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Temperature sensitivities of cytosolic malate dehydrogenases from native and invasive species of marine mussels (genus *Mytilus*): sequence–function linkages and correlations with biogeographic distribution

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Summary

The blue mussel *Mytilus galloprovincialis*, a native of the Mediterranean Sea, has invaded the west coast of North America in the past century, displacing the native blue mussel, *Mytilus trossulus*, from most of its former habitats in central and southern California. The invasive success of *M. galloprovincialis* is conjectured to be due, in part, to physiological adaptations that enable it to outperform *M. trossulus* at high temperatures. We have examined the structure and function of the enzyme cytosolic malate dehydrogenase (cMDH) from these species, as well as from the more distantly related ribbed mussel, *Mytilus californianus*, to characterize the effects of temperature on kinetic properties thought to exhibit thermal adaptation. The *M. trossulus* cMDH ortholog differs from the other cMDHs in a direction consistent with cold adaptation, as evidenced by a higher and more temperature-sensitive Michaelis–Menten constant for the cofactor NADH (K_m^{NADH}). This difference results from minor changes in sequence: the *M. trossulus* ortholog differs from the *M. galloprovincialis* ortholog by only two substitutions in the 334 amino acid monomer, and the *M. californianus* and *M. trossulus* orthologs differ by five substitutions. In each case, only one of these substitutions is non-conservative. To test the effects of individual substitutions on kinetic properties, we used site-directed mutagenesis to create recombinant cMDHs. Recombinant wild-type *M. trossulus* cMDH (rWT) has high K_m^{NADH} compared with mutants incorporating the non-conservative substitutions found in *M. californianus* and *M. galloprovincialis* – V114H and V114N, respectively – demonstrating that these mutations are responsible for the differences found in substrate affinity. Turnover number (k_{cat}) is also higher in rWT compared with the two mutants, consistent with cold adaptation in the *M. trossulus* ortholog. Conversely, rWT and V114H appear more thermostable than V114N. Based on a comparison of K_m^{NADH} and k_{cat} values among the orthologs, we propose that immersion temperatures are of greater selective importance in adapting kinetic properties than the more extreme temperatures that occur during emersion. The relative warm adaptation of *M. galloprovincialis* cMDH may be one of a suite of physiological characters that enhance the competitive ability of this invasive species in warm habitats.

Key words: cytosolic malate dehydrogenase, invasive species, *Mytilus*, temperature adaptation, site-directed mutagenesis.

Introduction

Coastal marine ecosystems are highly vulnerable to anthropogenically introduced invasive species, in part because movement of organisms between diverse and widely separated habitats can occur rapidly. High rates of translocation, most notably *via* the ballast water of ocean-going ships (Carlton and Geller, 1993), allow potential invaders repeated opportunities to colonize coastal habitats. Indeed, the result of such continual introductions over the past century has been a rapid increase in the number of successful colonizations by coastal and estuarine species (Cohen and Carlton, 1998). Clearly, however, not all species introduced into novel habitats colonize successfully, and a number of generalizations have been put forward to explain why certain invaders are able to compete successfully against aboriginal species (Kolar and Lodge, 2001; Lodge, 1993). These include high dispersal rates, phenotypic plasticity and pre-existing physiological suitability for the invaded habitat. Here, we explore whether biochemical adaptation to temperature potentially may play a role in invasion success, by comparing temperature sensitivity of the enzyme cytosolic malate dehydrogenase (cMDH) from three mussel congeners. Two of these mussels, *Mytilus trossulus* and *M. californianus*, are native to the west coast of North America and the third, *M. galloprovincialis*, is a species introduced there from the Mediterranean Sea.

Mytilus trossulus and *M. galloprovincialis* are members of

the 'blue mussel' complex, along with *M. edulis*, a species native to the North Atlantic Ocean (McDonald and Koehn, 1988). All three blue mussels are morphologically highly similar and occupy a range of intertidal and estuarine habitats. In the northern hemisphere, *M. trossulus* is found in the northern Pacific, where it originated, and in eastern Canada and the Baltic Sea. *Mytilus galloprovincialis* is native to the Mediterranean Sea and the eastern Atlantic north to the British Isles. Where distributions of blue mussels overlap, hybridization is common (Varvio et al., 1988; McDonald and Koehn, 1988). *Mytilus trossulus* appears to be the ancestral species of the blue mussel complex, with initial speciation into *M. edulis* occurring after 3.5 million years ago, when a transient opening of the Bering Strait allowed migrations of marine species along a corridor to the north of North America, predominantly from the Pacific to the Atlantic (Vermeij, 1993; Seed, 1992). Further speciation led to the separation of *M. galloprovincialis* from *M. edulis* after ~2 million years ago (Seed, 1992). Thus, in their native ranges, present-day *M. trossulus* and *M. galloprovincialis* occupy habitats with differing physicochemical attributes. In the Pacific, *M. trossulus* originally was found in bays, estuaries and rocky intertidal habitats from Baja California, Mexico, north along the west coast of North America to Alaska, and west to Japan (Suchanek et al., 1997). Within much of this range, individuals tended to be exposed to cooler water and more variable and lower salinity than individuals of *M. galloprovincialis*, which occupy the warmer and more saline waters of the Mediterranean. When *M. galloprovincialis* was introduced to southern California sometime in the first few decades of the last century (the site and date of entry are not clear, due to the extreme morphological similarity of the two species), it was able to out-compete and completely displace the native *M. trossulus* throughout the southern portion of its range, from Baja California north to the present-day *M. galloprovincialis–M. trossulus* hybrid zone, which extends from approximately Monterey Bay, California to Cape Mendocino, California (Rawson et al., 1999). North of the hybrid zone, *M. trossulus* dominates, and in most localities remains the sole blue mussel (Suchanek et al., 1997).

The presence of *M. galloprovincialis* in southern California and its success in displacing *M. trossulus* was only discovered in the 1980s, first through allozyme analysis (McDonald and Koehn, 1988) and later through mitochondrial DNA haplotyping (Geller et al., 1994). Based on the present distribution of *Mytilus* congeners in California and Japan, a number of authors have suggested that differences in temperature sensitivity may explain the successful invasion of *M. galloprovincialis* into southern California and the subsequent range partitioning of *M. galloprovincialis* to the south and *M. trossulus* to the north (Rawson et al., 1999; Suchanek et al., 1997; Gardner, 1994; Sarver and Foltz, 1993). Physiological studies support this conjecture. Hofmann and Somero (1996a) found differences between these congeners in induction temperatures of heat-shock protein synthesis, consistent with *M. trossulus* being the more cold-adapted species. Recently, Braby (2004) found differences in temperature-induced mortality and the effects of temperature on heart rate that are consistent with *M. galloprovincialis* being adapted to higher temperatures than *M. trossulus*.

To extend the analysis of temperature adaptation in blue mussels to the biochemical and molecular level, we have examined differences in temperature sensitivity in the enzyme cytosolic malate dehydrogenase (cMDH; EC 1.1.1.37, malate:NAD$^+$ oxidoreductase) in both species, as well as in the more distantly related ribbed mussel *M. californianus*. Cytosolic MDH – a dimeric enzyme distinct from the mitochondrial form central to the citric acid cycle – plays a role in a number of metabolic pathways, including the malate–aspartate (or NADH) shuttle, the acetate shuttle active in lipogenesis, amino acid synthesis and gluconeogenesis. We chose to examine cMDH in part because previous studies have shown molluscan cMDHs to exhibit distinct patterns of temperature adaptation in kinetic properties (Dahlhoff and Somero, 1991, 1993). In addition, the structure of pig cMDH has been determined through x-ray crystallography (Birktoft et al., 1989), which has helped to elucidate its catalytic cycle and to provide an interpretative framework to use in deducing how amino acid substitutions might affect function and stability.

