The Journal of Experimental Biology 200, 1839–1850 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB0596

AMINO ACID SEQUENCE DIFFERENCES CANNOT FULLY EXPLAIN INTERSPECIFIC VARIATION IN THERMAL SENSITIVITIES OF GOBIID FISH A_4-LACTATE DEHYDROGENASES (A_4-LDHS)

PETER A. FIELDS[1,2,*] AND GEORGE N. SOMERO[2]

[1]*Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093, USA* and [2]*Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950-3094, USA*

Accepted 18 April 1997

Summary

We compared the deduced amino acid sequences, heat stabilities and thermal sensitivities of a kinetic property, the apparent Michaelis–Menten constant (K_m) of pyruvate, of A_4-lactate dehydrogenase (A_4-LDH) in four species of goby fishes (Family Gobiidae), adapted to different temperatures, to examine how changes in primary structure influence the adaptation of enzymes. The effect of temperature on K_m of pyruvate reflected each species' environmental temperature. For the most eurythermal species, *Gillichthys seta*, which is endemic to shallow intertidal regions of the upper Gulf of California and encounters temperatures between approximately 9 and 40 °C, K_m of pyruvate was minimally affected by temperature, compared with the A_4-LDH orthologues from a less eurythermal congener, *G. mirabilis* (9–30 °C), a cold temperate goby, *Coryphopterus nicholsi* (10–18 °C) and a tropical species, *C. personatus* (25–32 °C). Heat denaturation profiles failed to correlate with habitat temperature; *G. mirabilis* A_4-LDH was most thermally stable, followed by the orthologues of *C. nicholsi* and *G. seta*. Complementary DNAs (cDNAs) encoding LDH-As of *G. seta*, Gulf of California and Pacific coast populations of *G. mirabilis* and *C. nicholsi* were isolated and sequenced, and the corresponding amino acid sequences deduced. The nucleotide sequences of LDH-A of the two populations of *G. mirabilis* were identical. Five nucleotide differences in the coding region and one amino acid substitution (at position 78) distinguished LDH-As of *G. mirabilis* and *C. nicholsi*. The substitution of a glycyl residue (*C. nicholsi*) for an alanyl residue (*G. mirabilis*) may account for the difference in thermal stability between these two orthologues. Comparisons of the LDH-A cDNAs of *G. mirabilis* and *G. seta* revealed four differences in nucleotide sequence in the coding region, but all nucleotide substitutions were synonymous. The identical deduced primary structures of the two enzymes suggested the possibility of different protein conformational variants ('conformers') in the two species. This hypothesis is supported by electrospray ionization mass spectrometry, which indicates that the masses of the A_4-LDH orthologues of the two species are the same within the resolution of the technique. To explore the possibility that the two enzymes were different conformers of the same primary structure, we treated purified *G. seta* and *G. mirabilis* A_4-LDHs with 3.0 mol l^{-1} urea or 6 mol l^{-1} guanidine–HCl and, after removing the denaturant, compared their kinetic properties and heat stabilities. Neither treatment had an effect on the A_4-LDH of *G. mirabilis*, but both converted the K_m *versus* temperature profile of the *G. seta* enzyme to that of the *G. mirabilis* A_4-LDH. The thermal stability of neither enzyme was affected. We propose, as has been suggested in several previous studies of A_4-LDH, that this enzyme can fold into a number of conformers with different stabilities and functional properties. The A_4-LDH of *G. seta* furnishes evidence that such conformers may provide an important mechanism for adaptation of proteins to temperature.

Key words: adaptation, *Coryphopterus nicholsi*, *Coryphopterus personatus*, *Gillichthys mirabilis*, *Gillichthys seta*, goby, temperature, lactate dehydrogenase.

Introduction

Temperature is a major factor limiting the distribution of ectothermic marine species (Hubbs, 1948; Fields *et al.* 1993), in part because it has such powerful effects on the structures and activities of enzymes and other proteins (Jaenicke, 1991; Somero, 1995). The role of temperature in affecting the evolution of proteins is indicated by studies that have compared interspecific homologues of enzymes (orthologous homologues, or orthologues) from species adapted to different

*e-mail: pfields@leland.stanford.edu

temperatures. Consistent interspecific differences in kinetic properties, such as Michaelis–Menten constants (K_m) and catalytic rate constants (k_{cat}), which appear to be temperature-adaptive, have been identified (Johnston and Walesby, 1977; Yancey and Somero, 1978; Graves and Somero, 1982; Yancey and Siebenaller, 1987; Dahlhoff and Somero, 1993; Somero, 1995; Holland *et al.* 1997). Thermal stability of structure also typically reflects adaptation temperature (Jaenicke, 1991; McFall-Ngai and Horwitz, 1990; Dahlhoff and Somero, 1993), although exceptions to this pattern have been found in closely related organisms (Place and Powers, 1984; Holland *et al.* 1997).

