Decreses in Activation Energy and Substrate Affinity in Cold-Adapted A₄-Lactate Dehydrogenase: Evidence from the Antarctic Notothenioid Fish Chaenocephalus aceratus

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Enzyme function is strongly affected by temperature, and orthologs from species adapted to different thermal environments often show temperature compensation in kinetic properties. Antarctic notothenioid fishes live in a habitat of constant, extreme cold (−1.86 ± 2°C), and orthologs of the enzyme A₄-lactate dehydrogenase (A₄-LDH) in these species have adapted to this environment through higher catalytic rates, lower Arrhenius activation energies (Ea), and increases in the apparent Michaelis constant for the substrate pyruvate (KₘPYR). Here, site-directed mutagenesis was used to determine which amino acid substitutions found in A₄-LDH of the notothenioid Chaenocephalus aceratus, with respect to orthologs from warm-adapted teleosts, are responsible for these adaptive changes in enzyme function. KₘPYR was measured in eight single and two double mutants, and Ea was tested in five single and two double mutants in the temperature range 0°C–20°C. Of the four mutants that had an effect on these parameters, two increased Ea but did not affect KₘPYR (Gly224Ser, Ala310Pro), and two increased both Ea and KₘPYR (Glu233Met, Gln317Val). The double mutants Glu233Met/Ala310Pro and Glu233Met/Gln317Val increased KₘPYR and Ea to levels not significantly different from the A₄-LDH of a warm-temperate fish (Gillluchthys mirabilis, habitat temperature 10°C–35°C). The four single mutants are associated with two α-helices that move during the catalytic cycle; those that affect Ea but not KₘPYR are further from the active site than those that affect both parameters. These results provide evidence that (1) cold adaptation in A₄-LDH involves changes in mobility of catalytically important molecular structures; (2) these changes may alter activation energy alone or activation energy and substrate affinity together; and (3) the extent to which these parameters are affected may depend on the location of the substitutions within the mobile α-helices, perhaps due to differences in proximity to the active site.

Introduction

Environmental temperature has a profound impact on species distributions, abundance, and survival, and the importance of temperature in limiting species distributions can be ascribed in part to its powerful effects on subcellular and molecular systems (Hochachka and Somero 2002). Enzyme function, in particular, is often extremely sensitive to temperature change, and this sensitivity is probably related to the balance maintained between flexibility and stability in discrete, relatively mobile regions of the polypeptides involved in catalysis. The resultant marginal stability is usually assumed to be a necessary attribute of enzymes (Jaenicke 1991; Zavodszky et al. 1998; Fields et al. 2002). This is because molecular flexibility is needed to maintain an appropriate catalytic rate, but stability is required to ensure active site geometry for substrate recognition and a useful lifetime before enzyme degradation occurs (Fields 2001). Changes in temperature tend to alter the balance between protein flexibility and stability; higher temperatures can make a protein overly flexible, reducing substrate affinity by disrupting the active site and ultimately leading to denaturation. Colder temperatures can make an enzyme overly stable, reducing catalytic rates below the range needed to maintain metabolic homeostasis in the cell (Fields 2001).

Over evolutionary time it is likely that functional attributes of the enzyme molecule will respond to selective pressure, in order to compensate for alterations in environmental temperature (Hochachka and Somero 2002). For example, a series of studies (Johnston and Whalesby 1977; Yancey and Somero 1978; Graves and Somero 1982; Yancey and Seibenaller 1987; Dahlhoff and Somero 1993; Holland, McFall-Ngai, and Somero 1997; Fields and Somero 1997, 1998; Johns and Somero 2004) has shown that kinetic properties of muscle-type lactate dehydrogenase (A₄-LDH; E.C. 1.1.1.27; lactate : NAD⁺ oxidoreductase) from ectothermic vertebrates are strongly correlated to environmental temperature. When the apparent Michaelis constants for the substrate pyruvate (KₘPYR) of A₄-LDH orthologs from a variety of species are measured at one temperature, values increase as habitat temperature decreases (Fields 2001; Hochachka and Somero 2002). Because KₘPYR is inversely related to substrate binding affinity, these results indicate that, when measured at the same temperature, A₄-LDH orthologs from cold-adapted species have lower substrate binding affinities than those from warm-adapted ones. However, when measured within each species’ habitat temperature range, KₘPYR values are comparable, indicating that the conservation of substrate binding affinity is important in temperature adaptation (Somero 1995). Similarly, when the catalytic rate constant, or turnover number (kcat), is measured at a common temperature, orthologs from more cold-adapted species again have higher values; that is, orthologs from more cold-adapted species catalyze the conversion of pyruvate to lactate faster than those from more warm-adapted species (Fields 2001; Hochachka and Somero 2002). These differences observed in catalytic rate appear adaptive, because they are found repeatedly in orthologs of species occurring at different temperatures. Modifications of kcat likely are due to changes in activation...
Cold Adaptation in Notothenioid A4-LDHs