We addressed the following questions: (1) are there differences in the effects of temperature on kinetic properties of cMDH orthologs of blue mussels that can be correlated with their different habitat temperatures; (2) how much divergence in amino acid sequence has occurred in these cMDH orthologs, which in the case of *M. trossulus* and *M. galloprovincialis* are separated by only ~3.5 million years and (3) can we identify amino acid substitutions responsible for any changes in function of cMDH that we may find? To answer these questions, we determined the amino acid sequence of cMDH orthologs from the three *Mytilus* congeners and aligned them to determine what changes had occurred to the enzyme during the speciation of these taxa. From these data, we created mutants of *M. trossulus* cMDH, substituting amino acids found in the orthologs of *M. galloprovincialis* and *M. californianus.* Finally, we measured kinetic parameters – Michaelis–Menten constants for the cofactor NADH (K_m^{NADH}), Arrhenius activation energies (E_a), turnover numbers (k_{cat}) and thermal stabilities – shown to be temperature sensitive in previous studies (Dahlhoff and Somero, 1993; Fields and Somero, 1997; Fields and Somero, 1998; Fields, 2001; Hochachka and Somero, 2002). We found that a single amino acid substitution is sufficient to explain the changes in kinetics of *M. galloprovincialis* and *M. californianus* cMDHs relative to the *M. trossulus* form. From these results, we argue that the cMDHs of *M. trossulus* and *M. galloprovincialis* provide an example of how adaptation at the biochemical level, as one part of a broad suite of adaptations, has the potential to facilitate successful colonization and competition by invasive marine ectotherms.

Materials and methods

Specimen collection

Mytilus trossulus Gould were collected from dock pilings in Yaquina Bay, Newport, OR, USA (44°38′ N, 124°03′ W) in July 2004 (Yaquina Bay is well north of the northern border of the *M. trossulus*–*M. galloprovincialis* hybrid zone). *Mytilus galloprovincialis* Lamarck were collected from Mission Bay, San Diego, CA, USA (32°46′ N, 117°14′ W; well south of the southern border of the hybrid zone) in October 2004. *Mytilus californianus* Conrad were collected from mussel beds in the exposed rocky intertidal zone at Hopkins Marine Station, Pacific Grove, CA, USA (36°37′ N, 121°54′ W) in July 2004. Individuals of *M. trossulus* and *M. galloprovincialis* were transported live to Hopkins Marine Station wrapped loosely in net bags, plastic and newspaper in insulated boxes. All individuals were held at 14°C in aquaria with no emersion and were fed diluted shellfish diet (Reed Mariculture, Campbell, CA, USA) as per manufacturer's instructions for a minimum of three weeks before sacrifice.

Purification of cytosolic malate dehydrogenase

Cytosolic MDH was purified from muscle tissue of *Mytilus* spp. following the protocol of Dahlhoff and Somero (1993). Approximately 1 g of tissue (mantle and adductor muscle) was excised from two to five individuals, depending on size, and pooled. Tissue was homogenized in five volumes of ice-cold potassium phosphate buffer (50 mmol l^{-1}, pH 6.8 at 4°C) with a rotor-stator homogenizer (Ultra-Turrax T8; IKA Works, Staufen, Germany) in three 10 s bursts at maximum speed; the sample was kept on ice for 1 min between each burst. Homogenate was centrifuged at 24 000 ***g*** for 30 min, and the supernatant was brought to 45% saturation with ammonium sulfate. After 30 min on ice, the sample was centrifuged at 24 000 ***g*** for 30 min, and the supernatant was decanted and brought to 80% saturation with ammonium sulfate. After a further 30 min on ice, the sample was centrifuged again at 24 000 ***g*** for 30 min, the supernatant was discarded, and the pellet was resuspended in 10 ml potassium phosphate buffer. Approximately 80% of the original MDH activity was found in the resuspended pellet.

The sample was dialyzed against TEB buffer [20 mmol l^{-1} Tris-HCl (pH 8.2 at 5°C), 10 μmol l^{-1} EDTA, 10 μmol l^{-1} 2-mercaptoethanol] overnight at 4°C. After dialysis, the sample was applied to a Matrex Red A dye affinity column (Millipore, Billerica, MA, USA) and washed with 1 l TEB plus 20 mmol l^{-1} KCl. The column was then washed sequentially with 100 ml TEB plus 20 mmol l^{-1} malate (pH 8.2 at 5°C); 100 ml TEB plus 20 mmol l^{-1} KCl; 100 ml TEB plus 1 mmol l^{-1} NADH; and 100 ml TEB plus 20 mmol l^{-1} KCl. The cMDH was eluted with a continuous gradient of TEB and TEB plus 100 mmol l^{-1} malate, 1 mmol l^{-1} NADH. Fractions were monitored for MDH activity, and active fractions (which generally eluted between 50 mmol l^{-1} malate, 0.5 mmol l^{-1} NADH and 90 mmol l^{-1} malate, 0.9 mmol l^{-1} NADH) were pooled, then concentrated and desalted against TEB using a Centriprep-50 centrifugal concentrator (Millipore).

Sequencing of cMDH

Total RNA was purified from hepatopancreas of a single individual from each species, using a phenol/chloroform extraction (Trizol reagent; Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. To obtain full-length sequences of the *cmdh* coding region, the purified total RNA was used as template for a Rapid Amplification of cDNA Ends (RACE) protocol (GeneRacer; Invitrogen), which inserts oligonucleotides of known sequence on the 5′ and 3′ ends of mRNA prior to reverse transcription. The resulting RACE cDNA was then used as template to amplify cMDH, employing 5′ or 3′ RACE primers provided in the GeneRacer kit and gene-specific primers designed by homology with *Crassostrea virginica* (eastern oyster) and *Nucella lapillus* (dogwhelk) (Kirby, 2000) cMDHs (accession numbers CV089210 and AF218065, respectively). Sequences of the gene-specific primers successfully used for *Mytilus cmdh* amplification, as well as other primers used in this study, are available from the corresponding author upon request.

Polymerase chain reaction (94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 56–64°C for 45 s and 72°C for 1–2.5 min, with a final 10 min extension at 72°C) was used to amplify *cmdh* cDNA, and products were examined using 1.2% agarose–ethidium bromide gels. Products showing a single band of the expected size were cleaned with exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, OH, USA) for 30 min at 37°C, followed by inactivation at 80°C for 15 min. Sequencing was performed using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Products of the sequencing reaction were run on an ABI 3700 automated DNA analyzer (Applied Biosystems), and contigs based on the resultant electropherograms were assembled using Sequencher software (GeneCodes Corp., Ann Arbor, MI, USA).

Site-directed mutagenesis of M. trossulus cMDH

Site-directed mutagenesis was performed on *M. trossulus* cMDH to examine the effects of the individual amino acid substitutions V114N and V114H on its kinetics. These mutations were chosen because they represent the only non-conservative changes between *M. trossulus* cMDH and either the *M. galloprovincialis* (V114N) or the *M. californianus* (V114H) ortholog. A full-length cDNA clone of *M. trossulus* cDNA was constructed with a *Bam*HI restriction site immediately 5′ to the start codon and an *Eco*RI site immediately 3′ to the stop codon. The gene was inserted into the pTrcHis vector (Invitrogen) using standard molecular biology techniques, creating a construct in which the expressed protein has a poly-His tag attached to the N-terminus of the cMDH monomer to facilitate purification.

After site-directed mutagenesis (QuikChange; Stratagene, La Jolla, CA, USA), pTrcHis vector containing either the *M. trossulus cmdh* insert (recombinant wild-type, rWT), mutant V114H or V114N was transformed into TOP10 chemically competent cells (Invitrogen) and plated on LB-agar containing 80 μg ml^{-1} ampicillin. Plasmids were purified from individual

colonies grown in 5 ml liquid LB/ampicillin (QiaQuick plasmid mini-prep; Qiagen, Valencia, CA, USA).