Elucidation of the changes in protein primary structure (amino acid sequence) that underlie adaptive differences in kinetic properties and thermal stabilities can provide important new insights in several contexts, including molecular biology, environmental physiology and evolutionary biology. These studies should reveal structure–function relationships that link specific amino acid substitutions to adaptive alterations in traits such as K_m and structural stability. Studies of orthologues from differently adapted species complement investigations that use laboratory-generated protein variants to deduce the effects of specific changes in primary structure on stability and function (e.g. Matthews *et al.* 1987). By revealing the amounts and types of sequence change that are needed to adapt a protein to a new thermal regime, insights into the mechanisms and rates of evolutionary change may also be possible.

To facilitate these types of comparative studies, both the species and enzyme to be studied must be carefully selected. It is essential to choose study species that are adapted to different temperature conditions, yet are close enough, phylogenetically, to ensure that the number of differences in amino acid sequence among orthologues is small enough to allow relatively straightforward interpretations of structure–function relationships. The ideal study system would appear to be a set of congeneric or confamilial species that have evolved in different thermal conditions. The enzyme to be studied should be one for which there is substantial information on primary and three-dimensional structure, to allow amino acid substitutions to be related to alterations in function and thermal stability.

With these considerations in mind, we initiated a study of A_4-lactate dehydrogenase (A_4-LDH; EC 1.1.1.27, NAD^+:lactate oxidoreductase) orthologues from fishes of the Family Gobiidae adapted to widely different thermal conditions. *Gillichthys mirabilis* is a eurythermal estuarine and mudflat species that occurs along the Pacific coast of North America from Tomales Bay, California, into northern Baja California and also has what is thought to be a disjunct population within the Gulf of California (Miller and Lea, 1972; Thomson *et al.* 1979). *G. mirabilis* encounters temperatures from approximately 9 to 30 °C and appears to avoid high temperatures by burrowing into the sediment (Barlow, 1961). Its congener, *G. seta*, is found exclusively in the shallow rocky intertidal zone of the northern Gulf of California (Thomson *et al.* 1979), where it encounters temperatures from approximately 9 to 40 °C and possibly higher. *Gillichthys seta* may be derived from *G. mirabilis* through progenesis (Barlow, 1961), and morphological similarities indicate that it is closely related to *G. mirabilis*.

The second species pair examined is from the genus *Coryphopterus*, which was divided by the development of the Isthmus of Panama between 3 and 4 million years ago (Coates *et al.* 1993; Knowlton *et al.* 1993). *Coryphopterus nicholsi* occurs from British Columbia to Baja California (Miller and Lea, 1972) and is found deeper at the southern extent of its range. Off the coast of southern California, *C. nicholsi* generally occurs below the thermocline at 20–30 m, where temperature varies annually from 10 to 18 °C (R. McConnaughey, Scripps Institution of Oceanography, personal communication). *Coryphopterus personatus* occurs in Bermuda, southern Florida and throughout the Caribbean (Zaneveld, 1983) to depths of 30 m. The sampled population from the Florida Keys encounters an annual temperature range of approximately 25–32 °C (Dr M. E. Clarke, University of Miami, personal communication). These four species provided an excellent study system for examining the evolution of A_4-LDH in different thermal conditions over varying periods of evolutionary time.

To compare the A_4-LDH orthologues of each species and of the Pacific coast and Gulf of California populations of *G. mirabilis*, we measured the effects of temperature on the K_m of pyruvate, a highly temperature-sensitive kinetic trait (Yancey and Somero, 1978). For all but *C. personatus*, we also had sufficient material to conduct thermal stability measurements and complementary DNA (cDNA) sequencing. We report that some of the differences in thermal sensitivity of the LDH-A orthologues can be explained by observed differences in sequence, but that differences in kinetics and thermal stabilities between the orthologues of *G. mirabilis* and *G. seta* may be due to alternative conformational variants (conformers) of an identical primary structure.