![Alignment of LDH-A consensus sequences from nine Antarctic notothenioid fishes (middle) and six temperate teleost fishes (bottom).](https://academic.oup.com/mbe/article-abstract/21/12/2246/1071047)

**Fig. 1.**—Alignment of LDH-A consensus sequences from nine Antarctic notothenioid fishes (middle) and six temperate teleost fishes (bottom). Secondary structural components of the LDH-A monomer are given in the top line. Nonconservative substitutions between the sequences are highlighted. Dashes represent agreement between the sequences; asterisks represent positions where three of the non-notothenioid consensus share an amino acid with the notothenioid consensus, and the other three share a conservative substitution with respect to the notothenioid consensus. Orthologs used in creating the consensus sequences are listed in the text. (Data from Fields and Somero 1998).

enthalpy, or, equivalently, in activation energy barriers to catalysis (Feller and Gerday 1997; Lonhienne, Gerday, and Feller 2000; Fields et al. 2002; Feller 2003), such that cold-adapted forms have reduced activation energies and can proceed through catalysis with a lower input of thermal energy from the medium.

The studies described above provide strong evidence of compensation to environmental temperature in A4-LDH thermal energy from the medium. Cold-adapted forms have reduced activation energies and Feller 2000; Fields et al. 2002; Feller 2003), such that enthalpy, or, equivalently, in activation energy barriers to catalysis, which may or may not involve concomitant reductions in substrate affinity; (2) adaptation to large changes in temperature may be achieved by one to a few amino acid substitutions (in the 332 residues composing the LDH-A monomer) outside the active site; and (3) these changes likely exert their effects by decreasing the energy input needed to alter the conformation of secondary structures that must move during catalysis, i.e., by decreasing activation energy.

**Materials and Methods**

**Collection of Specimens**

Individuals of Chaenocephalus aceratus, a teleost of the suborder Notothenioidei, were collected by otter trawl from the R/V Polar Duke near Palmer Station, Anvers Island, Antarctica from March to April 1997. Specimens were kept in ambient seawater (0°C–1°C) until killed, and white muscle samples were removed and immediately frozen at −70°C until used. Specimens of Gillichthys mirabilis were caught using baited minnow traps in Elkhorn Slough, approximately 20 km north of Monterey, Calif. They were kept in aquaria at 15°C until sacrificed, and whole bodies were frozen at −80°C.

**Site-Directed Mutagenesis**

Residues in the C. aceratus LDH-A monomer targeted for mutagenesis were selected by comparing a consensus of LDH-A amino acid sequences from nine species of Antarctic notothenioids with a consensus sequence derived from six species of warm-adapted teleosts (fig. 1; sequence data from Fields and Somero 1998). The notothenioid consensus was based on LDH-A sequences from Notothenia coriiceps, Lepidonotothen nudifrons, Gobionotothen gibberifrons, Dissostichus mawsoni, Chionodraco rastrosuspinus, Chaenocephalus aceratus, Champsoscephalus gunnari, Parachaenichthys charcoti, and Harpagifer antarcticus; the C. aceratus LDH-A sequence differs from the Antarctic notothenioid consensus at one position (Leu316Val). The non-notothenioid consensus was based on LDH-A orthologs we sequenced (the
eelpout *Austrolocus depressiceps*, the lampfish *Lamp- anyctus ritteri* [unpublished data], and the goby *Gillichthys mirabilis* [Fields and Somero 1997] and on sequences obtained from the literature (the killifish *Fundulus heteroclitus* [Quattro et al. 1995], and the barracudas *Sphyraena idiastes* and *S. luctosa* [Holland, McFall-Ngai, and Somero 1997]). The sequence of *G. mirabilis* LDH-A, which we used as a benchmark warm-adapted ortholog in this study (see below), differs from the non-notoenothienid consensus at four positions (Met12Ser, Pro16Ala, Pro310Ala, and Glu317Val).

There are 22 amino acid differences between the two consensus sequences (i.e., 93% identity; fig. 1), of which 12 are conservative. Because nonconservative substitutions are more likely than conservative ones to affect protein stability and function, the nonconservative substitutions were targeted for site-directed mutagenesis. We note, however, that although we focus on nonconservative substitutions in this study, conservative substitutions also have the potential to affect significantly the function of enzymes. Thus, the role of the 12 conservative mutations we have found in the cold adaptation of A\textsubscript{4}-LDH must be addressed in a future study.

