener, *M. galloprovincialis* (Fields et al., 2006).

The interspecific differences in thermal stability of the six cMDH orthologs show a patterning similar to that found for the thermal relationships of K_m^{NADH}. Residual activities of cMDHs from the most warm-adapted mid- to high-intertidal species, *L. gigantea*, *L. scabra* and *L. austrodigitalis*, are higher than those of orthologs of *L. scutum*, *L. pelta* and *L. digitalis* (Fig. 3). Although artifacts can occur in studies of thermal stability that use crude supernatant fractions like those used in this study (see Fields and Somero, 1998), the consistent trend noted in comparisons of congeners of *Lottia* argues for a strong, adaptation temperature-related difference in structural stability.

Two other studies present data that substantiate our conclusions regarding thermal adaptation in the congeners of *Lottia*. Wolcott (Wolcott, 1973) reported significant differences in thermal tolerance (LT_{50}) related to vertical position. Thermal tolerance of *L. scabra* was greater than that of the other mid- to high-intertidal species studied, *L. digitalis*; tolerance of *L. pelta* and *L. scutum* was lower than that of the two mid- to high-intertidal congeners. Following Wolcott's protocol, we determined the LT_{50} values of *L. digitalis* and *L. austrodigitalis*, which were 39.5–40.7°C and 40.5–41.7°C (95% confidence limits), respectively.

In a recent study (Dong et al., 2008), we reported differences in the synthesis of heat-shock protein 70 (Hsp70) in four congeners of *Lottia*. Two mid- to high-intertidal congeners, *L. scabra* and *L. austrodigitalis*, had higher constitutive levels of Hsp70 than two mid- to low-intertidal species, *L. pelta* and *L. scutum*, a pattern interpreted to indicate an elevated capacity to deal with sudden and unpredictable heat stress in the higher occurring congeners.

The differences found between *L. digitalis* and *L. austrodigitalis* in thermal tolerance and cMDH properties integrate well with recent observations of the range shifts of these two species. Crummett and Eernisse (Crummett and Eernisse, 2007) reported that *L. austrodigitalis* showed a clear northern range expansion at the expense of *L. digitalis* from 1977 to 1998 (Table 1). In the present study, among 231 individuals that were sampled from the intertidal rocky shore around HMS, 89% were *L. austrodigitalis* and 11% were *L. digitalis*. All 120 individuals collected at Bodega Bay were *L. digitalis*. The recent shift in distribution of these two limpets may be a reflection of the warming trend observed in coastal Central and Northern California (Barry et al., 1995; Nemani et al., 2001). At HMS, the mean near-shore water temperature has risen on average by 0.79°C over the past ~85 years, and peak summer water temperatures have risen by about 2.2°C over this period (Barry et al., 1995). Nemani and colleagues (Nemani et al., 2001) found that in the past 50 years mean air temperatures near the northern California coast have increased by more than 1.1°C.

The close relatedness of the cMDH sequences of certain of the congeners of *Lottia* (Table 2; Fig. 4) facilitates an interpretation of the differences in structural stability and cofactor affinity in terms of protein primary structure. The single difference in amino acid sequence between orthologs of *L. digitalis* and *L. austrodigitalis* at position 291 in the sequence enables additional hydrogen bonds to form in the warm-adapted species' subunit (Table 3). These additional hydrogen bonds, the lengthening of βL and the likely reduction in conformational entropy that results from replacing the glycine residue, which allows the greatest freedom of rotation around a peptide bond of any amino acid, with a serine residue provides the increased stabilization energy manifested in the reduced rate of denaturation at 42.5°C (Fig. 3).

In terms of how this single amino acid change might affect function, the three-dimensional structure of cMDH suggests that the substitution at site 291 could affect the mobility of regions of the molecule important in catalytic activity. This substitution (G291S) occurs near the C-terminus of βL. This β-strand, along with another highly variable β-strand, βM (Fig. 4), forms part of β-sheet III, which is important in catalytic conformational changes (Birktoft et al., 1989).

The potential adaptive significance of the variations in sequence between more distantly related cMDHs is harder to predict. Most variations in amino acid sequence in cMDH orthologs of *Lottia* congeners occur at residues which are at least partly exposed to solvent on the exterior of one subunit of the dimer of cMDH. As shown in Fig. 4, the highly variable regions of cMDH include most helices (αC′, α1F, α2F, α1G and αH) and two β-strands (βL and βM). The crystal structure of pig cMDH shows that, in the isolated cMDH monomer, all of the helices have at least one side exposed to the solvent on the exterior of the subunit (Birktoft et al., 1989). Upon dimerization, the helices αB, αC, α2F, α2C and α3G, most of which are less variable than the other helices in the subunit, have their solvent exposure reduced by subunit–subunit interactions. The lower variation in sequence in the helices involved in subunit–subunit interactions is a pattern of conservation one would anticipate because of the need for complementarity in structure between these interacting surfaces. The higher variation of solvent-exposed helices not involved in subunit–subunit interactions might be a reflection of selectively unimportant structural differences.

In the context of evolution and biogeographic range determinants, the key point in the structural analysis given above is that a single amino acid substitution within the 332 residue sequence can be sufficient to modify adaptively both K_m^{NADH} and protein stability. The finding that temperature-adaptive modifications of cMDH (this study) (Fields et al., 2006) and of a closely related enzyme, lactate dehydrogenase-A (Holland et al., 1997; Fields and Houseman, 2004; Johns and Somero, 2004), may be achieved by such minimal alterations in sequence may assist the development of predictive models that seek to estimate the rates at which adaptive change in proteins in the context of adapting to global warming might occur. From the current, limited database on orthologous proteins from differently thermally adapted species, one can tentatively conclude that many, and perhaps most, proteins must undergo sequence changes to adapt to a shift in ambient temperature, but the amount of evolutionary change in structure needed to effect adaptation in function and stability is small – less than 1% of the structure in the case of dehydrogenases. Furthermore, available data suggest two other important conclusions. The first is that these adaptive changes entail a common outcome, the alteration in mobility of regions of the protein involved in catalysis. The second is that there are many sites in the protein sequence at which alterations in conformational mobility can be induced by one or a few amino acid substitutions. Further exploration of the adaptive importance of substitutions in highly variable regions of the sequence that may influence conformational mobility may provide deeper insights into the rates at which adaptive change in proteins can occur, an important and unresolved issue in the analysis of climate change.

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