Materials and methods

Collection and care of specimens

Gillichthys seta Ginsburg were trapped by hand in the intertidal habitat approximately 3 km north of San Felipe, Baja California, Mexico. Gulf of California *G. mirabilis* Cooper were collected from a slough approximately 3 km south of San Felipe, and Pacific *G. mirabilis* were collected from a small slough approximately 10 km north of San Diego, CA. *G. mirabilis* were caught in minnow traps baited with chopped mackerel. All fish were transported to the laboratory at the Scripps Institution of Oceanography (SIO) in oxygenated sea water at ambient temperature. Fish were held at ambient temperature (approximately 15 °C) for over 3 months prior to use, and were fed fish pellets and minced mackerel twice per week.

Coryphopterus nicholsi Bean were collected using SCUBA in 20–30 m of water off the coast of La Jolla, CA. There was heavy mortality among the fish collected, so specimens were frozen at –80 °C immediately upon their arrival at SIO. *Coryphopterus*

personatus Jordan and Marsh were collected from the Florida Keys by Dr M. Elizabeth Clarke, of the University of Miami, and her associates. Fish were frozen at −80 °C upon capture, and were shipped to San Diego on solid carbon dioxide. Upon arrival, they were placed at −80 °C until used.

Determination of kinetics of A_4-LDH

Fish were decapitated after anesthetization in ice-cold sea water. Approximately 2 g of white muscle was dissected away from the body, with care being taken not to contaminate the sample with red muscle, skin or viscera. If 2 g could not be obtained from one fish, tissue from two or more individuals was pooled.

The A_4-LDH of each species was purified using agarose–oxamate column chromatography and dialyzed against 50 mmol l^{-1} potassium phosphate buffer, pH 6.8, as described in Yancey and Somero (1978). Complete separation of LDH from other proteins was shown by the presence of a single protein band on silver-stained sodium dodecyl sulfate polyacrylamide gels. Non-denaturing polyacrylamide gels stained for LDH activity also revealed a single band, indicating that the only isoenzyme present was A_4-LDH. Apparent Michaelis–Menten constants for pyruvate of A_4-LDH were determined using a Perkin Elmer Lambda 3B spectrophotometer equipped with a temperature-controlled cell attached to a Lauda RM6 recirculating water bath. The temperature of the cuvette sample was measured with a Digisense model 8528-20 thermocouple thermometer and maintained to within ±0.2 °C.

To ensure that pH levels in the experimental system corresponded to intracellular values over a wide range of temperatures, an imidazole chloride buffer (80 mmol l^{-1} imidazole chloride, 150 μmol l^{-1} NADH; pH 7.0 at 20 °C) was used during enzyme activity determinations (Yancey and Somero, 1978; Somero, 1981; Hochachka and Somero, 1984). For each K_m determination, eight concentrations of pyruvate were used: 0.083, 0.100, 0.125, 0.143, 0.167, 0.200, 0.250 and 0.333 mmol l^{-1} for K_m determinations at 10, 15, 20 and 25 °C, and 0.100, 0.125, 0.167, 0.200, 0.250, 0.333, 0.400 and 0.500 mmol l^{-1} for determinations at 30, 35 and 40 °C.

Each assay was initiated by addition of 10 μl of A_4-LDH to the imidazole/NADH/pyruvate solution, and the rate of decrease in absorbance was measured at 340 nm. Velocity determinations for the eight pyruvate concentrations at each experimental temperature were entered into a computer program (Wilman K_m-fitting; Brooks and Suelter, 1986) to calculate K_m using weighted linear regression as outlined by Wilkinson (1961).

Determination of thermal stabilities of A_4-LDH

The thermal stabilities of A_4-LDH from *G. mirabilis*, *G. seta* and *C. nicholsi* were determined at 45, 47.5, 50, 52.5 and 55 °C using enzyme purified as described above. 400 μl of enzyme in 50 mmol l^{-1} potassium phosphate buffer, pH 6.8, was added to 1.60 ml of thermal denaturation solution [1.4 g of KCl, 125 mg of bovine serum albumin (BSA), 100 ml of H_2O; Place and Powers, 1984] in a 13 mm×100 mm glass tube, and the tube was placed in a temperature-controlled Lauda RM6 recirculating water bath (±0.1 °C). Three 75 μl samples were removed at 0 (before incubation), 5, 10, 20, 40 and 60 min. The activity of the samples was measured as in the kinetics experiment, using 150 μmol l^{-1} NADH and 2 mmol l^{-1} pyruvate. No decrease in activity was found in the 0 min samples when held on ice for over 1 h. The three values for each time point were averaged and divided by the mean value for 0 min to allow comparison of residual activities.

