

**MECHANISMS OF ADAPTATION TO TEMPERATURE
IN FISH LDHS: FROM ANTARCTIC ICE
TO THE MEXICAN DESERT**

Peter A. Fields
Hopkins Marine Station, Stanford University
Oceanview Blvd., Pacific Grove, CA 93950 USA
Phone: 001-831-655-6238/Fax: 001-831-375-0793/email:
pfields@leland.stanford.edu

George N. Somero
Hopkins Marine Station, Stanford University
Oceanview Blvd., Pacific Grove, CA 93950 USA
Phone:001-831-655-6243/Fax: 001-831-0793/email:
somero@leland.stanford.edu

Introduction

Fish occur in environments representing extremes of temperature, pressure, salinity and desiccation, and to survive these extremes they have evolved a broad array of physiological and biochemical modifications. While the internal milieu can be shielded from some physical factors, such as osmotic stress, others, including pressure and temperature, necessarily affect the organism on the molecular level, and it is here that adaptations to these stresses are most fruitfully studied. In order to understand better how enzymes evolve to cope with varying thermal environments, we have examined the glycolytic enzyme A₄-lactate dehydrogenase (A₄-LDH, EC 1.1.1.27, NAD⁺:lactate oxidoreductase) from a diverse array of fishes. In each study, we have compared orthologs from closely related species that occur in differing thermal habitats, in order to ensure that the differences in structure and function we find are adaptive, and not accidents of phylogenetic history. Fish taxa we have studied include notothenioid species from polar Antarctic and temperate South American waters (Fields and Somero, 1998), barracudas (genus *Sphyraena*; Holland et al.,1997) from temperate to tropical waters, and gobies (*Gillichthys* and *Coryphopterus*; Fields and Somero, 1997) from the temperate California subtidal to the intertidal bordering the Mexican Sonoran desert. Our goal has been to determine how much change in environmental temperature is necessary to induce a change in A₄-LDH function, as measured by apparent Michaelis constants for the substrate pyruvate (K_m^{PYR}), and turnover number (k_{cat}). In addition we have described the

changes in structure (i.e., amino acid sequence) responsible for these modifications.

Antarctic notothenioids have adapted to their polar niche in the ~15my since Antarctic waters dropped to below 0°C. In this short period of time their A₄-LDHs have responded by developing lower substrate affinities (higher K_m 's) and higher turnover rates (higher k_{cat} 's), as compared to South American notothenioids living in water at ~5-10°C. These functional changes can be ascribed to a limited number of substitutions within the primary structure of A₄-LDH. Similarly, barracuda congeners, some of which live in waters with mean temperatures only ~5°C apart, also show adaptive changes in kinetic parameters. In this case, due to the very limited number of amino acid substitutions among the orthologs, changes responsible for modifying K_m^{PYR} can be identified unambiguously. Among the gobies we studied, again the close relatedness of the species ensured that only a few amino acid substitutions were responsible for the kinetic differences seen between the *Coryphopterus* and *Gillichthys* A₄-LDH orthologs. Surprisingly, two congeners of *Gillichthys*, *G. mirabilis* and *G. seta*, possess orthologs that show distinct substrate affinities and thermal stabilities, and yet have no differences in amino acid sequence. This suggests that enzymes of very closely related species may adapt to changing thermal environments via mechanisms other than alteration of primary structure.

Methods

Sphyraena lucasana and *S. argentea* were collected by hook and line at Guaymas, Mexico (average water temperature 23°C) and La Jolla, CA, USA (18°C), respectively. *Gillichthys mirabilis* and *G. seta* were collected near San Felipe, Baja California Norte, Mexico. *Gillichthys mirabilis* was collected using baited minnow traps from an estuary where temperatures were measured at >30°C, and *G. seta* was collected by dip net from a cobble beach where water temperature as the tide rose exceeded 40°C. Individuals of all species were frozen on dry ice at the site of collection and transported back to the laboratory, where they were stored at -80°C until used. Antarctic notothenioid fishes (habitat temperature ~-1°C) were collected by otter trawl at depths of approximately 100 m from the R/V Polar Duke near Palmer Station, Antarctica. Specimens were frozen immediately at -80°C, shipped on dry ice to the United States, and stored at -80°C until use. Dr. Ian Johnston of the Gatty Marine Laboratory caught South American notothenioid fishes in Patagonia, and he provided the white muscle samples used in this study as a kind gift.