Upon confirmation of the presence of the desired mutation by automated DNA sequencing, 125 ml of LB/ampicillin was inoculated and grown to log phase. Isopropylthio-B-D-galactoside (IPTG) was added to a final concentration of 1 mmol l^{-1}, and cells were allowed to express protein overnight. To purify the polyHis-tagged recombinant cMDH, cells were pelleted at 5000 ***g*** for 10 min, and the pellet was resuspended in 5 ml CellLytic B (Sigma, St Louis, MO, USA) per gram wet cell paste. Extraction was performed according to the manufacturer's instructions, and the final supernatant was filtered (0.45 μm). The supernatant was then passed over a 1 ml TALON cobalt His-tag affinity column (BD Biosciences, San Jose, CA, USA), which binds the polyHis-tag attached to the N-terminus of the recombinant cMDH and allows rapid purification. After the TALON column was washed (25 ml of 50 mmol l^{-1} potassium phosphate, pH 7.0, 300 mmol l^{-1} NaCl), the purified cMDH was eluted with 3.5 ml wash buffer plus 150 mmol l^{-1} imidazole. Fractions with highest MDH activity were pooled and desalted into storage buffer (50 mmol l^{-1} potassium phosphate, pH 6.8) using PD-10 desalting columns (Amersham Biosciences, Piscataway, NJ, USA).

Measurement of cMDH K_m^{NADH} and E_a

Michaelis constants of NADH (K_m^{NADH}) and maximal velocities (V_{max}) were determined at five temperatures – 5, 15, 25, 35 and 45°C – for the three tissue-purified cMDH orthologs, as well as rWT *M. trossulus* cMDH and the mutants V114N and V114H. Substrate saturation curves were generated at each temperature using a reaction cocktail containing 80 mmol l^{-1} imidazole-Cl (pH 7.2 at 15°C), 150 μmol l^{-1} oxaloacetic acid (OAA) and 7.5–75 μmol l^{-1} NADH (Dahlhoff and Somero, 1993). Imidazole was used as the buffer for these assays because its pH–temperature relationship is similar to that of cytosol, and it maintains the appropriate protonation state of histidyl residues (alpha-stat regulation) (Reeves, 1977; Yancey and Somero, 1978), thereby ensuring physiologically realistic enzyme function across a broad range of temperatures.

Michaelis constants were calculated from initial velocity measurements at different [NADH] using Wilman4 software (Brooks and Suelter, 1986), which employs a nonlinear weighting scheme for V_{max} and K_m calculation (Wilkinson, 1961). Activation energies (E_a) were calculated from V_{max} data at different temperatures using the Arrhenius equation:

$$V_{max} = A\ e^{(-Ea/RT)}\ , \quad (1)$$

where A is a constant dependent on the reaction under consideration, and $-E_a$ is proportional to the slope of the line relating $\log_e (V_{max})$ to the reciprocal of absolute temperature. Before calculating E_a, data were standardized by converting V_{max} values for each ortholog to 1.00 at 0°C and correcting values at higher temperatures by the appropriate conversion factor for each ortholog. This standardization removes differences in elevation of the Arrhenius regression lines, which has no effect on E_a, and allows differences in slope to be seen more easily.

Measurement of rWT and mutant cMDH k_{cat} values

Turnover numbers (k_{cat} values) were determined for rWT *M. trossulus* cMDH, as well as the mutants V114H and V114N, using the equation:

$$k_{cat} = V_{max}\ /\ [E]\ , \quad (2)$$

where [E] is enzyme concentration. Because the tissue-purified cMDHs did not attain a sufficient level of purity according to denaturing polyacrylamide gel electrophoresis (SDS-PAGE), [E] could not be obtained, and k_{cat} values were not calculated for these enzymes. Purity of each recombinant enzyme was confirmed by SDS-PAGE, followed by silver staining (data not shown). Enzyme concentrations of the recombinant proteins were determined using a modified Bradford protein assay (Pierce Biochemicals, Rockford, IL, USA). The enzyme molecular masses used to calculate molarity included the N-terminal poly-histidine leader that was added to each recombinant protein.

Thermal denaturation profiles

Thermal denaturation assays were used to determine the effects of mutations V114H and V114N on the structural stability of rWT *M. trossulus* cMDH. After purification, each recombinant enzyme was 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 tested in triplicate for activity. Residual activity was defined as the ratio between the mean activity at time t and the mean activity at time 0.

Statistics

Assays for K_m^{NADH}, k_{cat}, E_a and thermal denaturation were run in triplicate, and results are reported as means ± s.d. To determine whether K_m^{NADH} or k_{cat} values of the enzymes were significantly different at each temperature tested, we used a one-way analysis of variance, followed by a Student–Newman–Keuls multiple comparisons test with α=0.05. To test for significant differences in rate of heat denaturation and in the calculation of E_a, we compared slopes using an analysis of covariance (aoctool; MATLAB; The Mathworks, Inc., Natick, MA, USA), followed by a Tukey–Kramer multiple comparisons test (α=0.05).

Molecular modeling

We visualized the mutations at residue 114 within the three-dimensional structure of the cMDH monomer by creating a homology model using pig apo-cMDH (PDB accession number 4MDH) (Birktoft et al., 1989) as a template onto which the primary structure of *M. trossulus* cMDH was threaded. SwissModel software (Schwede et al., 2003; Guex and Pietsch, 1997) was used to create the model, and mutations were modeled using SwissPDBViewer (Guex and Pietsch, 1997). The resulting structures were visualized with SwissPDBViewer and VMD software (Humphrey et al., 1996).

Results

cmdh nucleotide and deduced amino acid sequences

An alignment of the 1002 bp coding region of *cmdh* cDNA from *M. trossulus* (GenBank accession no. DQ149969), *M. galloprovincialis* (DQ149970) and *M. californianus* (DQ149971) is given in Fig. 1. The genes from the two most closely related species, *M. trossulus* and *M. galloprovincialis*, differ at 41 sites, yielding an identity of 95.9%. The more distantly related *M. californianus* differs from *M. trossulus* at 62 sites and from *M. galloprovincialis* at 53 sites (93.8% and 94.7% identities, respectively). A number of ambiguities occur in the sequences, as described in the figure legend. These ambiguities are caused by double peaks in the electropherograms produced by the automated sequencer, which suggests allelic variation at these sites. In all instances, however, the ambiguous bases result in synonymous codons and so have no effect on the amino acid sequences.

The presence of potential allelic variation in the individuals we sampled suggests that there may be greater variation in cMDHs within each of the populations. A broader survey, especially including multiple individuals from each species at different latitudes and across the hybrid zone, might reveal cMDH alleles coding for differing amino acid sequences. This area of enquiry may prove fruitful in future studies.

Amino acid sequences of each cMDH ortholog were deduced from the nucleotide sequences given above, and an alignment of these amino acid sequences is shown in Fig. 2. There are five amino acid substitutions between *M. trossulus* and *M. californianus* (R66K, V114H, S118T, S206T and D222E), four between *M. galloprovincialis* and *M. californianus* (R66K, N114H, S206T and D222E) and two between the closely related *M. trossulus* and *M. galloprovincialis* (V114N and S118T). All but one of the amino acid differences among the orthologs are conservative, maintaining charge (arginine to lysine at position 66, and aspartic acid to glutamic acid at position 222) or polarity (serine to threonine at positions 118 and 206). The single non-conservative mutation occurs at position 114, where *M.*