From the 10 nonconservative substitutions found, we designed eight single mutants (all mutants are given in the cold-adapted [notoenothienoid] → warm-adapted [non-notoenothienoid] direction): 75His, Ala187Gly, Gly213Thr, Gln217Lys, Gly224Ser, Glu233Met, Ala310Pro, and Gln317Val. In addition, we produced two double mutants (Glu233Met/Ala310Pro and Glu233Met/Gln317Val) by combining mutations that showed effects on A\textsubscript{4}-LDH kinetics in pilot studies, to determine whether such combinations might reveal synergistic effects among the substitutions we have found in the cold adaptation of A\textsubscript{4}-LDH must be addressed in a future study.

Complementary DNA from white epaxial skeletal muscle of *C. aceratus* was created through reverse transcription of mRNA using Superscript II reverse transcriptase (Promega, Madison, Wis.) and polythymidine primers. The sequences of the 5' and 3' untranslated regions (UTRs) of *ldh-a* cDNA were determined using RACE PCR (Generacer; Invitrogen, Carlsbad, Calif.) and gene-specific primers originally derived from the sequence of barracuda *ldh-a* (Holland et al. 1997; for the 5' UTR: 5'-CCTCAGTCTCCAGGGCAACATAG-3'; for the 3' UTR: 5'-TACACCTCTGAGGCCATGCTTG-3'). After sequences for the UTRs were obtained, primers designed from within the 5' UTR (5'-TCTAACTGAACAACTC-3') and 3' UTR (5'-CCCTCCTCTGTA-CCTTACTG-3') of *C. aceratus* *ldh-a* were used to amplify the entire coding sequence. The PCR protocol used for the amplification consisted of 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 90 s; a final extension at 72°C for 10 min was followed by rapid cooling to 4°C. Amplified *ldh-a* cDNA was purified (Qiaquick PCR purification, Qiagen Inc., Valencia, Calif.) and cloned into pAlter-Ex1 (Altered Sites II in vitro Mutagenesis System, Promega) and used to transform repair-minus strain ES1301 of *Escherichia coli* (mutS). After growth to log phase, plasmids were purified (Qiaprep miniprep kit, Qiagen) and used to transform *E. coli* JM109 cells. For each mutant, appropriate antibiotic resistance and automated nucleotide sequencing (BigDye Terminator Cycle Sequencing, Applied Biosystems, Foster City, Calif.) of the *ldh-a* insert confirmed mutagenesis.

### A\textsubscript{4}-LDH Expression

The pAlter-Ex1 plasmid contains a tac promoter and a ribosome binding site upstream of the insert. Transformed JM109 cells were grown to log phase (OD\textsubscript{600} = 0.400–0.600) in 800 ml LB broth, and expression of recombinant A\textsubscript{4}-LDH was induced with IPTG. The culture was incubated for another 6–12 h, and the cells were collected by centrifugation at 4,000×g for 20 min. Cells were lysed with CelLytic B II (Sigma, St. Louis, M.O.) following the manufacturer’s instructions. After centrifugation at 12,000×g and 4°C for 1 h, LDH activity was found in the supernatant. Supernatant was dialyzed (10,000 MWCO) overnight against 50 mM potassium phosphate pH 6.8, and LDH assays were performed without further purification. Activity-stained native polyacrylamide gel electrophoresis (Brewer 1970) indicated that no other isoforms of LDH were present in the supernatants (data not shown).

### Measurement of K\textsubscript{m} and Arrhenius Activation Energy

Kinetic assays were performed on a Shimadzu model UV1601 or a Pharmacia Ultraspec 2100pro UV-Visible spectrophotometer, each with a temperature-controlled cuvette holder (±0.2°C). Substrate saturation curves were derived for each A\textsubscript{4}-LDH mutant, as well as for recombinant wild type *C. aceratus* A\textsubscript{4}-LDH (rWT), at 0°C, 5°C, 10°C, 15°C, and 20°C. Measurements were also made for A\textsubscript{4}-LDH purified (Yancey and Somero 1978) from white muscle of *C. aceratus* (WT) and the warm-temperate goby *Gillichthys mirabilis*. Three replicates of six pyruvate concentrations were used at each temperature; the cofactor NADH was saturating (150 μM) for all assays. Replicate measurements were made using a single enzyme preparation for each of the samples except recombinant wild type, for which triplicate measurements were made on four independently produced samples, totaling 12 replicates.