A₄-LDH was purified from each species using affinity chromatography (Yancey and Somero, 1978). White muscle tissue was homogenized in 50 mM potassium phosphate, pH 6.8, and centrifuged at 10,000 g for 30 min. Potassium chloride and NADH were added to the homogenate supernatant at concentrations of 500 mM and 200 μM, respectively, and the supernatant was passed across an oxamate-sepharose column. After washing with excess NADH, pure A₄-LDH was eluted with 10 mM pyruvate in buffer. Fractions with high A₄-LDH activity were pooled and dialyzed overnight against 4 L 50 mM potassium phosphate, pH 6.8.

The parameters K_m^{PYR} and k_{cat} were determined by measuring the activity of each enzyme spectrophotometrically. Assays were performed in a temperature-controlled cuvette, with 80 mM imidazole (pH 7.0 at 20°C), 150 μM NADH, and eight concentrations of pyruvate chosen to straddle the expected K_m^{PYR} value. Reaction rates were measured at 340 nm, and these rates were input into a computer program (Wilman4; Brooks and Suelter, 1986) along with the corresponding pyruvate concentrations to determine K_m^{PYR} and maximal velocity (V_{max}). To derive k_{cat} from V_{max} , LDH concentrations were measured using a spectrophotometric assay based on Coomassie staining (Pierce Coomassie Plus, Rockford, IL, USA).

In order to obtain derived amino acid sequences of LDH-A from each species, mRNA was purified from white muscle tissue using the Dynabeads poly-thymidine magnetic bead protocol (Dyna, Great Neck, NY, USA). Complementary DNA was prepared from the mRNA using reverse transcriptase, and LDH-A cDNA was amplified using primers based on barracuda (*Sphyraena* spp.) LDH-A (Holland et al., 1997). PCR-amplified LDH-A cDNA was then sequenced using a dye-terminator protocol (Applied Biosystems Prism dye terminator kit) on an Applied Biosystems 373A automated sequencer. Resultant LDH-A sequences were aligned and analyzed using GCG (Oxford Molecular Group, Campbell, CA, USA) and DNASTar (DNASTar, Inc., Madison, WI, USA) software.

Results and Discussion

These studies have had three related goals: i) To describe how A₄-LDH kinetic parameters such as K_m^{PYR} and k_{cat} vary with environmental temperature in marine fishes, ii) To determine the structural differences (i.e., amino acid

substitutions) in A₄-LDH orthologs that underlie adaptively modified reaction kinetics, and iii) To explain how these amino acid substitutions among orthologs lead to changes in the kinetic parameters we have measured.

It is clear from measurements of both K_m^{PYR} and k_{cat} that the kinetics of A₄-LDH of marine fishes, like other vertebrates that have been studied (Somero, 1995), are adaptively modified in response to changes in environmental temperature. Figure 1 shows K_m^{PYR} values for representative Antarctic and South American notothenioids (Fields and Somero, 1998), *S. lucasana* and *S. argentea* (Holland et al., 1997) and *G. mirabilis* and *G. seta* (Fields and Somero, 1997) A₄-LDH orthologs in relation to measurement temperature. At any measurement temperature the A₄-LDH orthologs from more cold-adapted species have higher K_m^{PYR} values, indicating a lower affinity to the substrate pyruvate. However,

