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Hot spots in cold adaptation: Localized increases in conformational flexibility in lactate dehydrogenase A_4 orthologs of Antarctic notothenioid fishes

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ABSTRACT **To elucidate mechanisms of enzymatic adaptation to extreme cold, we determined kinetic properties, thermal stabilities, and deduced amino acid sequences of lactate dehydrogenase A_4 (A_4-LDH) from nine Antarctic (−1.86 to 1°C) and three South American (4 to 10°C) notothenioid teleosts. Higher Michaelis–Menten constants (K_m) and catalytic rate constants (k_{cat}) distinguish orthologs of Antarctic from those of South American species, but no relationship exists between adaptation temperature and the rate at which activity is lost because of heat denaturation. In all species, active site residues are conserved fully, and differences in k_{cat} and K_m are caused by substitutions elsewhere in the molecule. Within geographic groups, identical kinetic properties are generated by different substitutions. By combining our data with A_4-LDH sequences for other vertebrates and information on roles played by localized conformational changes in setting k_{cat}, we conclude that notothenioid A_4-LDHs have adapted to cold temperatures by increases in flexibility in small areas of the molecule that affect the mobility of adjacent active-site structures. Using these findings, we propose a model that explains linked temperature-adaptive variation in K_m and k_{cat}. Changes in sequence that increase flexibility of regions of the enzyme involved in catalytic conformational changes may reduce energy (enthalpy) barriers to these rate-governing shifts in conformation and, thereby, increase k_{cat}. However, at a common temperature of measurement, the higher configurational entropy of a cold-adapted enzyme may foster conformations that bind ligands poorly, leading to high K_m values relative to warm-adapted orthologs.**

Because of its pervasive effects, temperature plays a critical role in the evolution of organisms at all levels of biological organization, including macromolecular systems such as proteins (1). To study temperature adaptation of proteins, it is advantageous to compare differently adapted yet closely related species. This ensures that divergence times are short and that there are apt to be few differences in amino acid sequence among orthologs other than temperature-adaptive ones (2, 3). Furthermore, by comparing closely related species that have evolved under identical conditions of temperature, it may be possible to determine whether several types of amino acid substitutions can lead to the same end-result of adaptive modification in kinetic properties and structural stability.

An excellent study system for investigating adaptation of proteins to low temperature is provided by fishes of the teleost suborder Notothenioidei. Notothenioids have radiated to become the most speciose and numerically abundant fish taxa found south of the Antarctic polar front (4). Although hypothesized to be a group that originally was demersal, contemporary notothenioids inhabit many ecological niches in the Antarctic Ocean and normally live at temperatures between the freezing point of seawater, −1.86°C, and +1 to 2°C. Additionally, a number of notothenioid species are found exclusively north of the Antarctic polar front in New Zealand, Australia, and in southern South America, where they encounter water temperatures ≈5–10°C warmer than Antarctic species do (5). Although there is no fossil record for this suborder, the initial isolation and speciation of the notothenioid stock is assumed to have occurred as the Cretaceous Gondwanan landmass split up and the Drake passage between South America and Antarctica opened, allowing for the first time a circumpolar West Wind Drift that isolated Antarctica climatologically and oceanographically (4, 6). These geological events and the subsequent cooling of the Antarctic Ocean, which occurred between 20–25 million years ago and 10–14 million years ago, respectively, are theorized to have provided ancestral notothenioids an opportunity to occupy and adapt to an extremely cold and thermally stable environment. Molecular phylogenies support this scenario and suggest divergence dates for nonbovichtid notothenioids of 5–15 million years ago (7, 8). The extent to which Antarctic notothenioids have adapted to low, stable temperatures is revealed by their extreme stenothermy (9, 10), the presence of antifreeze glycoproteins in their blood (11), the loss in icefishes (family Channichthyidae) of hemoglobin (12, 13) and myoglobin (14), and other physiological adaptations (15).

To investigate how an enzymatic protein is adapted to low temperatures, we compared in nine Antarctic and three South American notothenioid species orthologs of A_4-lactate dehydrogenase (A_4-LDH; EC 1.1.1.27), a homotetrameric enzyme that converts pyruvate to lactate during anaerobic glycolysis. For all 12 orthologs, effects of temperature on the apparent Michaelis–Menten constant of pyruvate (K_m^{PYR}), resistance to heat denaturation, and deduced amino acid sequences were determined. Catalytic rate constants (k_{cat}) were measured for six species as well.

Although acute increases in temperature generally raise K_m values, for a given enzyme-substrate pair, K_m is highly conserved among differently adapted species at normal body temperatures (2, 3, 16, 17). For example, K_m^{PYR} for A_4-LDH is conserved within a narrow range (≈0.15–0.35 mM) at physiological temperatures. Similarly, at any single temperature of measurement, k_{cat} values tend to be higher for enzymes of cold-adapted than for those of warm-adapted species, such that rates of catalysis at physiological temperatures are partially temperature-compensated (18, 19).