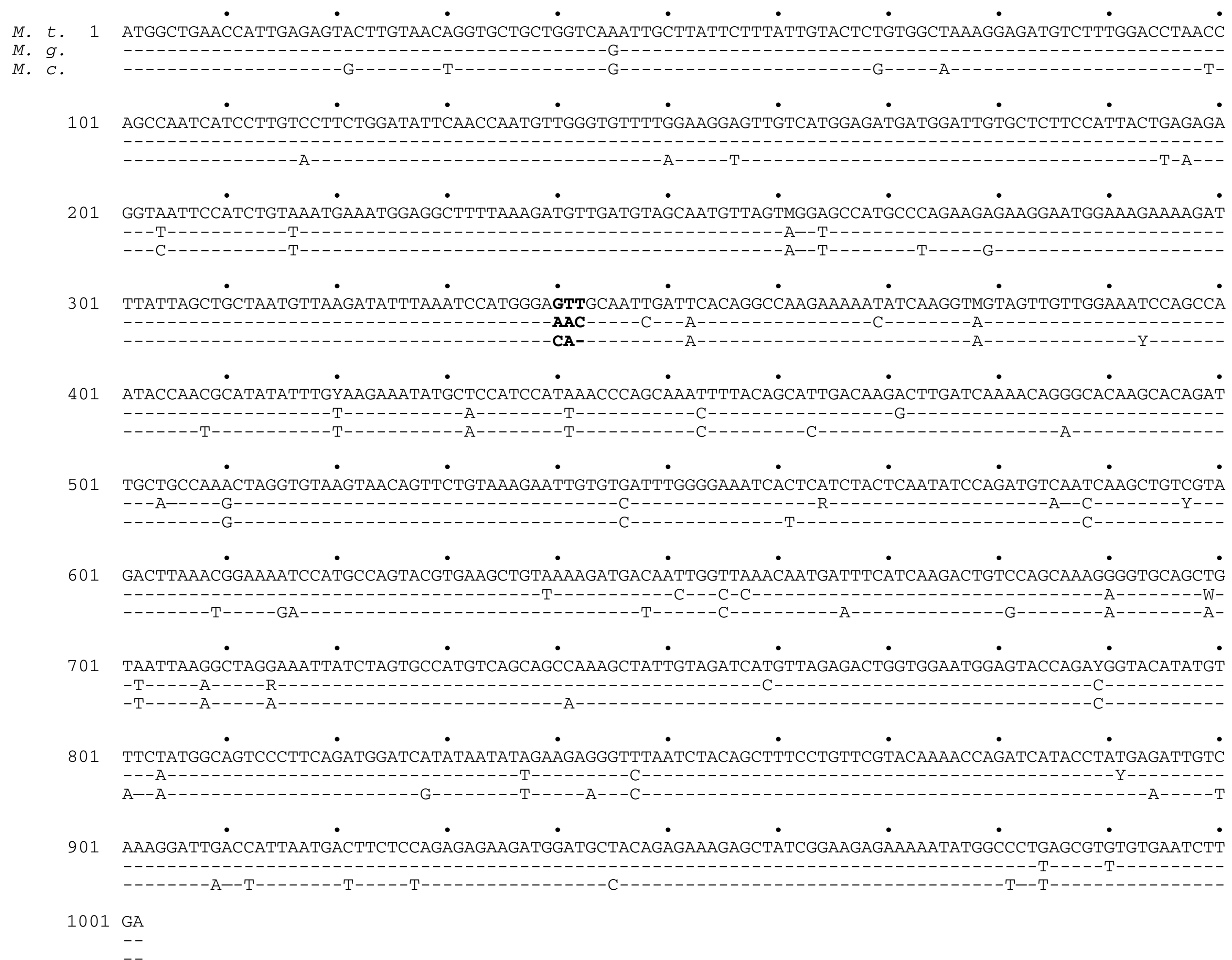

Fig. 1. Nucleotide sequences of the coding regions of *cmdh* cDNAs from *Mytilus trossulus* (*M. t.*), *M. galloprovincialis* (*M. g.*) and *M. californianus* (*M. c.*). Dashes represent identity with the *M. trossulus* sequence. The codons for the non-conservative substitutions at residue 114 in each ortholog are highlighted. In a number of positions, nucleotide ambiguities (i.e. double peaks) were noted in the source electropherograms: M=A or C; R=A or G; Y=C or T; W=A or T; all ambiguities are synonymous in the deduced amino acid sequence.

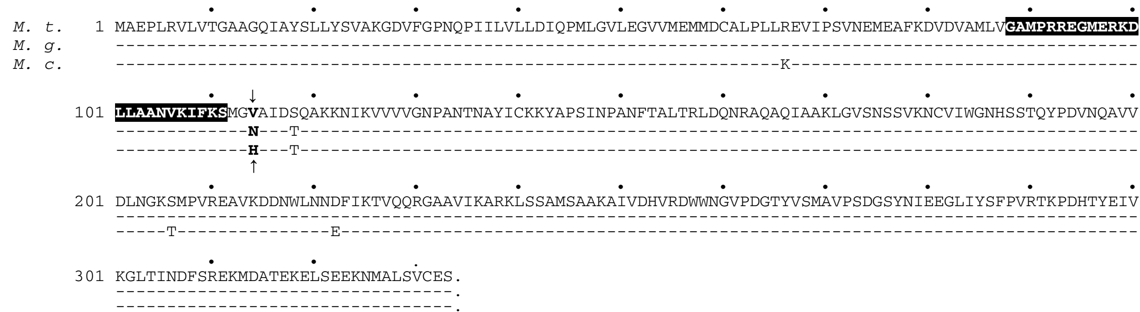

Fig. 2. Amino acid sequences of cMDHs from *Mytilus trossulus* (*M. t.*), *M. galloprovincialis* (*M. g.*) and *M. californianus* (*M. c.*), deduced from the nucleotide sequences given in Fig. 1. Dashes represent identity with the *M. trossulus* sequence. The single non-conservative substitution at position 114 between *M. trossulus* and the other two congeners is indicated by the arrows. The catalytic loop region of cMDH (see Discussion) is highlighted in black.

trossulus cMDH has a non-polar valine, *M. galloprovincialis* cMDH has a polar asparagine, and *M. californianus* cMDH has a charged histidine. It is remarkable that residues with such different properties occur at the same site in the three cMDH orthologs; these substitutions are based on mutations in three consecutive nucleotides between the *M. trossulus* and *M. galloprovincialis cmdh* genes (GTT to AAC at positions 340–342 in Fig. 1) and two consecutive mutations between the *M. trossulus* and *M. californianus* genes (GTT to CAT). The cluster of mutations within this codon is especially notable because of the high level of identity in the remainder of the coding sequence – the average number of substitutions per base varies from 0.041 (41/1002) between the *M. trossulus* and *M. galloprovincialis cmdh* genes to 0.062 (62/1002) between the *M. trossulus* and *M. californianus cmdh* genes. In addition, the three consecutive mutations responsible for the substitution at position 114 between *M. galloprovincialis* and *M. trossulus* indicate strongly that this substitution did not occur recently; that is, it is unlikely that the substitution arose in the California population of *M. galloprovincialis* subsequent to its introduction in the first half of the twentieth century.

K_m^{NADH} and E_a of cMDH orthologs and mutants

Fig. 3A shows K_m^{NADH} values measured from 5 to 45°C for cMDHs purified from muscle tissue of *M. californianus, M. trossulus* and *M. galloprovincialis*. Although K_m^{NADH} values are similar among the orthologs at lower temperatures, ranging from 8.5 μmol l^{-1} NADH for *M. californianus* cMDH to 12.4 μmol l^{-1} for the *M. trossulus* ortholog at 5°C, statistically significant differences in K_m^{NADH} occur at higher temperatures. It is especially notable that the K_m^{NADH} values for *M. trossulus* cMDH are significantly higher than those for the *M. galloprovincialis* ortholog from 15 to 35°C, because these two members of the blue mussel species complex are so closely related and their orthologs differ by only two amino acid substitutions. At 45°C, the K_m^{NADH} values are no longer significantly different, but this is because of the large errors associated with the measurements at this high and potentially denaturing temperature (see below).