For each assay, conversion of NADH to NAD\textsuperscript{+} was monitored at 340 nm and converted to reaction rate. These data were used to determine three K\textsubscript{m} values and maximal velocity (V\textsubscript{max}) values at each temperature, using Wilman K\textsubscript{m}-fitting software (Brooks and Sueltzer 1986) and nonlinear regression analysis (Wilkinson 1961). Activation energy was determined from V\textsubscript{max} data according to the Arrhenius equation, where the slope of the plot (ln [V\textsubscript{max}] vs. 1/K) can be related to E\textsubscript{a} by the equation E\textsubscript{a} = −slope × R, where R is the universal gas constant (8.3144 J mol\textsuperscript{−1} K\textsuperscript{−1}).

### Statistics

Each K\textsubscript{m} value reported represents the mean ± SE for three replicates, except rWT, which comprises...
12 replicates. To determine whether orthologs had significantly different $K_m$ values, an analysis of variance was used (anova1; MATLAB; Mathworks, Natick, Mass.), followed by a Tukey-Kramer multiple comparisons test (multcompare; MATLAB) with $\alpha = 0.05$. These tests were performed on the $K_m$ values of each ortholog measured at 10°C. To determine whether $E_a$ values were different among orthologs, an analysis of covariance was performed on the regressions of the Arrhenius plots ($\ln [V_{max}]$ vs. $1/K$). Because slope in this type of plot is proportional to $E_a$, statistically significant differences in slope indicate orthologs that possess different $E_a$ values. Analysis of covariance was performed with the aoctool module of MATLAB software, and Tukey-Kramer multiple comparisons tests ($\alpha = 0.05$) were performed with the multcompare module.

**Molecular Modeling**

To better understand how the mutations found in $A_4$-LDH of the Antarctic notothenioids affect the catalytic process, we created a molecular model using SwissModel software (Guex and Pietsch 1997; Schwede et al. 2003). Dogfish (Abad-Zapatero et al. 1987) and pig (Dunn et al. 1991) $A_4$-LDH structures (PDB accession codes 6LDH and 9LDT, respectively) were used as templates on which the $C. aceratus$ LDH-A primary structure was threaded. Because of the high homology, both in primary and tertiary structure, among vertebrate $A_4$-LDHs, we consider the resultant homology model of $C. aceratus$ $A_4$-LDH to be an accurate representation of the structure of the LDH-A monomer. This model was visualized with Rasmol (Sayle and Milner-White 1995) and DeepView software (Guex and Pietsch 1997) to locate the position of the mutations with respect to the active site and to mobile structures in the molecule involved in subunit interactions and catalysis.

**Results**

**Effects of Mutations on $K_m$ of Chaenocephalus aceratus $A_4$-LDH**

The effects of specific mutations on $A_4$-LDH substrate affinity were initially determined by measuring $K_m$ values at 10°C. Preliminary results from the three single mutants His75, Ala187Gly, and Gln217Lys indicated that they did not have altered $K_m$ values at 10°C with respect to WT (data not shown). Although we recognize that these mutations may affect enzyme function in other, potentially temperature-sensitive ways, we chose to exclude these mutants from further study to reduce the number of enzymes examined. Other mutants that did alter substrate affinity in initial tests were examined in more detail. The results of these experiments, in which $K_m$ of each mutant was measured from 0°C–20°C, are shown in figure 2. Figure 2 also shows $K_m$ values for WT, the recombinant wild type (rWT), and the ortholog of *Gillichthys mirabilis*, a goby fish that experiences habitat temperatures from 10°C–35°C (Barlow 1961; Fields and Somero 1997). A multiple comparisons test of the means of all $A_4$-LDHs measured at 10°C (fig. 3) shows that $K_m$ values of the $C. aceratus$ WT and rWT forms are not significantly different from one another ($n = 3$ for all means, except rWT $n = 12$).
of the enzyme for pyruvate. Large and statistically significant differences in $K_m$ values between the C. aceratus and G. mirabilis orthologs (figs. 2 and 3) illustrate the extent to which this kinetic parameter can be altered by selection in different thermal environments.

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Two double mutants also were created to determine whether there were synergistic interactions between substitutions in C. aceratus $A_4$-LDH. The mutant Glu233Met/Ala310Pro has $K_m$ values not significantly different from the single mutant Glu233Met (figs. 2 and 3). This suggests that the mutation Ala310Pro exerts no effect on $K_m$, whether singly or in conjunction with Glu233Met. The double mutant Gln317Val/Glu233Met produced $K_m$ values between those of the single mutants Gln317Val and Glu233Met but not significantly different from either one (fig. 3).