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Abbreviation: A_4-LDH, A_4-lactate dehydrogenase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF079819–AF079830).

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It also has been found that the temperatures at which enzymes denature, as indicated by loss of catalytic activity, generally are correlated with adaptation temperature (17). Such differences in protein thermal stability are conjectured to be related to the need for the entire enzyme molecule to maintain the appropriate conformational flexibility at physiological temperatures, allowing the conformational changes that accompany binding and catalysis to occur at appropriate rates (17, 20). However, this correlation may break down when closely related species and differently adapted conspecifics are compared (2, 3, 21), suggesting that refinement of this hypothesis is required. It may be more appropriate to examine flexibility in the specific regions of an enzyme that govern the energetics of the conformational changes necessary for binding and catalysis. To this end, we have used existing information about the three-dimensional structure (22) and catalytically important conformational changes of A_4-LDH (23, 24) to interpret the differences in kinetic properties and amino acid sequence we have found among the A_4-LDHs of notothenioids and other vertebrates.

MATERIALS AND METHODS

Collection of Specimens. Antarctic notothenioids from four families (Nototheniidae: *Notothenia coriiceps*, *Lepidonotothen nudifrons*, *Gobionotothen gibberifrons*, and *Dissostichus mawsoni*; Channichthyidae: *Chionodraco rastrospinosus*, *Chaenocephalus aceratus*, and *Champsocephalus gunnari*; Bathydraconidae: *Parachaenichthys charcoti*; and Harpagiferidae: *Harpagifer antarcticus*) were collected by otter trawl from the R/V *Polar Duke* near Anvers Island on the Antarctic Peninsula in March and April, 1997. Muscle samples from three South American notothenioids, the nototheniids *Paranotothenia magellanica* and *Patagonotothen tessellata* and the eleginopsid *Eleginops maclovinus*, were collected in Patagonia. All Antarctic fish were held in ambient seawater (0–1°C) until killed, and muscle samples for A_4-LDH and mRNA purification immediately were placed at −70°C. Muscle samples from South American specimens were shipped on dry ice and stored at −70°C. Blue rockfish (*Sebastes mystinus*) were collected in Monterey Bay, CA, longjawed mudsuckers *(Gillichthys mirabilis*) were trapped near San Diego, and desert iguanas (*Dipsosaurus dorsalis*) were collected in the Mojave Desert, CA. The latter three species were used only for k_{cat} measurements.

Purification of A_4-LDH and Measurement of Apparent K_m^{PYR}, k_{cat}, and Thermal Stability. A_4-LDH was purified from 2 to 5 g of white epaxial skeletal muscle or leg muscle (*D. dorsalis*) by using an oxamate-agarose affinity column (16). Activity staining of native PAGE gels showed a single band of LDH activity, indicating that only the A_4-LDH isoform was present.

Apparent K_m^{PYR} was measured for A_4-LDH of each notothenioid species at 0, 5, 10, 15 and 20°C by using an imidazole buffer (80 mM imidazole·HCl, pH 7.0 at 20°C; refs. 16 and 25). Assays were performed in a thermostatted (±0.2°C) Perkin–Elmer Lambda 3B spectrophotometer. Three replicates of eight pyruvate concentrations (0.1, 0.125, 0.167, 0.2, 0.25, 0.333, 0.4 and 0.5 mM) were used to determine K_m^{PYR} values at each temperature. A computer program (WILMAN K_m-FITTING; ref. 26) calculated K_m^{PYR} and SD by using weighted linear regression (27).

k_{cat} values were determined at 0°C on a freshly purified enzyme, to minimize artifacts from denaturation. Theoretical maximal velocities were normalized to protein concentration to derive k_{cat} values. Protein was quantified by using the Bradford method (Bio-Rad).

To quantify thermal stability, residual activity of purified A_4-LDH after incubation at 50°C for 5, 10, 20, 40, and 60 min (20) was measured at 20°C in an imidazole buffer (80 mM imidazole·HCl/2 mM pyruvate/0.15 mM NADH, pH 7.0 at 20°C). Residual activity was determined by dividing the average of three replicates of activity at each time point by the