Fig. 3B shows K_m^{NADH} data for rWT *M. trossulus* cMDH, as well as the two mutants of that enzyme we created based on the non-conservative mutations noted between the species, V114N (corresponding to *M. galloprovincialis* cMDH) and V114H (corresponding to the *M. californianus* ortholog). At all temperatures, measured rWT *M. trossulus* cMDH K_m^{NADH} values are significantly higher than those of V114N. In addition, V114H has significantly higher K_m^{NADH} values than V114N at all temperatures higher than 5°C. This pattern, when compared with that shown in Fig. 3A, suggests that the non-conservative mutations at residue 114 are sufficient to cause the differences in temperature sensitivity of K_m^{NADH} we have noted among the three orthologs. This result is further supported by the data in Fig. 3C, which compare K_m^{NADH} values measured at 35°C among the six enzymes we tested. These results illustrate first that expression in *E. coli* does not affect K_m^{NADH} of *M. trossulus* cMDH (i.e. the K_m^{NADH} of rWT at 35°C, 38.85±3.39 μmol l^{-1} NADH, is not significantly different from that of the tissue-purified enzyme, 42.33±4.47 μmol l^{-1} NADH) and second that the mutations V114H and V114N modify the K_m^{NADH} of *M. trossulus* cMDH to values not different from those measured in the *M. californianus* and *M. galloprovincialis* orthologs, respectively.

Arrhenius plots of V_{max} for each of the *Mytilus* cMDH orthologs and recombinant enzymes are shown in Fig. 4. Arrhenius activation energies were determined from these plots using the relationship E_a=–slope/R, where R is the universal gas constant. According to the Arrhenius equation, the lines relating $\log_e V_{max}$ to the reciprocal of absolute temperature should be linear (i.e. the activity of the enzymes should increase exponentially); this is true for all enzymes tested here except mutant V114N, which showed a decrease in activity at 45°C (see Fig. 4). Data at this temperature for V114N therefore were excluded from E_a calculations. An analysis of covariance (α=0.05) performed on the six slopes indicated that none of the slopes were significantly different from the mean. We therefore cannot state that the E_a value of any of the six enzymes is significantly different from the others but, given the magnitude of variation associated with these measurements (95% confidence intervals of the slopes ranged

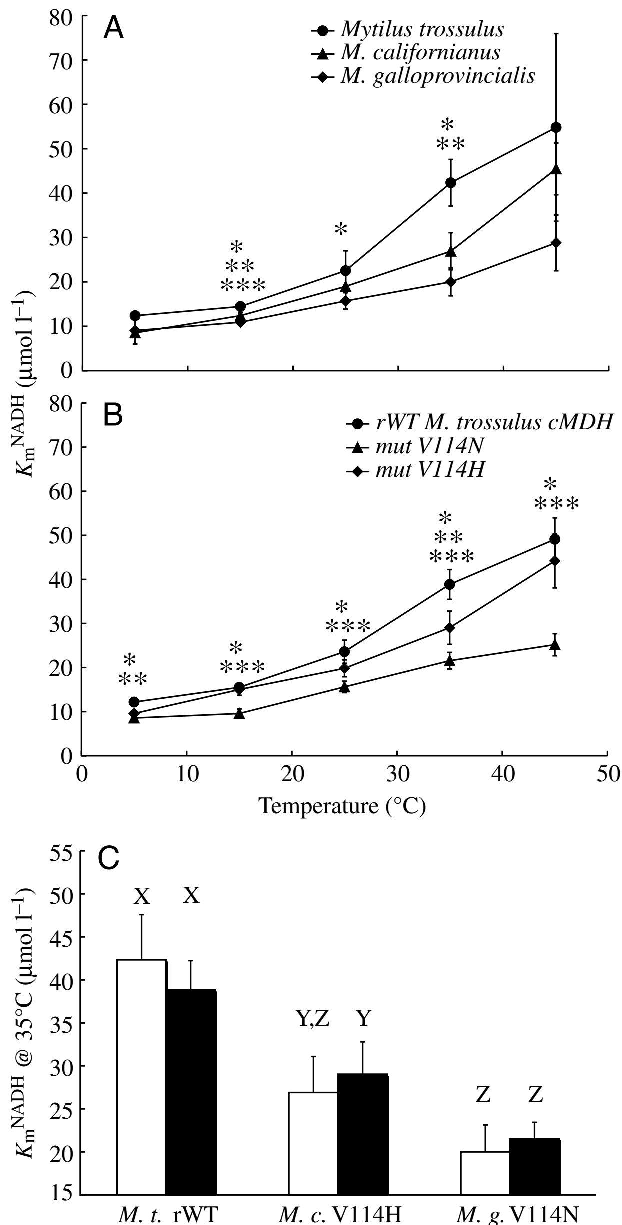

Fig. 3. Michaelis–Menten constants for the cofactor NADH of cMDHs either purified from muscle tissue or expressed recombinantly. (A) K_m^{NADH} values for cMDHs purified from muscle tissue of *M. trossulus*, *M. galloprovincialis* and *M. californianus*, measured from 5 to 45°C [* indicates a significant difference (α=0.05) between *M. trossulus* and *M. galloprovincialis* cMDHs; ** between *M. trossulus* and *M. californianus*; and *** between *M. californianus* and *M. galloprovincialis*]. (B) K_m^{NADH} values of recombinant *M. trossulus* cMDH (rWT) and mutants of *M. trossulus* cMDH replacing the residue at position 114 with that found in *M. galloprovincialis* (V114N) or *M. californianus* (V114H), measured from 5 to 45°C [* indicates a significant difference (α=0.05) between rWT and V114N cMDHs, ** between rWT and V114H; and *** between V114H and V114N]. (C) A comparison of K_m^{NADH} values measured at 35°C between *M. trossulus* (*M. t.*) cMDH and rWT, *M. galloprovincialis* (*M. g.*) cMDH and mutant V114N, and *M. californianus* (*M. c.*) cMDH and mutant V114H. K_m^{NADH} values sharing a letter designation (X,Y,Z) are not significantly different (α=0.05); all other comparisons are significantly different.

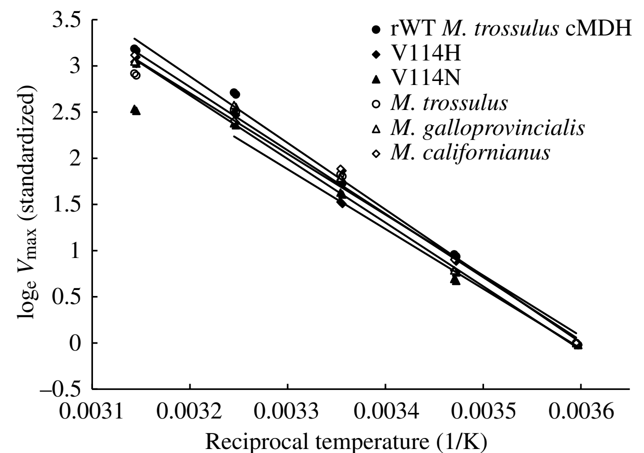

Fig. 4. Arrhenius activation energies (E_a) of *M. trossulus, M. galloprovincialis* and *M. californianus* cMDHs, as well as rWT *M. trossulus* cMDH, mutants V114H and V114N. V_{max} values were standardized to 1.00 at 0°C before $\log_e$-transformation, to aid in visualizing differences among the slopes. E_a is proportional to the negative slope of the regression relating $\log_e V_{max}$ to the reciprocal of absolute temperature. An analysis of covariance indicates that none of the E_a values are significantly different (α=0.05).

from 2.6 to 3.7% of the E_a values), it is possible that small but thermodynamically significant differences in E_a do exist.

k_{cat} *values of recombinant cMDHs*

Turnover numbers were calculated for rWT *M. trossulus* cMDH and mutants V114H and V114N from 5 to 35°C and are shown in Fig. 5. At all temperatures, the k_{cat} values of the three recombinant enzymes are significantly different from one another, except those for rWT and V114H (*M. californianus*) at 5°C. As with K_m^{NADH} values, rWT has the highest and most temperature-sensitive k_{cat} values, mutant V114H is intermediate and V114N has the lowest values. The heavy lines in Fig. 5 highlight the k_{cat} values of rWT and V114N within the common immersion temperature ranges of *M. trossulus* (~5–15°C) and *M. galloprovincialis* (~13–25°C), respectively. The k_{cat} values are highly similar within these ranges, suggesting that the single mutation at position 114 results in temperature compensation of catalytic rate in these orthologs.