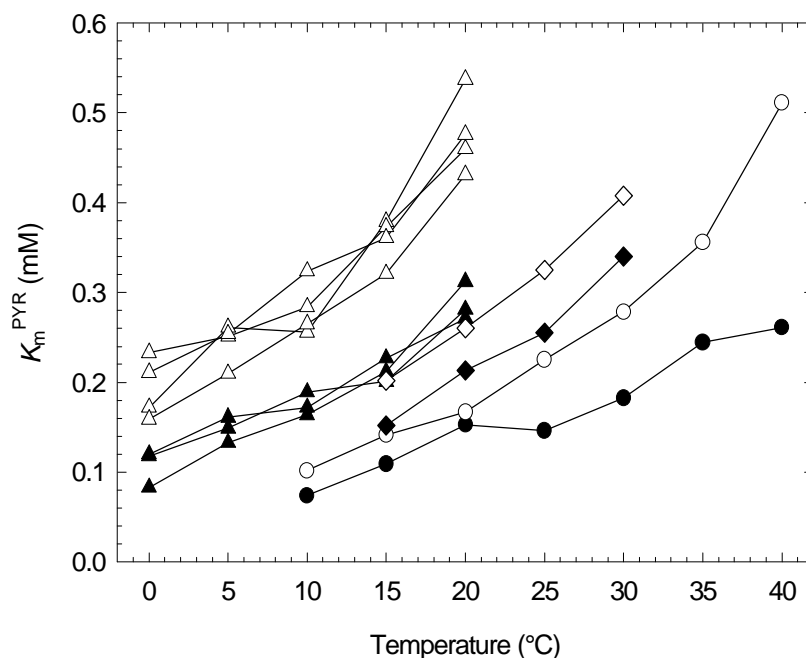


Figure 1. K_m^{PYR} of A₄-LDHs vs. temperature. Antarctic notothenioids -- open triangles; South American notothenioids -- closed triangles; *Sphyræna argentea* -- open diamonds; *S. lucasana* -- closed diamonds; *Gillichthys mirabilis* -- open circles; *G. seta* -- closed circles.

when compared at physiological temperatures (i.e., 0°C for Antarctic notothenioids, 5-10°C for South American notothenioids, 13-23°C for *S. argentea*, 16-28°C for *S. lucasana*, 20-30°C for *G. mirabilis* and 20-40°C for *G. seta*), the K_m^{PYR} values are comparable, indicating compensation to the amount of thermal energy present in the environment.

We also compared k_{cat} values for some of these species, as is shown in Figure 2. In this figure, all k_{cat} values have been corrected to 0°C using a Q_{10} of 2.0. As with K_m^{PYR} values, k_{cat} 's also show compensation for the temperatures each species experiences. In this case, the rate at which any A₄-LDH ortholog catalyzes its reaction is correlated with environmental temperature; the more cold-adapted enzymes have higher k_{cat} values.

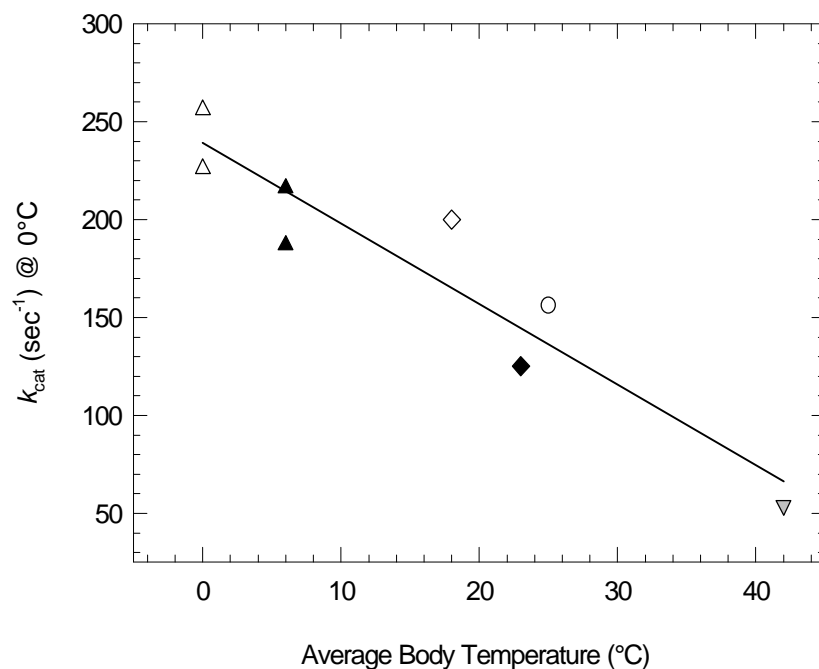


Figure 2. k_{cat} 's of A₄-LDHs (at 0°C) vs. habitat temperature. Symbols as in Figure 1; stippled upside-down triangle -- *Dipsosaurus dorsalis* (desert iguana).