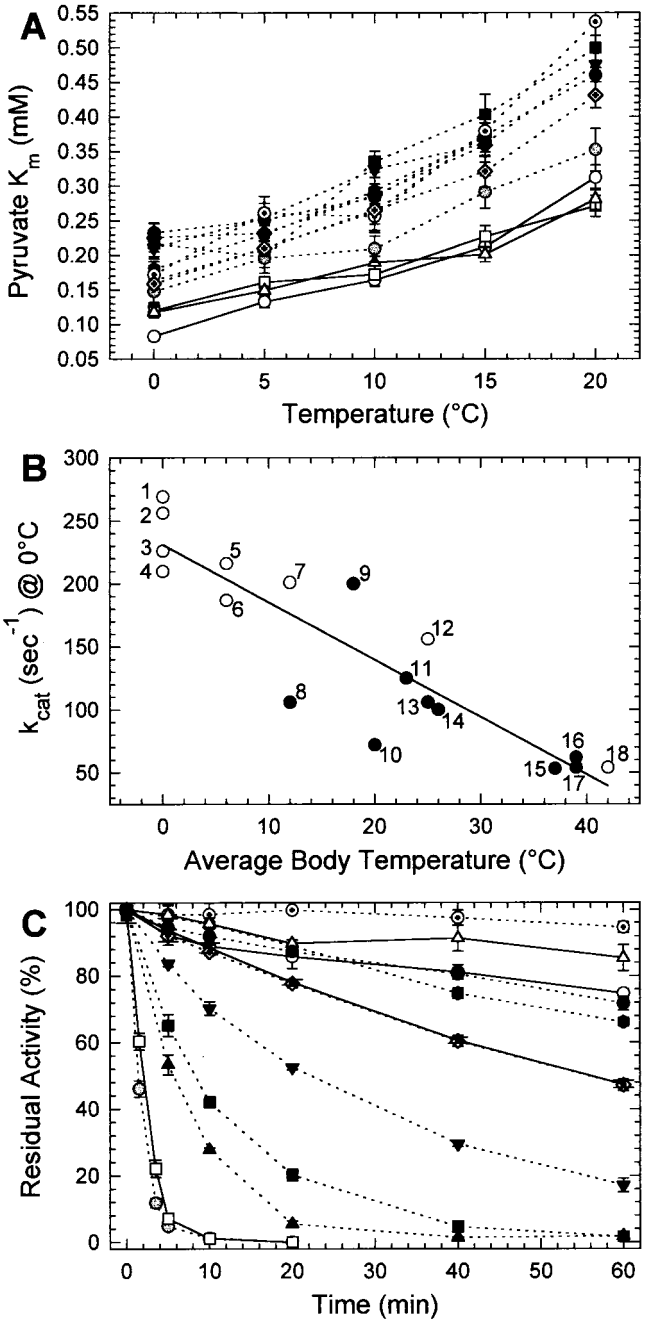

FIG. 1. (*A*) Apparent K_m^{PYR} of A_4-LDH from 12 species of notothenioids: *N. coriiceps* (■); *L. nudifrons* (×); *G. gibberifrons* (◍); *D. mawsoni* (⊙); *C. rastrospinosus* (●); *C. aceratus* (●); *C. gunnari* (▲); *P. charcoti* (▼); *H. antarcticus* (◇); *P. magellanica* (○); *P. tessellata* (□); *E. maclovinus* (△). (*B*) Correlation between average body temperature and k_{cat} of A_4-LDH measured at 0°C. Open symbols represent data collected in this study; those taken from earlier studies are shown by closed symbols. 1, *P. charcoti*; 2, *L. nudifrons*; 3, *C. gunnari*; 4, *H. antarcticus*; 5, *P. tessellata*; 6, *E. maclovinus*; 7, *S. mystinus* (rockfish); 8, *Hippoglossus stenolepis* (halibut) (28); 9, *Sphyraena argentea* (barracuda) (18); 10, *Squalus acanthias* (dogfish) (28); 11, *Sphyraena lucasana* (18); 12, *Gillichthys mirabilis* (goby) (2); 13, *Thunnus thynnus* (bluefin tuna) (28); 14, *Sphyraena ensis* (18); 15, *Bos taurus* (cow) (28); 16, *Gallus gallus* (chicken) (28); 17, *Meleagris gallopavo* (turkey) (28); 18, *D. dorsalis* (desert iguana). Linear regression: $y = -4.6x - 231$; $r^2 = 0.81$. (*C*) Thermal denaturation profiles of A_4-LDH from 12 species of notothenioids, incubated at 50°C. Symbols are as in *A*.

average of three replicates of a control sample, which had been held on ice.

Deduced Amino Acid Sequences. Messenger RNA was purified from ≈50 mg of white muscle tissue by using the Dynabead mRNA Direct system (Dynal, Great Neck, NY). Preparation of cDNA from mRNA and amplification of the *ldh-a* message was performed in a single-tube reaction (Access Reverse Transcription–PCR System, Promega) by using primers derived from barracuda (*Sphyraena* spp.) *ldh-a* sequences (3). Sequencing gels were run on an Applied Biosystems 373A automatic DNA sequencer by using fluorescent dye-labeled terminators (Applied Biosystems Prism dye terminator cycle sequencing kit, Perkin–Elmer). Resultant sequences were aligned and analyzed by using the GCG software package (Oxford Molecular Group, Campbell, CA). The three-dimensional structure of dogfish LDH-A monomer was retrieved from the Protein Data Bank, Biology Department, Brookhaven National Laboratory (Upton, NY; accession no. 6LDH; ref. 22) and was viewed with the RASMOL software package (Glaxo).