Thermal denaturation profiles

Fig. 6 shows the loss of activity in rWT *M. trossulus* cMDH and the mutants V114N and V114H due to thermal denaturation at 42.5°C. The highly linear regressions of the semi-log plots (r^2=0.991, 0.987 and 0.993 for rWT, V114N and V114H, respectively) indicate that the denaturation process follows first-order kinetics. The slopes of the regressions of rWT and V114H are not significantly different (α=0.05), but the slope of the V114N regression is significantly steeper than those of the other two forms, i.e. V114N loses activity at a

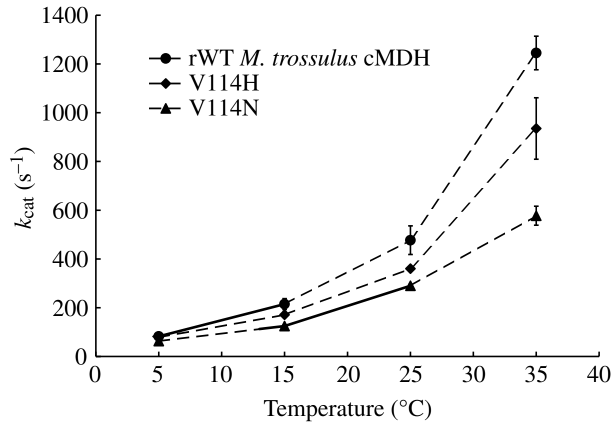

Fig. 5. Turnover numbers (k_{cat}) of rWT *M. trossulus* cMDH and the mutants V114N and V114H from 5 to 35°C. Mutations V114N and V114H significantly reduce the catalytic rate relative to rWT at all temperatures except 5°C for V114H. Solid bars indicate the physiological immersion temperature ranges of *M. trossulus* and *M. galloprovincialis*, where rWT and V114N exhibit similar catalytic rates.

significantly higher rate. Calculated from the regression equations, the half-lives of the enzymes at 42.5°C are 20.9 min for rWT, 19.8 min for V114H and 14.2 min for V114N. Given the relatively temperature-insensitive K_m^{NADH} values (Fig. 3B) and low k_{cat} (Fig. 5) of the V114N mutant, both of which suggest increased molecular stability, it is interesting to note that this enzyme has the highest rate of activity loss at 42.5°C.

Molecular modeling

A three-dimensional model of one monomer of *M. trossulus* cMDH is shown in Fig. 7A. The positions of all amino acid substitutions between the *M. trossulus* and the *M. californianus* and *M. galloprovincialis* orthologs are indicated. In addition, residues in the active site that are directly involved in OAA reduction are highlighted. Residue 114, which we have shown to play an important role in altering the temperature sensitivity of the *Mytilus* cMDH orthologs, sits at the edge of the 'catalytic loop'. This structure moves substantially during catalysis in

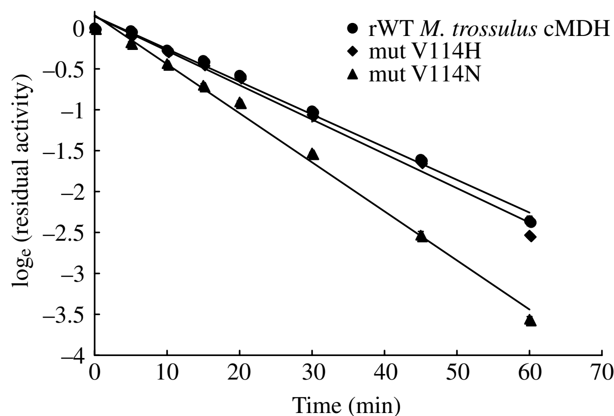

Fig. 6. Thermal denaturation profiles of rWT *M. trossulus* cMDH and the mutants V114H and V114N. Samples were held at 42.5°C for the indicated time before residual activity was measured in triplicate. The rWT *M. trossulus* and V114H mutants are not significantly different in stability, but the V114N mutant denatures at a significantly higher rate than the other two forms.

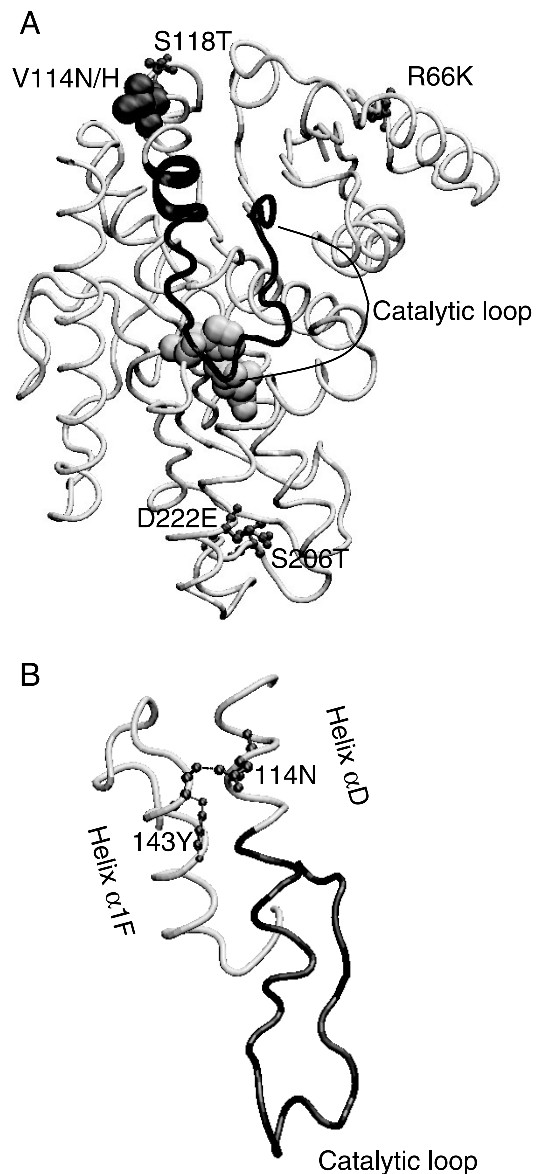

Fig. 7. (A) A model of one monomer of *M. trossulus* cMDH, based on the structure of the pig ortholog (PDB accession number 4MDH). All residues that differ between the *M. trossulus* and the *M. galloprovincialis* or *M. californianus* orthologs are labeled; the non-conservative mutation at position 114 is shown in dark spacefill. Representative active site residues are shown in light spacefill, and the highly mobile catalytic loop, which must move by ~10 Å during catalysis, is black. (B) A magnified view of mutant V114N, showing the relationship of that residue to the catalytic loop (black), as well as the hydrogen bond that may form between the amide nitrogen of the asparagine side chain and the carbonyl oxygen of 143Y on the neighboring helix α1F. Models were visualized with VMD (Humphrey et al., 1996).

order to close over the active site and create the catalytic vacuole in which reduction of OAA takes place (Gerstein and Chothia, 1991). Because changes in conformation associated with substrate binding and loop closure are likely to be rate limiting in 2-hydroxy acid dehydrogenases such as cMDH (Dunn et al., 1991), changes in the flexibility of the catalytic loop region have the potential to explain differences both in K_m^{NADH} and k_{cat} of the cMDHs examined here (see Discussion).

To further explore the effect of changes at position 114 in the *M. trossulus* ortholog, we used SwissPDBViewer software to model the effects of mutations V114N and V114H. Insertion of asparagine at 114 (Fig. 7B) allows the formation of a hydrogen bond between the terminal amide nitrogen of the asparagine side chain and the backbone carbonyl oxygen of a tyrosinyl residue on the neighboring α1F helix, 143Y. It is possible that this additional hydrogen bond, absent in rWT *M. trossulus* cMDH, leads to stabilization of the catalytic loop region and thus is responsible for the changes noted in K_m^{NADH} and k_{cat} of the V114N mutant. Although the V114H mutant also reduces K_m^{NADH} and k_{cat} with respect to rWT, no additional hydrogen bonds or salt bridges are apparent after this mutation.