Sequencing of LDH-A cDNA

Total RNA was purified from white muscle tissue of *G. mirabilis*, *G. seta* and *C. nicholsi* using a guanidinium thiocyanate–phenol extraction method adapted from the method of Chomczynski and Sacchi (1987). To separate messenger RNA (mRNA) from total RNA, microscopic (2.8 mm) magnetized beads to which 25-mers of poly-deoxythymidylate had been covalently attached (Dynabeads; Dynal, Inc.) were used to bind reversibly the poly-adenylate tails of the mRNA molecules (Hornes and Korsnes, 1990). Complementary DNA was synthesized from the purified mRNA using the RACE procedure (rapid amplification of cDNA ends; Frohman *et al.* 1988). Amplification of LDH-A cDNA from the synthesized cDNA thus obtained was accomplished using the polymerase chain reaction (PCR; Mullis and Faloona, 1987). LDH-A-specific primers were designed on the basis of the LDH-A sequence of barracuda (genus *Sphyraena*; Holland *et al.* 1997). These primers were adequate to amplify the coding region of *ldh-a*, but were not able to provide amplification of the 5′-noncoding region. This region was amplified by single-strand ligation of cDNA (Tessier *et al.* 1986; Edwards *et al.* 1991) using reagents provided with the 5′-amplifinder race kit from Clontech Laboratories, Inc.

The complementary DNA sequence was obtained through the dye-labeled ddNTP terminator protocol (Smith *et al.* 1987*a*,*b*) using a prism ready reaction dyedeoxy terminator cycle sequencing kit from Applied Biosystems and the Applied Biosystems model 373A automated sequencer.

Treatment of A_4-LDH with urea and guanidine–HCl

The effects of incubation in 3.0 mol l^{-1} urea on A_4-LDH kinetics and thermal stabilities were determined for *G. mirabilis*, *G. seta* and *C. nicholsi*. In addition, the effects of denaturation in 6 mol l^{-1} guanidine–HCl (Gdn–HCl) on A_4-LDH thermal stability were examined for *G. mirabilis* and *G. seta*. For urea treatment, 1.13 ml of A_4-LDH in 50 mmol l^{-1} potassium phosphate buffer, pH 6.8, was mixed with 0.20 ml of 10 mg ml^{-1} BSA, to which 0.667 ml of 9 mol l^{-1} urea was added, for a final urea concentration of 3 mol l^{-1}. The mixture was allowed to incubate for 15 min at room temperature, and then urea was removed by centrifugal filtration in a Centricon 30 concentrator (Amicon). The BSA served to prevent loss of enzyme activity during the filtration step due to adherence of enzyme to the Centricon filter. After concentration to

approximately 100 μl, the retentate was diluted with 1 ml of 50 mmol l^{-1} potassium phosphate buffer (pH 6.8 at 20 °C) and re-filtered; this process was repeated three times, and the retentate was returned to a final volume of 1.13 ml. Kinetics and thermal stabilities were then determined for the urea-treated A_4-LDH as described above.

To induce denaturation by Gdn–HCl, 0.833 ml of A_4-LDH in 50 mmol l^{-1} potassium phosphate buffer, pH 6.8, was mixed with 1.67 ml of 9 mol l^{-1} Gdn–HCl and 10 mmol l^{-1} 2-mercaptoethanol (2-ME), to give a final Gdn–HCl concentration of 6 mol l^{-1}. The sample was held at room temperature for 1 h. It was then diluted to a final volume of 150 ml (i.e. 60-fold dilution) with 50 mmol l^{-1} Tris–HCl, 5 mmol l^{-1} 2-ME and 350 μmol l^{-1} NADH, pH 7.5. This was held at room temperature for 48 h to permit renaturation. The sample was then concentrated using an Amicon model 52 ultrafiltration cell with a BSA-pretreated (1 mg ml^{-1}) YM30 filter. After concentration to 5 ml, the sample was washed twice with 50 mmol l^{-1} potassium phosphate buffer, pH 6.8, concentrated to 5 ml, and further concentrated with a BSA-pretreated Centricon-30 to approximately 1 ml. Thermal stabilities were then determined for the A_4-LDH samples as described above.