RESULTS

Apparent K_m^{PYR} and Catalytic Rate Constants. At all temperatures of measurement, K_m^{PYR} was higher for A_4-LDHs of the Antarctic than for the South American species (Fig. 1*A*). This trend is consistent with previous studies of K_m^{PYR} vs. temperature relationships in A_4-LDHs of vertebrates adapted to widely different temperatures (2, 3, 16). The average k_{cat} value for orthologs of Antarctic notothenioids is 240 s^{-1} (Fig. 1*B*), a value that is >4-fold higher than the A_4-LDH k_{cat} for the most warm-adapted species studied, the desert iguana *D. dorsalis* (54 s^{-1}), and is ≈1.5 times higher than the k_{cat} determined for the A_4-LDH of a warm-temperate fish, *G. mirabilis* (156 s^{-1}). The k_{cat} of the ortholog of the cold-temperate fish *S. mystinus* (201 s^{-1}), which encounters water temperatures as low as ≈5°C, is only slightly lower than the k_{cat} values of the A_4-LDHs of Antarctic fishes. The A_4-LDH k_{cat} of one of the South American notothenioids, *P. tessellata,* is within the range of values found for orthologs of the Antarctic species (216 s^{-1}), but the k_{cat} of the ortholog of the more distantly related notothenioid *E. maclovinus* is ≈35% lower (187 s^{-1}).

Thermal Stability. Thermal stabilities of notothenioid A_4-LDHs did not show the consistent differences between Antarctic and South American species that were found for kinetic properties (Fig. 1*C*). After 20 min incubation at 50°C, A_4-LDHs of *P. tessellata* and *G. gibberifrons* had no residual activity whereas the *D. mawsoni* ortholog retained most of its original activity. The other orthologs fell within these two extremes but with no relation to environmental temperature, phylogenetic relationship, or K_m^{PYR} and k_{cat} values.

Deduced Amino Acid Sequences. The deduced amino acid sequences for 11 of 12 notothenioid A_4-LDHs are highly similar, as expected, given the inferred divergence times of the taxa. All of the species studied, except *E. maclovinus*, have one to four differences with respect to the 331-residue notothenioid A_4-LDH consensus sequence (Fig. 2). Only *C. aceratus* and *C. rastrospinosus* share identical primary structures. Substitutions occur at numerous sites in the molecule but are not distributed randomly. No substitutions occur in the catalytic loop region (amino acids 96–113; ref. 22) or in substrate and cofactor binding residues found elsewhere in the sequence (22, 30–32). (Note that the residue numbering system used in this paper is based on the notothenioid consensus sequence except