Figure 5 illustrates the Arrhenius activation energy ($E_a$) for A4-LDHs from Chaenocephalus aceratus, WT, Gillichthys mirabilis, and seven mutants. The regression line is included to illustrate the trend of decreasing $E_a$ with increasing $K_m$ values; however, this correlation is only marginally significant ($P = 0.0632$; see text).

Effects of Mutations on Arrhenius Activation Energy of Chaenocephalus aceratus $A_4$-LDH

The Arrhenius activation energy ($E_a$) for each of the mutants was calculated using $V_{max}$ values collected from 0°C–20°C. Figure 4 gives $E_a$ values ±95% confidence intervals for each enzyme and shows that mutants Gly213Thr and Gly224Ser have $E_a$ values not significantly different from WT or rWT; the other three single mutants (Glu233Met, Ala310Pro, and Gln317Val) and the two double mutants (Glu233Met/Ala310Pro and Glu233Met/Gln317Val) have $E_a$ values significantly higher than that of C. aceratus $A_4$-LDH, but not significantly different from the warm-adapted G. mirabilis ortholog.

Figure 4 shows $E_a$ plotted against $K_m$ values at 10°C for WT, rWT, the G. mirabilis ortholog, and each of the mutants. Although there is a general trend toward lower $E_a$ values as $K_m$ increases (i.e., as substrate affinity decreases), the correlation is only marginally significant ($r = 0.608; P = 0.0632$; Zar 1984). These results indicate that activation energy and substrate binding affinity may change independently, such that in some mutants activation energy is altered without a substantial effect on $K_m$.
this is true whether the shift is from WT to a single mutant or from the single mutant Glu233Met to a double mutant (i.e., Glu233Met/Ala310Pro; see fig. 5).

However, although Ea can change without a shift in $K_m^{PYR}$, in none of the mutants tested does $K_m^{PYR}$ change without a concomitant shift in Ea. A second set of mutants (Gln317Val, Glu233Met, and the double mutants including Glu233Met) do shift both $K_m^{PYR}$ and Ea. In each of these cases Ea increases as $K_m^{PYR}$ decreases, suggesting that these mutations, and Glu233Met in particular, modify kinetics to increase both binding affinity and activation energy barriers to catalysis. The double mutants containing the Glu233Met substitution modify both Ea and $K_m^{PYR}$ to values that are not significantly different from the A4-LDH of the warm temperate goby G. mirabilis.

In sum, these results indicate that the mutant Glu233Met strongly affects both Ea and $K_m^{PYR}$, increasing the former and decreasing the latter; Gln317Val strongly affects Ea and moderately affects $K_m^{PYR}$, and Ala310Pro strongly affects Ea without affecting $K_m^{PYR}$. The changes—decreases in substrate affinity and activation energy barriers—are in the direction predicted if the substitutions serve to adapt C. aceratus A4-LDH to function at low temperatures. Double mutants of these substitutions have no synergistic effects, and the other mutants tested have no significant effect on Ea or $K_m^{PYR}$ (except Gly224Ser, which increased $K_m^{PYR}$).

Molecular Modeling

A three-dimensional model of one monomer of C. aceratus A4-LDH is shown in figure 6. The mutations that affect the kinetic properties of the enzyme are associated with one of two $\alpha$-helices ($\alpha_{1G-2G}$ and $\alpha_H$) that form the margins of the cleft by which ligands can enter the active site. As discussed below, these helices must move during catalysis (Gerstein and Chothia 1991). Each of the effective mutants is associated with one of these helices, but the mutations that increase Ea without affecting substrate affinity (Gly224Ser and Ala310Pro) are further from the active site on these helices than the substitutions (Glu233Met and Gln317Val) that increase both kinetic parameters.

Discussion

A number of researchers have argued that there is a link between temperature adaptation in enzyme function and changes in the amount of flexibility inherent in the enzyme molecule (Somero 1995; Feller and Gerday 1997; Fields and Somero 1998; Zavodszyk et al. 1998; Lonhienne, Gerday, and Feller 2000; D’Amico et al. 2002; Feller 2003). Although inherent changes in molecular flexibility do not appear to be the only method by which enzymes may adapt to cold (see, for example, Kim et al. 1999; Gerike, Danson, and Hough 2001; Svingor et al. 2001), a number of mutagenesis and modeling studies have confirmed that increased flexibility associated with decreases in activation energy often characterize cold-adapted enzymes (such studies include Tsigos et al. 2001; Collins et al. 2003; Georlette et al. 2003; Xu et al. 2003).