Discussion

Temperature adaptation in Mytilus cMDHs

The higher K_m^{NADH} and k_{cat} values of *M. trossulus* cMDH suggest that this ortholog is more cold-adapted than the orthologs of either *M. galloprovincialis* or *M. californianus*. We base this inference on the results of numerous comparative studies of marine ectotherms that examined temperature sensitivity of 2-hydroxy acid dehydrogenases, i.e. cMDH and the closely related, structurally homologous enzyme A_4-lactate dehydrogenase (A_4-LDH; this enzyme catalyzes the reduction of pyruvate to lactate using NADH as a cofactor). Such studies repeatedly have shown that the enzyme kinetic parameters K_m and k_{cat} shift in a stereotypical manner during temperature adaptation, in order to conserve substrate affinities and catalytic rate within an optimal range at physiological temperatures (Hochachka and Somero, 2002). For example, studies using A_4-LDH from marine teleosts have shown that, as species adapt to colder temperatures, the enzyme tends to show lower substrate affinities (i.e. higher $K_m^{PYRUVATE}$) and higher catalytic rates at any measurement temperature (e.g. Yancey and Siebenaller, 1987; Holland et al., 1997; Fields and Somero, 1998; Johns and Somero, 2004). These changes counteract the acute effect of lower temperature to reduce both K_m and k_{cat}. Similarly, a study on the cMDHs of 11 species of marine invertebrates, including some collected from hydrothermal vents (Dahlhoff and Somero, 1991), showed a clear inverse correlation between K_m^{NADH} values and habitat temperature. A second study (Dahlhoff and Somero, 1993), focusing on cMDHs from congeners of the mollusk *Haliotis* (abalone) occurring in different thermal environments along the west coast of North America, also showed a significant increase in K_m^{NADH} as habitat temperature decreased. Thus, the findings of these earlier studies provide strong support for our inference that the higher K_m^{NADH} and k_{cat} values of *M. trossulus* cMDH indicate cold adaptation with respect to the orthologs of *M. californianus* or *M. galloprovincialis*.

The finding that *M. trossulus* cMDH is cold-adapted relative to that of *M. galloprovincialis* corresponds with previous work on physiological responses to temperature in these species. A study by Hilbish et al. (1994) showed that *M. galloprovincialis* has a three-fold higher clearance rate (i.e. feeding rate) and a higher metabolic rate than *M. trossulus* at 23°C, which they argued would translate into an energetic advantage in warmer habitats and might explain the success of *M. galloprovincialis* in displacing the native *M. trossulus* in southern California. Hofmann and Somero (1996a) showed that induction of heat-shock protein 70 (Hsp70) occurred at a lower temperature in *M. trossulus* than in *M. galloprovincialis*, a further indication of differences in optimal habitat temperature. A more recent study (Braby, 2004) has shown that, when exposed to a rapid, steady increase in temperature (0.1 deg. min^{-1}), heart function in *M. galloprovincialis* is maintained to a higher temperature than in *M. trossulus*. Furthermore, during long-term exposure to elevated temperature (14 or 21°C), mortality rates for *M. galloprovincialis* were essentially zero, while *M. trossulus* mortality exceeded 40% (14°C) or 60% (21°C) (Braby, 2004).

Combined, these data on diverse physiological processes suggest that *M. galloprovincialis* is adapted to warmer habitat temperatures than *M. trossulus* and thus provide a potential mechanism for the success of the invasive species in displacing *M. trossulus* in the southern, warmer portion of its original range. This conclusion is strengthened by a closer examination of the cMDH kinetics presented here. If K_m^{NADH} and k_{cat} are compared within the temperature range each species experiences during immersion in its native habitat, we see that the values of each correspond closely. Within the western Mediterranean, *M. galloprovincialis* experiences an annual seawater temperature range of approximately 13–25°C (Pickard, 1968), while *M. trossulus* in the eastern Pacific experiences water temperatures of ~5–15°C (Suchanek et al., 1997; Ricketts et al., 1968). [Note that Suchanek et al. (1997) suggest that an annual mean sea surface temperature of 13–14°C represents the border between southern populations of *M. galloprovincialis* and northern populations of *M. trossulus* both in California and in a second area of overlap in Japan.] Despite the large interspecific differences shown in substrate affinity of the mytilid cMDHs (Fig. 3A), within the habitat temperature ranges given above, the K_m^{NADH} values of cMDH from *M. trossulus* and *M. galloprovincialis* are very similar. Interpolating from the data given in Fig. 3A, the K_m^{NADH} range of *M. trossulus* cMDH is ~12.4–14.5 μmol l^{-1} NADH, while the *M. galloprovincialis* ortholog ranges from 10.5 to 15.7 μmol l^{-1} NADH. These values are also quite similar to values measured for *Haliotis* cMDHs within their habitat temperature ranges, 11–21 μmol l^{-1} NADH (Dahlhoff and Somero, 1993). Our results thus suggest a large degree of compensation to environmental temperature in the substrate

affinity of the cMDHs of the two blue mussels, which is due to the single amino acid substitution valine → asparagine at position 114.

Substrate affinity data from the cMDH of the third mussel we examined, *M. californianus*, are intermediate to those of the *M. trossulus* and *M. galloprovincialis* forms (Fig. 3A). Because *M. californianus* has a much broader latitudinal distribution along the west coast of N. America – extending from Southern Baja California, Mexico to the Aleutian Islands (Sagarin and Gaines, 2002) – it necessarily occupies a wider thermal range than its congeners. The K_m^{NADH} values we have measured for *M. californianus* cMDH are not surprising, then, considering that at the southern end of its range it experiences temperatures at least as warm as those experienced by the invasive *M. galloprovincialis*, and at the northern extreme it must tolerate temperatures as low as those experienced by *M. trossulus*. As with the V114N mutation between *M. trossulus* and *M. galloprovincialis*, the single mutation V114H appears sufficient to explain the differences in K_m^{NADH} we have found between the *M. trossulus* and *M. californianus* cMDHs (Fig. 3B,C).

Although k_{cat} values were not measured for tissue-purified cMDHs, we expect that the measurements made with rWT *M. trossulus* cMDH and the mutants V114H and V114N accurately represent the k_{cat} values of the native enzymes, because of the similarity between the tissue-purified and recombinant enzymes in the other kinetic parameters tested, K_m^{NADH} and E_a. Comparing the k_{cat} values of rWT and V114N, which contains the *M. galloprovincialis* substitution at position 114, we see compensation to temperature in catalytic rate (Fig. 5) similar to that noted above for substrate affinity. From 5 to 15°C, rWT k_{cat} ranges from 83 to 215 s^{-1}; from 13–25°C the k_{cat} of V114N increases from 112 to 290 s^{-1}. Again, these results suggest that the V114N substitution has adapted the cMDH of *M. galloprovincialis* for function at warmer temperatures than the *M. trossulus* ortholog. Fig. 5 also indicates that mutant V114H has k_{cat} values intermediate to those of rWT and V114N, providing further evidence that the *M. californianus* ortholog has kinetic properties intermediate to those of *M. trossulus* and *M. galloprovincialis*, and that these differences are due to the single amino acid substitution.