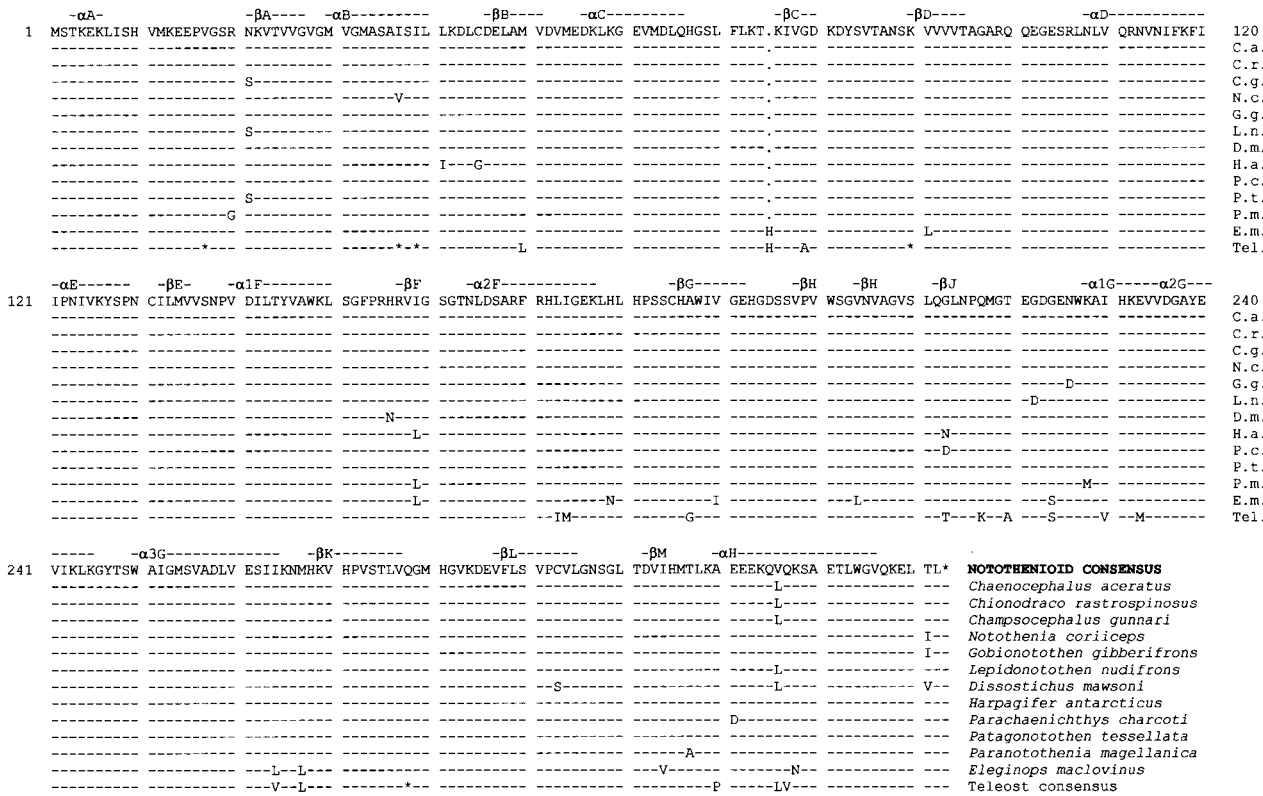

FIG. 2. Deduced amino acid sequences of LDH-A for 12 species of notothenioid fishes. The top line provides the consensus sequence, a dash indicates identity with the consensus, and a dot indicates a deletion. The bottom line is the consensus sequence of LDH-A for six non-notothenioid teleost species [the killifish *Fundulus heteroclitus* (29), the goby *G. mirabilis* (2), the barracudas *Sphyraena idiastes* and *S. lucasana* (3), the eelpout *Austrolycos depressiceps*, and the lampfish *Lampanyctus ritteri* (P.A.F. and G.N.S., unpublished data)]. In the teleost consensus sequence, an asterisk indicates residues evenly divided among the species; in each case, one of the residue pairs agrees with the notothenioid consensus, and the substituted residue is chemically similar.

in the case of His-75, which is missing in all notothenioid sequences except that of *E. maclovinus*.)

Orthologs of two South American nototheniid species, *Patagonotothen tessellata* and *Paranotothenia magellanica*, do not vary from the consensus sequence to a greater extent than do the Antarctic orthologs, possessing one and four differences, respectively. This is noteworthy, given the difference in K_m^{PYR} values between South American and Antarctic forms and the congruence in K_m^{PYR} within geographic groupings (Fig. 1*A*). In contrast, the sequence of A_4-LDH of the third South American species, *E. maclovinus*, which recently has been reassigned from the family Notothenioidae to a new family (Eleginopsidae; ref. 33), is notably divergent from the rest of the species examined. It has 11 differences with respect to the consensus sequence, including His-75, a position where the rest of the notothenioids share a deletion. This histidine is conserved in all other teleosts for which the A_4-LDH sequence is known (2, 3).

DISCUSSION

Using these data on kinetics, thermal stabilities, and deduced amino acid sequences of A_4-LDH, plus information on changes in enzyme conformation essential for the catalytic process, we discuss the following points: (*i*) The divergent evolutionary thermal histories of the Antarctic and South American species have led to temperature-adaptive changes in A_4-LDH kinetics but not in resistance to heat inactivation. (*ii*) Comparisons among notothenioid A_4-LDH orthologs indicate that only minor differences in primary structure outside the active site are necessary for modification of kinetic properties. (*iii*) The cold-adapted kinetic properties of notothenioid A_4-LDHs appear to have arisen through changes in conformational flexibility in areas of the molecule that control structural movements known to be rate-limiting for catalysis. (*iv*) These findings suggest a model of enzymatic adaptation to temperature in which cold-adapted orthologs possess higher conformational entropy, that is, occupy a broader distribution of conformational states at a given temperature than do warm-adapted orthologs.

Temperature-Adaptive Variation. For all notothenioid species, similar amino acid sequences lead to two distinct patterns of K_m^{PYR} vs. temperature relationships that correlate with the habitat temperature of each species (Fig. 1*A*). A number of studies have shown that A_4-LDH orthologs of vertebrates maintain K_m^{PYR} values within a relatively narrow range of pyruvate concentration when measurements are made across the ranges of body temperature the species normally experience (2, 3, 16). This pattern is repeated in the Antarctic and South American notothenioids. The polar species (-1.86 to $+1$°C; refs. 4, 34, and 35) have A_4-LDH orthologs with higher K_m^{PYR} values at any given temperature than do the South American species (4 to 10°C; refs. 5 and 36). When K_m^{PYR} values within the environmental temperature range of each species are compared across species, however, it becomes apparent that the affinity of the enzyme for pyruvate has been conserved.