The reasoning behind this supposition is that for many enzymes, including A4-LDH (Dunn et al. 1991), the rate-limiting step to catalysis is a conformational shift during binding of ligands or release of product. According to this view, at colder temperatures, where less thermal energy is available in the environment to drive catalytically necessary conformational changes, catalytic rates are maintained at optimal levels—i.e., temperature compensation is produced—by decreasing the energy barriers to shifts between conformational states. We further have argued (Fields and Somero 1998; Fields 2001) that results
of this increase in conformational entropy in an ortholog adapted to cold compared to a warm-adapted ortholog include: (1) a decrease in Ea and an increase in $k_{cat}$, as lower energy barriers allow the cold-adapted form to proceed through the catalytic cycle more quickly; and (2) an increase in $K_m$, as increased conformational flexibility causes the cold-adapted ortholog to spend more time in conformational microstates that are not optimal for substrate binding. This model has received support from a recent study by D’Amico, Gerday, and Feller (2001), who found a decrease in $K_m$ and $k_{cat}$ (i.e., loss of cold adaptation) in mutants of a psychrophilic 3-amylose that had been designed to increase thermostability through increased molecular rigidity.

In our earlier study (Fields and Somero 1998) we showed that Antarctic notothenioid A2-LDHs indeed displayed increased $K_m^{Pyr}$ and $k_{cat}$ with respect to a group of more warm-adapted teleost orthologs. The Antarctic notothenioids, which spend their entire life cycle at extremely cold temperatures, have A2-LDH orthologs with the highest $K_m^{Pyr}$ and $k_{cat}$ values yet found. Here we have shown that a single amino acid substitution, Glu233Met (notothenioid → non-notothenioid), is sufficient to decrease $K_m^{Pyr}$ and increase $k_{cat}$ of a notothenioid A2-LDH to a level comparable to those of orthologs from much more warm-adapted teleosts.

There is, however, an alternate explanation for the differences we have found in the apparent $K_m^{Pyr}$ and $E_a$ of our recombinant A2-LDHs with respect to WT: it is possible that differences in the intracellular milieu between teleost white muscle and E. coli, such as changes in osmolality or osmolyte composition, could lead to modifications in the final conformation of the homotramer. These subtle structural changes might affect the kinetics of the expressed enzymes, irrespective of the presence of amino acid substitutions. There is growing evidence that A2-LDH kinetics indeed can be affected by the folding environment in vivo, either in individuals of the same species acclimated to different temperatures (e.g., cod, see Zakhartsev et al. 2003; loach, see Ozernyuk, Klyachko, and Polosukhina 1994), or in confamilial whose LDH-A primary structures are identical but whose kinetics are different (Gilleithys spp., see Fields and Somero 1997; Fields et al. 2002). However, in the present study we have shown that WT C. aceratus A2-LDH (that is, enzyme extracted directly from white muscle) has identical $K_m^{Pyr}$ and $E_a$ values to rWT A2-LDH expressed in E. coli (figs. 2–5). We take this as evidence that differences in kinetics between WT and our A2-LDH mutants indeed are due to the amino acid substitutions per se, and not to differences in cytosolic composition between E. coli and C. aceratus.

Antarctic notothenioid fishes are an especially appropriate model for testing cold adaptation, because of their rapid and recent invasion of the extremely cold polar marine habitat. Before ~25 MYA Antarctica was a part of the Gondwanan supercontinent, attached to South America by what is today the Antarctic peninsula. Before this time, the coastal waters around Antarctica were relatively temperate (~5°C–20°C). After 25 MYA, however, Antarctica separated from South America, opening the Drake Passage and allowing formation of the circum-polar current that isolated the region from more temperate waters. From the middle Miocene to the present, water temperatures around the Antarctic continent dropped to 0°C or below (Eastman 1993; Clarke and Johnston 1996), creating a novel environment where selection for cold adaptation was strong. These geological events indicate that the speciation of the Antarctic notothenioids likely occurred around 25 MYA or later, and molecular phylogenies of the Antarctic notothenioid families suggest an even more recent radiation. Comparisons of 12S and 16S mitochondrial rRNA genes indicate that Antarctic notothenioids diverged relatively recently (~10–15 MYA) in response to the rapid changes in the thermal environment around Antarctica (Bargelloni et al. 1994; Clarke and Johnston 1996). As a result of this recent speciation, the relatively few amino acid substitutions found in the Antarctic notothenioid LDH-A sequences relative to the non-notothenioid consensus (22/332, or 7%) are likely the products of positive selection for function in the cold polar environment rather than of neutral variation.