The conservation of K_m^{NADH} and k_{cat} exhibited by the cMDHs of these *Mytilus* congeners at temperatures corresponding to the common seawater temperatures of their habitats suggests that it is the temperatures experienced during immersion that select for the thermal optima of enzymes. During emersion at low tide, mussel body temperatures may exceed 35°C in the summer and approach 0°C in the winter, and the maximal and minimal body temperatures reached during emersion may be similar among the three species (Hofmann and Somero, 1995; Hofmann and Somero, 1996b; G.N.S., personal observations). However, at these extreme body temperatures, cardiac activity may cease and valve (shell) closure may occur (Braby, 2004), and it is likely that metabolic processes are downregulated during these periods, both due to temperature stress and reduced access to oxygen (Hofmann and Somero, 1996b). Normal physiological activities may only resume after re-immersion with the rising tide, when body temperatures moderate and the gills are again bathed with seawater so ventilation and feeding can resume (see Hofmann and Somero, 1996b). Thus, because metabolically demanding activities occur mainly during immersion, and some level of metabolic depression may occur during emersion, we propose that the cooler temperatures characteristic of immersion, rather than the extreme temperatures attained during emersion, are the temperatures that are of primary importance in selecting for enzyme kinetic properties like K_m^{NADH} and k_{cat}.

One curious and unexpected result of this work is the lower *in vitro* thermal stability of the V114N mutant in comparison to rWT *M. trossulus* cMDH (Fig. 6), suggesting that the *M. galloprovincialis* ortholog is less stable than the *M. trossulus* form. *A priori*, proteins from more heat-tolerant species would be expected to exhibit greater thermal stability than those from species occupying colder habitats. However, this assumption has not been universally supported in past studies (e.g. Place and Powers, 1984; Holland et al., 1997; Fields and Somero, 1998), suggesting that enzyme orthologs, especially from closely related species, do not necessarily modify global stability in order to adapt their kinetic properties to moderate changes in environmental temperature. A possible explanation for these results is based on limitations in the techniques employed to measure protein thermal stability. When denaturation studies employ loss of activity as the index of denaturation, as in the present case and in the majority of other comparative studies of protein thermal stability, transient and localized unfolding at high but not fully denaturing temperatures may lead to enzyme aggregation and precipitation, with concomitant loss of activity. Such loss of activity may not provide a valid index of the intrinsic thermodynamic stability of proteins or the stability under *in vivo* conditions, where protein concentrations are much higher than under the *in vitro* conditions used in stability measurements (see Fields and Somero, 1998; Fields, 2001). For these reasons, measurements of *in vitro* protein stability may fail to manifest the effects of changes in amino acid sequence that are responsible for adaptation in kinetic properties. Given the findings described above regarding the higher levels of Hsp70 in *M. trossulus* relative to *M. galloprovincialis* (Hofmann and Somero, 1996a), and the resulting implication that *M. trossulus* experiences a higher burden of stress-induced protein unfolding, the physiological importance of the reduced thermal stability in *M. galloprovincialis* cMDH remains unclear.

The role of mutation V114N in altering the kinetics of cMDH

In order to understand better the mechanism by which mutations at position 114 affect the kinetics of *M. trossulus* cMDH, we created a three-dimensional model of one monomer, based on the crystallographic structure of pig cMDH (Fig. 7A) (Birktoft et al., 1989). Using SwissPDBViewer software, we mutated the model to insert either a histidine or asparagine in place of the valine at position 114 and examined the potential interactions of these mutants with neighboring

amino acids. Based on this analysis, V114N (the mutant corresponding to *M. galloprovincialis* cMDH) potentially creates a hydrogen bond that could act as a source of stabilization and thus affect enzyme function. Due to the relatively long side chain of asparagine, the terminal amide group is able to interact with residues on a neighboring α-helix, α1F (see Fig. 7A,B), and the amide nitrogen may form a hydrogen bond with the backbone carbonyl oxygen of tyrosine 143. Residue 114 borders a region of the molecule, the catalytic loop, that must close down over the active site upon substrate binding to create a hydrophobic vacuole (Figs 2, 7A,B). The structure and movement of the catalytic loop has been examined in the closely related 2-hydroxy acid dehydrogenases MDH (Goward and Nicholls, 1994; Birktoft et al., 1982) and lactate dehydrogenase (LDH) (Gerstein and Chothia, 1991). According to a detailed description of catalytic loop closure given by Gerstein and Chothia (1991), combined with LDH and MDH alignments provided by Goward and Nicholls (1994), the catalytic loop region of mytilid cMDHs extends from residue 88 to 111 (Figs 2, 7B). During catalysis, the C-terminal segment of helix αD closest to residue 114 moves rigidly, but the N-terminal segment of this helix is part of the loop proper and deforms in order to allow the loop to close (Gerstein and Chothia, 1991).

Conformational changes associated with substrate binding and loop closure have been shown to be rate-limiting for catalysis in LDH (Dunn et al., 1991), and, if this is true for the structurally closely related MDH, changes in the flexibility of catalytically important mobile regions such as the catalytic loop could have a strong effect on k_{cat}. Concomitantly, however, increases in flexibility in the catalytic loop, which contains residues that interact with substrate upon binding (Goward and Nicholls, 1994), may reduce substrate affinity (i.e. increase K_m). This is because the apo-enzyme of the more flexible, cold-adapted ortholog can occupy a broader range of conformations, some of which may be less compatible with substrate binding. Such changes in the flexibility of mobile regions surrounding the active site have been argued to affect temperature adaptation in A_4-LDHs of notothenioid fishes (Fields and Somero, 1998; Fields and Houseman, 2004) and damselfishes (Johns and Somero, 2004), but in these previous studies the structures showing temperature-adaptive substitutions were α-helices structurally unrelated to the catalytic loop region. Thus, this is the first instance in which structural changes associated with the catalytic loop region have been shown to affect the kinetics of MDH or LDH in a temperature-adaptive manner.

Despite the addition of a positive charge, no hydrogen bonds or short-range electrostatic interactions appear to be created through mutation V114H (corresponding to *M. californianus* cMDH). Thus, the mechanism relating the mutation V114H to an increase in localized stability around helix αD is unclear. However, one potential mechanism relating V114H to a change in the flexibility of the catalytic loop involves insertion of the positive charge of the histidine side chain near the C-terminus of helix αD. This type of mutation has a tendency to stabilize the helix dipole (Richardson and Richardson, 1988; Kelly et al., 1993) and therefore may affect the propensity of the helix to deform, which is necessary during the closing of the catalytic loop (Gerstein and Chothia, 1991). Although this hypothetical mechanism potentially could lead to changes in catalytic rate and substrate affinity, studies testing the hypothesis need to be performed.

The above argument relating changes in the kinetics of the *M. galloprovincialis* cMDH to increased stability in the region around helix αD, caused by a putative additional hydrogen bond between 114N and 143Y (Fig. 7A), appears to be contradictory to the evidence presented regarding the lower global stability of mutant V114N relative to rWT *M. trossulus* cMDH. In other words, how could mutant V114N both cause an increase in local structural stability, reducing catalytic rate but increasing substrate affinity (Figs 3, 5), and simultaneously lead to a decrease in thermal stability of the magnitude shown in Fig. 6? A possible explanation is that the stabilization of the additional hydrogen bond is not sufficient to protect the molecule from initial localized unfolding at higher temperatures – note the drop in activity of mutant V114N at 45°C relative to the other enzymes in Fig. 4. Once unfolding begins, the presence of asparagine at position 114, rather than valine or histidine, may more readily lead to unfolding intermediates that are more likely to irreversibly aggregate and precipitate. That is, 114N may provide increased stability to the region adjacent to helix αD at low and intermediate temperatures but may more readily induce irreversible loss of activity at temperatures above 40°C.

Conclusions

Our data indicate that the mutations at position 114 in the mytilid cMDHs are responsible for the changes we have found in the kinetics of the three orthologs. The decrease in K_m^{NADH} and k_{cat} seen in *M. galloprovincialis* relative to *M. trossulus* may result from an additional hydrogen bond between 114N and 143Y that increases stabilization of the catalytic loop region, whose movement is likely to be rate limiting to catalysis. This minor change in structure poises *M. galloprovincialis* cMDH for function at warmer temperatures and may be part of a broad suite of physiological, biochemical and molecular adaptations that has allowed this species to displace its congener, *M. trossulus*, throughout the warmer part of the latter's original range in North America and Japan.

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