k_{cat} values for notothenioid A_4-LDHs also show a high degree of temperature compensation when compared with orthologs of more warm-adapted species (Fig. 1*B*). By using a temperature coefficient (Q_{10}) of 2.0 to adjust k_{cat} values determined at higher temperatures to a common temperature of 0°C, the estimated k_{cat} values for orthologs of the endotherms chicken, turkey, and cow are 62, 54, and 53 s^{-1}, respectively (28). For A_4-LDHs of other species of fish, k_{cat} values at 0°C are 106 s^{-1} for bluefin tuna and halibut, 72 s^{-1} for dogfish (28), and 200, 125, and 100 s^{-1} for orthologs of cold-temperate, subtropical, and tropical barracuda fishes, respectively (18).

Among the A_4-LDHs of notothenioids, heat denaturation of enzymatic activity (Fig. 1*C*) is not correlated with habitat temperature or values of K_m^{PYR} and k_{cat}. Although a positive correlation between thermal stability of A_4-LDH and habitat temperatures has been found for vertebrates whose body temperatures range from -1.86 to $\approx$40°C by using the physical technique of acrylamide quenching (37), a number of studies that measured residual activity after heat denaturation have shown that the correlation does not always extend to comparisons among closely related species (2, 3, 21). It should be noted that measurements of global stability by using the latter method do not give a free energy of unfolding and may be contaminated by effects of aggregation that are not related to the free energy of stabilization. Thus, heat denaturation of activity may be at best a crude measure of the role played by altered conformational flexibility in adapting kinetic properties to temperature.

Intra-notothenioid Sequence Comparisons. Two primary conclusions can be drawn by comparing kinetic and sequence data among the notothenioid A_4-LDHs. First, similar kinetic properties characterize orthologs with different primary structures. Among the Antarctic notothenioids, none of the 15 amino acid differences among the nine species is correlated with differences in K_m^{PYR} or k_{cat}, except in the case of A_4-LDH of *G. gibberifrons*, which exhibits a flatter K_m^{PYR} vs. temperature response than orthologs of other Antarctic species. Likewise, for orthologs of the three South American notothenioids, different sequences are associated with similar K_m^{PYR} and k_{cat} values. Although orthologs of two of the South American species, *Patagonotothen tessellata* and *Paranotothenia magellanica*, have identical K_m^{PYR} vs. temperature profiles (Fig. 1*A*), none of their differences with consensus sequence is shared. *Patagonotothen tessellata* has only one such difference: Asn21Ser. *Paranotothenia magellanica* has four: Arg20Gly, Leu159Ile, Lys228Met, and Thr307Ala. Three of these are not conservative, yet, as a group, they result in the same K_m^{PYR} profile as that produced by the Asn21Ser substitution of *P. tessellata*. The finding that different sequences yield similar kinetics suggests either that some substitutions are without effect on kinetics (although they may modify thermal stability) or that comparable shifts in kinetic properties can be achieved by different suites of amino acid substitutions.

Second, similar primary structures can lead to different kinetic properties. This is illustrated by the comparison between the South American notothenioid *P. tessellata* and the Antarctic *C. gunnari*. As described above, *P. tessellata* diverges from the notothenioid consensus sequence by only one residue (Asn21Ser); *C. gunnari* has the same substitution plus one other, Val316Leu. However, both the K_m^{PYR} vs. temperature profiles (Fig. 1*A*) and thermal stabilities (Fig. 1*C*) of these orthologs are notably different. That such a chemically conservative substitution outside the active site of the enzyme would have a significant impact on the kinetics and thermal stability of the molecule might be considered unlikely, but a precedent for this type of effect has been found in barracuda A_4-LDHs (3), in which a single amino acid substitution at position 8 led to adaptive differences in K_m^{PYR} between congeners. These results indicate that minor modifications distant from the active site may lead to adaptive changes in the function of enzymes. A mechanism by which such modifications might affect rates of catalysis is discussed below.

Structure and Function: Notothenioid vs. Non-notothenioid Sequences. The mechanism by which LDH catalyzes the reduction of pyruvate has been conserved strongly, and, as a result, there is little difference in the orientation and interactions among enzyme and ligands in ternary-LDH from organisms as divergent as bacteria, dogfish, and humans (38). This finding, along with the remarkable conservation of active site residues among A_4-LDHs of disparate taxa (24), indicates that adaptive changes in kinetic properties are unlikely to occur