If modifications in molecular flexibility indeed are necessary during temperature adaptation, then amino acid substitutions with effects on enzyme kinetics, such as $K_m$ and Ea, should be localized to areas of the enzyme that must move during catalysis. Based on this conceptual framework, we (Fields and Somero 1998) hypothesized that temperature-adaptive changes in notothenioid A2-LDH structure were likely to reside near one or more of three specific structures in the molecule that had been described as “major movers” during catalysis (Gerstein and Chothia 1991): the “catalytic loop” region N-terminal to and including part of helix a2D, the region surrounding helix a1G-2G, and the region encompassing helix a2H at the C-terminus of the molecule (see figs. 1 and 6). These regions form the channel through which NADH and pyruvate must pass to access the active site (fig. 6). After ligands are bound, these three regions close together, creating a catalytic vacuole separated from the surrounding medium, in which the hydride transfer takes place. These structures must open again to allow release of lactate and NAD$^+$ and to return the enzyme to the apo form (Abad-Zapatero et al. 1987; Gerstein and Chothia 1991; Dunn et al. 1991).

Examination of figure 1 reveals that the “catalytic loop” region, although it moves the greatest amount during the catalytic cycle (~15Å; Gerstein and Chothia 1991), shows no differences between the Antarctic notothenioid and the non-notothenioid teleost LDH-A consensus sequences. Therefore, changes in the flexibility of this region cannot be responsible for cold adaptation in the Antarctic notothenioid orthologs. In contrast, there are a number of substitutions between the two consensus sequences near both the a1G-2G and the a2H helices. The β1-α1G loop region is directly N-terminal to the α1G-2G helix, a relatively disordered structure (Abad-Zapatero et al. 1987) that interacts with the solvent and may play a role in controlling the mobility of the catalytically important α1G-2G helix (Fields and Somero 1998). The 14 residues in the β1-α1G loop immediately N-terminal to the α1G-2G helix contain five differences between the two consensus...
sequences (36%, compared to 7% in the entire molecule). Of these, two (Gly213Thr and Gly 224Ser) involved replacement of polar residues in the warm-adapted forms with glycyI residues in the notothenioid orthologs. The substitution of glycyI residues in the cold-adaptive forms is suggestive, because addition of these residues increases the flexibility of the peptide backbone (Matthews, Nicholson, and Becktel 1987), and increased flexibility in the Jβ-1G loop region might confer greater mobility on the presumably rigid 1G-2G helix, allowing it more readily to undergo catalytically necessary conformational shifts. The results of site-directed mutagenesis replacing Gly213 or Gly224 with Thr or Ser, respectively, indicate that Eα of the mutant A4-LDHs indeed increases, suggesting that the loss of flexibility in the Jβ-1G loop region caused by either of these substitutions is sufficient to increase the activation energy barriers to catalysis. Importantly, however, neither of these mutants decreased K_m^PYR, and Gly224Ser instead increased it, suggesting that these mutants either have no effect on substrate binding affinity—presumably because they are far from the active site in a relatively loosely constrained loop—or may subtly disrupt the geometry of the binding site through effects on active site residues (e.g., Thr220) at the C-terminal end of helix 1G-2G. A third mutant from the same region, Gln217Lys, was examined in a preliminary study and showed no effect on K_m^PYR at 10°C. Although this mutant, which causes a loss of charge in the cold-adapted direction, might affect mobility of the region through long-range electrostatic effects or interactions with the solvent, preliminary tests suggested this is unlikely.

Interestingly, two other studies on teleost A4-LDHs implicated the Jβ-1G loop as a region important in temperature adaptation. Johns and Somero (2004) examined temperature adaptation in tropical and temperate damselfishes (Chromis spp.) and found that the relatively cold-adapted C. punctipinnis possesses an A4-LDH with lower K_m^PYR and K_m^act relative to the tropical orthologs and that these changes in kinetics can be attributed to one amino acid difference, Thr219Ala (temperate → tropical). The same substitution occurs in the notothenioids (Thr220Ala in fig. 1), but mutational analyses have not yet been performed on this residue. Holland, McFall-Ngai, and Somero (1997) examined A4-LDHs in temperate barracudas (Sphyraena spp.) and found that adaptive changes in kinetics could be explained by another single substitution, Asp8Gly, in the temperate → subtropical (S. argentea → S. lucasana) direction. This position appears far removed from the Jβ-1G loop, but in the quaternary structure of the A4-LDH homotrimer the αA helix, in which residue 8 resides, interacts with the Jβ-1G region of a neighboring monomer. Thus, both of these studies, along with the work presented herein, suggest strongly that the Jβ-1G loop region is an important site for temperature adaptation in A4-LDH, likely through effects on the mobility of the neighboring 1G-2G helix.