In order better to understand these phenomena of temperature compensation in the reaction kinetics of A₄-LDHs, we compared the primary structures (amino acid sequences) of the orthologs of each species. Despite the clear relationship between adaptation temperature and enzyme function, we were not able to identify any general modifications of protein structure that might underlie the changes in kinetics we measured. Table 1 shows a series of structural parameters for A₄-LDH orthologs from fishes, arranged in order of increasing environmental temperature. Each of the attributes listed has been hypothesized to have some effect on enzyme function at different temperatures, usually by modifying the rigidity of the molecule in question. For example, it is expected that cold-adapted enzymes will have more glycyl residues than orthologs from

warmer-adapted orthologs, because glycine, which has no side chain to sterically limit rotation of the polypeptide backbone, allows greater flexibility of protein structure (Matthews et al., 1987). Conversely, it has been hypothesized that cold-adapted enzymes will possess fewer prolyl residues, because the unique structure of the prolyl pyrrolidine ring restricts the rotational freedom of the peptide backbone (Creighton, 1993).

Other structural modifications that are expected to affect enzyme function at different temperatures include the amount of hydrophobic residues, the number of polar residues capable of hydrogen bonding, and the number of charged residues involved in salt bridges (Feller and Gerday, 1997; Jaenicke, 1991). On a secondary structural level, increased proportions of unstructured loops and turns may also lead to greater polypeptide flexibility and increased catalytic efficiency at cold temperatures (Davail et al., 1994).

An examination of each of the columns in Table 1 shows that regardless of the structural parameter chosen, there is no correlation between gross structural features and adaptation to temperature. In other words, at least in the case of A₄-LDH orthologs, there is no particular structural component that has been modified in all species during adaptation to different thermal environments. From these results we can reach two conclusions: i) That in order to alter kinetics to the degree measured in the above studies, the necessary modifications in A₄-LDH structure are relatively small (small enough to be missed by the large-scale comparisons described in Table 1), and ii) that structural adaptation to environmental temperature change can occur a number of different ways. In other words, small substitutions at widely varying sites along the A₄-LDH structure can have similar effects on kinetic parameters.

Table 1. Comparison of polypeptide attributes of LDH-As from some marine and freshwater fishes. All values are given as percent of the entire LDH-A molecule.

Species	Avg. Habitat T°	Helix + sheet	Turn + coil	Glycyl %	Prolyl %	Amino Acid Type				
						Charged	Acidic	Basic	Polar	Hydrophobic
<i>Chaenocephalus aceratus</i> ¹	-1	85.8	14.2	8.8	3.0	27.8	10.9	10.3	22.4	36.9
<i>Lycodichthys dearborni</i> ²	-1	85.5	14.5	8.4	3.0	27.4	10.5	10.2	22.6	37.4
<i>Eleginops maclovinus</i> ¹	7	86.5	13.6	8.4	3.0	27.4	10.8	9.9	23.2	37.1
<i>Coryphopterus nicholsti</i> ³	14	86.8	13.3	8.4	2.7	28.3	10.5	10.8	22.0	37.7
<i>Fundulus heteroclitus</i> ⁵	15	87.1	13.0	8.1	3.0	28.3	10.8	10.2	22.3	38.0
<i>Cyprinus carpio</i> ⁶	15	83.4	16.6	8.1	3.3	27.9	10.8	10.8	23.1	37.2
<i>Sphyraena argentea</i> ⁴	18	82.5	17.5	8.4	3.3	28.9	10.5	11.1	22.9	36.1
<i>Gillichthys mirabilis</i> ³	20	87.1	13.0	8.1	2.7	28.3	10.5	10.8	22.0	38.0
<i>Sphyraena lucasana</i> ⁴	23	86.5	13.6	8.7	3.3	28.6	10.8	10.8	21.1	37.0

1 - Fields and Somero (1998) *C.a.* Accession #: AAC63277, *E.m.*: AAC63283; 2 - AAD48489; 3 - Fields and Somero (1997) *C.n.*: AAC31199, *G.m.*: AAC28855; 4 - Holland et al., (1997) *S.a.*: AAB38886, *S.l.*: AAB38888, 5 - Quattro et al., (1995) Q92055; 6 - AAD40736.