through modification of direct interactions among substrates and enzyme. Instead, it is changes in residues outside the active site that appear to alter the kinetics of A_4-LDH during adaptation to new temperatures (2, 3). It is likely that such substitutions exert their effects on catalytic rate and binding through changes in the flexibility and mobility of different areas of the enzyme. This is supported by evidence indicating that, compared with rate-limiting conformational changes during substrate binding, hydride transfer from NADH to pyruvate is quite rapid ($>$1,000 s^{-1}; ref. 23). Thus, changes in k_{cat} must be mediated by altered flexibility of A_4-LDH substructures rather than by changes in the rate of substrate reduction. Gerstein and Chothia (24) showed that A_4-LDH is composed of concentric shells of mobility, with the static core packed at the center of the tetramer and increasingly mobile structures occurring nearer the surface, culminating with the catalytic loop, which travels 15 Å when shifting from the apo- to the ternary form. We use this description of A_4-LDH to develop a model in which notothenioid orthologs have evolved higher k_{cat} and K_m^{PYR} values by increasing the flexibility of structures important in the binding and release of substrates.

To determine likely sites of change during notothenioid evolution, we have compared the notothenioid A_4-LDH consensus amino acid sequence with a consensus sequence derived from orthologs of other teleosts (Fig. 2). Such a comparison reveals two major areas of divergence within the sequences: the C-terminal α-helix of the LDH-A monomer, αH, and the extended loop region connecting strand βH to helix α1G (see Figs. 2 and 3).

Helix αH forms one margin of the active site into which cofactor and substrate enter before the catalytic loop closes (Fig. 3). Helix αH is in close contact with the αD helix of the catalytic loop, and αD must slide past αH during catalytic loop closure (24). In the majority of teleosts, as well as in dogfish, a Pro residue initiates the αH helix. The steric limitations imposed by this Pro may restrict the number of possible positions of αH relative to αD. In notothenioids, Pro has been replaced by Ala, which, through the easing of rotational constraints, should allow greater flexibility to this region. Additionally, in the notothenioid αH the teleost pattern of Leu316-Val317 has become Leu/Val316-Gln317, which may reduce hydrophobic interactions between αH and residues on the surface of the opposing αD helix.

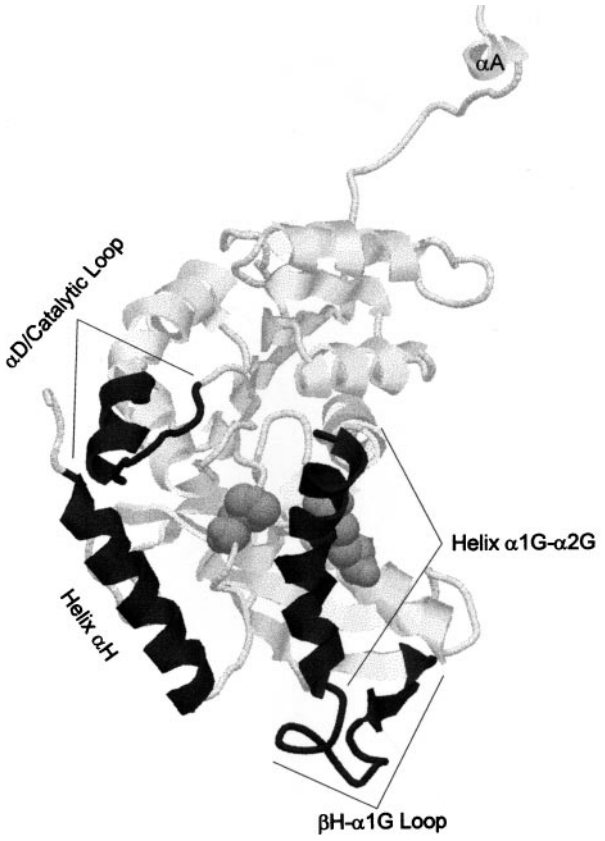

FIG. 3. Cartoon of dogfish A_4-LDH structure after Abad-Zapatero *et al*. (22). One monomer is shown, with the secondary structure discussed in the text labeled. His193 and Arg169 are space-filled to indicate the location of the active site.

The movement of αH is one of the conformational events that are necessary for binding and release of ligands and that are rate-limiting to catalysis (23). If changes found in notothenioid αH add flexibility to the LDH molecule in the vicinity of the catalytic loop and active site, then reduced interference between αH and αD could lead to a more rapid closure of the catalytic loop on ligand binding, thereby increasing k_{cat}.

A second area of variability in sequence between A_4-LDHs of notothenioids and other teleosts occurs in a disordered loop region between strand βH and helix α1G (amino acids 207–226), which includes strand βJ (Figs. 2 and 3). This region is variable within the teleosts (3) as well as among other vertebrates for which LDH-A amino acid sequences are known (24). The βH-α1G loop connects a relatively static area of the active site (including residues Asp-166, Arg-169, and His-193) with catalytically important residues, including Tyr-239 and Thr-248, that are located on helix α1G/α2G. Like the movements of αH and αD, a conformational change involving an inward shift of α1G/α2G on ligand binding is necessary for enzyme function (23, 24). In the dogfish apo-LDH-A crystal structure (22), the βH-α1G loop is constrained poorly and has the highest temperature factor of the entire molecule, indicating that it is relatively flexible. Portions of the loop are exposed to the solvent, and other areas interact hydrophobically with the N-terminal αA helix of a neighboring LDH-A monomer. Helix α1G/α2G presumably remains rigid because of hydrogen bonding, but its shift as a whole during catalysis is caused partly by the flexibility of the βH-α1G loop region.