The second mobile helix of A4-LDH, αH, also had a substitution at its N-terminal end in notothenioid orthologs, with potential effects on mobility. Ala310Pro is a suggestive substitution, because prolyl residues uniquely constrain rotational freedom through the creation of a pyrrolidine ring between the α carbon and the amide nitrogen of the peptide backbone. The loss of this constraint in notothenioid A4-LDHs, with respect to the non-notothenioid consensus, suggested a mechanism by which helix αH would achieve greater structural mobility and hence reduce the activation energy necessary for catalysis. Indeed, results indicate that replacement of the notothenioid prolyl by the non-notothenioid prolyl at residue 310 increases Eα significantly (figs. 4 and 5). However, as with the addition of glycyI residues in the Jβ-1G region, this increase in mobility has no significant effect on K_m^PYR of the mutant. Again, this separation of effects on activation energy from binding affinity may be due to the position of Ala310Pro, far enough from the active site to avoid modifying active site geometry but directly associated with a structure, helix αH, that must move during catalysis.

It must be noted, however, that the presence of a prolyl residue at position 310 is not universal among vertebrate A4-LDHs, and a number of orthologs that would be predicted to be “warm-adapted” have an alternate residue at this site. Examples of species with such residues include, among others, humans (310Ser; accession number I110_A), the green iguana (Iguana iguana; 310Ser; accession number AA05098), and goby fishes such as the species used as a warm-adapted benchmark in this paper (G. mirabilis). It is clear, therefore, that despite the effects on K_m^PYR of substituting proline for alanine at this position in C. aceratus A4-LDH, the loss of 310Pro alone cannot be considered indicative of cold adaptation in A4-LDH orthologs.

In contrast to Ala310Pro, two other mutants, Gln317Val and Gly233Met, each affected both K_m^PYR and Eα of C. aceratus A4-LDH. Gln317Val had a moderate effect on both parameters, whereas Gly233Met increased both to a level comparable to those of an ortholog from the warm-temperate goby G. mirabilis (figs. 3–5). Unlike the mutants described above, which were located at the ends of the mobile helices 1G-2G and αH farthest from the active site, Gly233Met and Gln317Val are positioned within each of these helices, respectively. They therefore occur closer to the active site, and they may be in a position to impact the affinity of the enzyme for substrate. Homology modeling (fig. 6) indicates that the side chains of these two residues project into the medium, rather than into the catalytic vacuole. In addition, in no other LDH studied do residues in homologous positions interact with substrate or cofactor. Thus, it is unlikely that either affects K_m^PYR through interactions with pyruvate or NADH, and their effects must be indirect.

A number of mechanisms potentially might explain the effects of Gly233Met and Gln317Val on the K_m^PYR and Eα of C. aceratus A4-LDH, including (1) altered interactions of the mobile α-helices with the aqueous medium; (2) modified interactions between these residues, positioned on either edge of the cleft leading to the active site, and the ligands pyruvate and NADH; or (3) modified long-range electrostatic interactions that lead to changes in the mobility of these structures. The relative importance of these mechanisms is unclear, and further research must be performed to determine why structural changes in these
specific locations lead to the changes noted in the $K_{m,\text{PYR}}$ and $Ea$ of A$_4$-LDH.

Conclusion

The data presented in this paper support the hypothesis that amino acid substitutions associated with the mobile helices x1G-2G and x1H are responsible for the cold-adaptive changes in kinetics found in nototenioid A$_2$-LDHs. In addition, these results show that adaptive substitutions relatively distant from the active site, but associated with mobile structures (i.e., Gly213Thr, Gly224Ser, and Ala310Pro), affect activation energy barriers to catalysis, with cold-adapted orthologs having lower Ea values, but do not affect substrate affinity, as measured by $K_{m,\text{PYR}}$. In contrast, substitutions associated with mobile structures and positioned relatively close to the active site (i.e., Glu233Met and Gly 317Val) affect both $K_{m,\text{PYR}}$ and Ea. Further research will be necessary to determine whether similar amino acid substitutions, associated with catalytically important mobile secondary structures, can be found in orthologs of other cytosolic enzymes adapted to extreme cold.

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