These conclusions can be illustrated more fully by describing the amino acid modifications underlying A₄-LDH temperature adaptation in three groups of marine fishes: Barracudas (genus *Sphyraena*), notothenioids and gobies. In the case of the two *Sphyraena* species shown in Figures 1 and 2 and Table 1 (Holland et al., 1997), the K_m^{PYR} of *S. argentea* A₄-LDH is higher than that of *S. lucasana* at all temperatures tested, but is identical when measured at each species' body temperature ($K_m^{\text{PYR}} = 0.24$ mM pyruvate, 18°C for *S. argentea*, 23°C for *S. lucasana*). These findings indicate adaptation to environmental temperature in each species' A₄-LDH. The deduced amino acid sequences of the LDH-A of these two congeners reveal four amino acid differences in the 331-amino-acid-long molecule. Of these four, site directed mutagenesis studies have shown that only two play a role in modifying the kinetics of the catalytic reaction. These two are, in the direction *S. argentea* → *S. lucasana*, Ala61Val and Ser68Gly. An *a priori* examination of these substitutions would not suggest the presence of temperature adaptation, based on our current limited understanding of protein thermodynamics. Neither of these positions is involved in binding substrate or cofactor, and both substitutions are chemically conservative (that is, each change retains the chemical nature of the original residue, nonpolar to nonpolar and polar to polar, respectively). Thus the function of the enzyme has been adaptively modified by one or two subtle amino acid residue changes outside the active site of A₄-LDH, although the temperatures the species experience are only ~5°C apart.

A study of notothenioid A₄-LDH orthologs provides conclusions similar to those of the *Sphyraena* study described above. Again, adaptive differences in kinetic parameters, both K_m^{PYR} and k_{cat} , were found in Antarctic notothenioids when compared to South American notothenioids and temperate teleosts in general (Fields and Somero, 1998). In this study, nine Antarctic and three South American notothenioid LDH-A cDNAs were sequenced, and in order to allow an analysis of cold-adaptation in the group as a whole, a consensus sequence of all notothenioid LDH-As was compared to a consensus sequence of orthologs from temperate non-notothenioid teleosts. This comparison revealed 17 amino acid differences, of which nine are non-conservative. We initially hypothesized that some of these non-conservative substitutions would be more important than others in modifying the kinetics of the extremely cold-adapted Antarctic

notothenioid A₄-LDHs. These putatively important amino acid changes include additional glycines and fewer prolines in areas of the molecule responsible for controlling the motion of the active site. However, site-directed mutagenesis showed that modification of these amino acids has little effect on K_m^{PYR} of A₄-LDH. Instead, as in the *Sphyraena* study, two conservative substitutions outside the active site seem sufficient to explain the increased K_m^{PYR} found in Antarctic notothenioid A₄-LDHs.

The above two studies illustrate that only very minor changes in structure are necessary to alter kinetics of A₄-LDH in a temperature-adaptive manner. The final study -- of goby confamilials (Fields and Somero, 1997) -- adds support to this conclusion, and further suggests that such adaptive modifications may be possible with no change at all in the primary structure of the molecule. *Gillichthys mirabilis* lives in estuaries where water temperature may exceed 30°C, and *Coryphopterus nicholsi* occurs in the subtidal zone where temperatures remain between 10 and 18°C. A comparison of the K_m^{PYR} s of A₄-LDHs of these gobies shows the expected temperature compensation (data not shown). As with the studies described above, this difference in kinetics can be traced to a minor substitution, Ala78Gly (from *G. mirabilis* to *C. nicholsi*), which is outside the active site.

A further comparison within the gobies, between *G. mirabilis* and its congener *G. seta*, leads to a more puzzling result. *Gillichthys seta* lives in the rocky intertidal of Northern Baja California, where water temperatures can rise above 40°C. As shown in Figure 1, its A₄-LDH K_m^{PYR} values reflect adaptation to its environment, when compared to those of *G. mirabilis*. Surprisingly, however, the deduced amino acid sequences of the A₄-LDH orthologs of these congeners are identical. The underlying nucleotide sequences show synonymous mutations that have been sequenced repeatedly, indicating that the identity of the amino acid structures is not a spurious finding. Thus, we are left with the conundrum of identical structures providing adaptively different kinetics.