Differences between notothenioids and other teleosts in this loop region include Thr213Gly and Thr/Ser224Gly substitutions, which, in notothenioids, increase the number of Gly residues in the 20-residue region from 3 to 5. In orthologs of cow and chicken, only two glycyls are in this loop (see ref. 3). It should be noted that the substitution at position 224 has not occurred in the ortholog of the South American species *E. maclovinus*, which has a low k_{cat} relative to other notothenioid A_4-LDHs. Because glycyls increase the conformational entropy of a peptide (39), the additional glycyl residues in notothenioid fishes may serve to enhance the flexibility of this region and thus provide the α1G/α2G helix greater mobility.

Further differences between the consensus sequences of notothenioids and non-notothenioid teleosts in this region are Ala220Thr and Met233Glu. Each of these substitutions serves to add a polar group and may destabilize the region by either enhancing interactions with the solvent (40) or reducing hydrophobic interactions with the αA of the neighboring monomer. These effects, in turn, should further increase the flexibility of the loop and, consequently, the mobility of the α1G/α2G helix.

Helix α1G/α2G sits across the active site entrance from αH, with the catalytic loop closing between them (Fig. 3). It has been shown that the catalytic loop region is conserved strongly in all LDHs (22, 24), in part because certain residues in the catalytic loop are involved in binding to substrate. Presumably, these conserved residues, as well as others in the active site (e.g., His193 and Arg168), cannot be altered during adaptation to cold temperature without loss of enzymatic activity. Therefore, if increased flexibility in the active site region is necessary for cold-adapted LDHs to achieve high k_{cat} values at low temperatures, areas most likely to show adaptive substitutions

would be those that help to govern the ease of movement of catalytically important elements of secondary structure such as α1G/α2G, αH, and the catalytic loop.

Conformational Flexibility and Protein Adaptation to Temperature. We have proposed that the sequence changes underlying adaptation of notothenioid A_4-LDHs to cold temperature allow a higher degree of flexibility in areas that move during catalysis and that an increase in flexibility in these areas should increase k_{cat} by reducing the energetic cost of conformational change from the apo- to the holoenzyme. In addition, these findings form the basis of a model that explains the covariation observed in K_m^{PYR} and k_{cat} among orthologs from different species. We summarize this model as follows.

The flexibility in any enzyme, including A_4-LDH, necessary for function causes each molecule to occupy an ensemble of conformational states (41, 42). A change in temperature is likely to alter the number of states available, the rate at which molecules shift among these states, and the time spent in any given state. Starting from these basic principles, we postulate that differences among orthologs in K_m and k_{cat} derive from inherent differences in conformational entropy, that is, in the number of conformational states accessible to an ortholog at any given temperature.

According to this hypothesis, temperature-adaptive increases in k_{cat} will occur concomitantly with increases in K_m. This can be seen by comparing cold- and warm-adapted enzymes at a common temperature. The higher conformational mobility (for example, in αH, α1G/α2G, and the catalytic loop of A_4-LDH) required for rapid catalysis (high k_{cat}) in the cold-adapted form will lead to a higher number of conformational states available to the molecule and, as a result, to a larger proportion of enzyme conformations that bind substrates poorly or not at all. This will yield a higher K_m in the cold-adapted enzyme. Conversely, at this common temperature, the enzyme adapted to function at high temperatures will have less inherent conformational flexibility, the proportion of binding-competent states will be higher, and K_m (as well as k_{cat}) will be lower.

The inherent differences in conformational flexibility between cold- and warm-adapted enzymes also will be manifested in their responses to acute temperature changes. Q_{10} is a direct measure of the rise in k_{cat} with temperature and reflects thermal effects on rate-limiting conformational changes. Q_{10} values of cold-adapted orthologs are lower than those of warm-adapted ones (43), indicating lower activation enthalpies ($\Delta H^{\ddagger}$) that we argue are caused by increased localized flexibility. Conversely, activation entropies ($\Delta S^{\ddagger}$) for cold-adapted enzymes are higher (43), revealing the greater degree of ordering these orthologs must undergo to form the ternary complex. In summary, sequence changes outside the active site can alter the enthalpy and entropy of conformational changes of importance in binding and catalysis; these effects should play a critical role in evolutionary adaptation of k_{cat} and K_m, and in establishing the responses of these kinetic parameters to acute temperature changes.

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