A possible solution to this mystery is suggested by a series of experiments in which both orthologs were exposed to mild denaturing conditions (3M urea) and then allowed to renature (Fields and Somero, 1997). After this treatment K_m^{PYR} values of the A₄-LDH of *G. mirabilis* were unchanged, but the ortholog of *G. seta* now showed K_m^{PYR} values indistinguishable from those of *G. mirabilis*. These experiments suggest that the differences in kinetics between the congeners are based on different conformations of the same molecule, and that these differences can be partially erased by unfolding and refolding.

Conclusions

The three examples of A₄-LDH adaptation to temperature described above illustrate that the "rules" we expect proteins to follow when adapting to different temperatures -- e.g., changes in hydrophobicity, modifications in secondary structure, etc. -- do not necessarily apply when comparisons are made amongst closely-related species. Further, those differences ultimately responsible for large adaptive modifications in kinetics can be quite subtle (i.e., both conservative and far from the active site). These findings agree with other studies (e.g., Giver et al., 1998; Kotik and Huber, 1993), as well as theoretical considerations (Jaenicke, 1991), that suggest there are many ways to modify the kinetics of an enzyme, whether those modifications are performed in a laboratory or are evolutionarily mediated. Thus, if adaptation to novel environments ultimately depends upon random mutations within proteins, perhaps the multiple pathways apparently available to these molecules makes getting from "here" to "there" more likely than once was expected.

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References

- Brooks, S.P.J. and C.H. Suelter. 1986. Estimating enzyme kinetic parameters: A computer program for linear regression and nonparametric analysis. *Int. J. Bio-med. Comput.* 19:89-99.
- Creighton, T.E. 1993. *Proteins: Structures and Molecular Properties*. (W.H. Freeman, New York.)
- Davail, S., G. Feller, E. Narinx and C. Gerday. 1994. Cold adaptation of proteins: Purification, characterization, and sequence of the heat-labile subtilisin from the Antarctic psychrophile *Bacillus* TA41. *J. Biol. Chem.* 269:17448-17453.

- Feller, G. and C. Gerday. 1997. Psychrophilic enzymes: Molecular basis of cold adaptation. *Cell mol. Life sci.* 53:830-841.
- Fields, P.A. and G.N. Somero. 1998. Hot spots in cold adaptation: Localized increases in conformational flexibility in lactate dehydrogenase A₄ orthologs of Antarctic notothenioid fishes. *Proc. Natl. Acad. Sci. USA* 95:11476-11481.
- Fields, P.A. and G.N. Somero. 1997. Amino acid differences cannot fully explain interspecific variation in thermal sensitivities of gobiid fish A₄-lactate dehydrogenases. *J. Exp. Biol.* 200:1839-1850.
- Giver, L., A. Gershenson, P.-O. Freskgard and F.H. Arnold. 1998. Directed evolution of a thermostable esterase. *Proc. Natl. Acad. Sci. USA* 95:12809-12813.
- Holland, L.Z., M. McFall-Ngai and G.N. Somero. 1997. Evolution of lactate dehydrogenase-A homologs of barracuda fishes (genus *Sphyræna*) from different thermal environments: Differences in kinetic properties and thermal stability are due to amino acid substitutions outside the active site. *Biochemistry* 36:3207-3215.
- Jaenicke, R. 1991. Protein stability and molecular adaptation to extreme conditions. *Eur. J. Biochem.* 202:715-728.
- Kotik, M. and H. Zuber. 1993. Mutations that significantly change the stability, flexibility, and quaternary structure of the l-lactate dehydrogenase from *Bacillus megaterium*. *Eur. J. Biochem.* 211:267-280.
- Matthews, B.W., H. Nicholson and W.J. Becktel. 1987. Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc. Natl. Acad. Sci. USA* 84:6663-6667.
- Quattro, J.M., D.D. Pollock, M. Powell, H.A. Woods and D.A. Powers. 1995. Evolutionary relations among vertebrate muscle-type lactate dehydrogenases. *Mol. Mar. Biol. Biotechnol.* 4:224-231.
- Somero, G.N. 1995. Proteins and temperature. *Annu. Rev. Physiol.* 57:43-68.

Yancey, P.H. and G.N. Somero. 1978. Temperature dependence of intracellular pH: its role in the conservation of pyruvate apparent K_m values of vertebrate lactate dehydrogenases. *J. Comp. Physiol.* 125B:129-134.