Mass determination by electrospray ionization mass spectrometry

Molecular masses were determined for the LDH-A monomers of *G. mirabilis* and *G. seta* in order to determine whether the enzyme orthologues contain different post-translational modifications. To prepare enzyme samples for electrospray ionization (ESI) mass spectrometry, approximately 72 μg (2 nmol) of each was dialyzed against 20 mmol l^{-1} of the volatile buffer triethanolamine HCl. The samples were then concentrated under vacuum to a final volume of 25 μl.

These samples were sent to Peptidogenic Research, Inc. (Livermore, CA), where mass determinations were performed on a Perkin Elmer API-Plus mass spectrometer.

Results

Kinetics of A_4-LDH

The results of the kinetics studies for the *Gillichthys* congeners are shown in Fig. 1A, and the results for the *Coryphopterus* congeners are given in Fig. 1B. As can be seen in the inset of Fig. 1A, there was no significant difference in the A_4-LDH kinetics of the two populations of *G. mirabilis* within the temperature range examined (log-transform analysis of covariance, F=0.751, P>0.50); thus, kinetic data from these populations were pooled. When compared with the values for *G. mirabilis*, the affinity of *G. seta* A_4-LDH for pyruvate remains significantly higher (i.e. the K_m remains lower) at temperatures greater than 20 °C (see legend to Fig. 1). However, when the K_m values are compared within the environmental temperature range of each species (solid lines, Fig. 1A), it is apparent that the orthologues of A_4-LDH maintain similar pyruvate affinities. These differences in K_m of pyruvate values are not due to the effects of acclimatization, because specimens were held for over 3 months at a common temperature (approximately 15 °C) prior to being killed. Further, we obtained identical K_m values for enzymes purified from specimens collected and immediately frozen in the field at different seasons.

Fig. 1B shows that the A_4-LDH of *C. personatus* has a greater affinity for pyruvate than does that of *C. nicholsi* at all temperatures tested except 40 °C. Although small sample sizes preclude determination of statistical significance at individual temperatures, a paired-sample *t*-test indicates that *C. nicholsi* A_4-LDH has significantly higher K_m values (t=6.534; P<0.01). Again, however, an examination of K_m values within the normal thermal range of each species (solid lines, Fig. 1B) reveals similar pyruvate affinities.

Thermal stabilities of A_4-LDH

Residual activities of A_4-LDH from *G. mirabilis*, *G. seta* and *C. nicholsi* after incubation at 50 °C are shown in Fig. 2. *G. mirabilis* A_4-LDH retained greater activity than the other two orthologues at this temperature, possessing 75.3 % of its original activity after 60 min, compared with 23.3 % for *C. nicholsi* and 8.4 % for *G. seta*. Thus, it appears that the mechanisms responsible for producing relatively temperature-insensitive kinetics in the *G. seta* enzyme, when compared with the *G. mirabilis* orthologue (Fig. 1A), do not lead to a higher resistance to heat denaturation. The absence of a linkage between thermal effects on kinetic properties and structural stability is also indicated by the comparison between the orthologues of *G. mirabilis* and *C. nicholsi*, in which identical effects of temperature on K_m of pyruvate (Fig. 1A,B) are associated with different thermal stabilities.

cDNA sequences and deduced amino acid sequences of LDH-A

Nucleotide sequence differences of *ldh-a* among the species of goby studied are given in Fig. 3. There was no difference in nucleotide sequence between the two populations of *G. mirabilis*. Between *G. seta* and *G. mirabilis*, there were a total of four base differences in the coding region, three of which occur in the first 100 bases (positions 15, 30 and 69). The final substitution occurred at position 648. In addition, there was one base difference in the 31 bases sequenced in the 5′-noncoding region (not shown). The nucleotide sequence of *C. nicholsi* did not show much greater disparity in comparison with *G. mirabilis* than did the *G. seta* sequence. In the coding region there were five base differences, which are concentrated in the first few hundred base pairs (at positions 126, 195, 231, 233 and 234); there were also three substitutions in the 5′-noncoding region.

The differences in amino acid sequences of the LDH-A protein deduced from the nucleotide sequences described above are also given in Fig. 3. Because there are no base differences in the LDH-A cDNA sequence of the *G. mirabilis* populations, there are no amino acid differences. Remarkably, however,

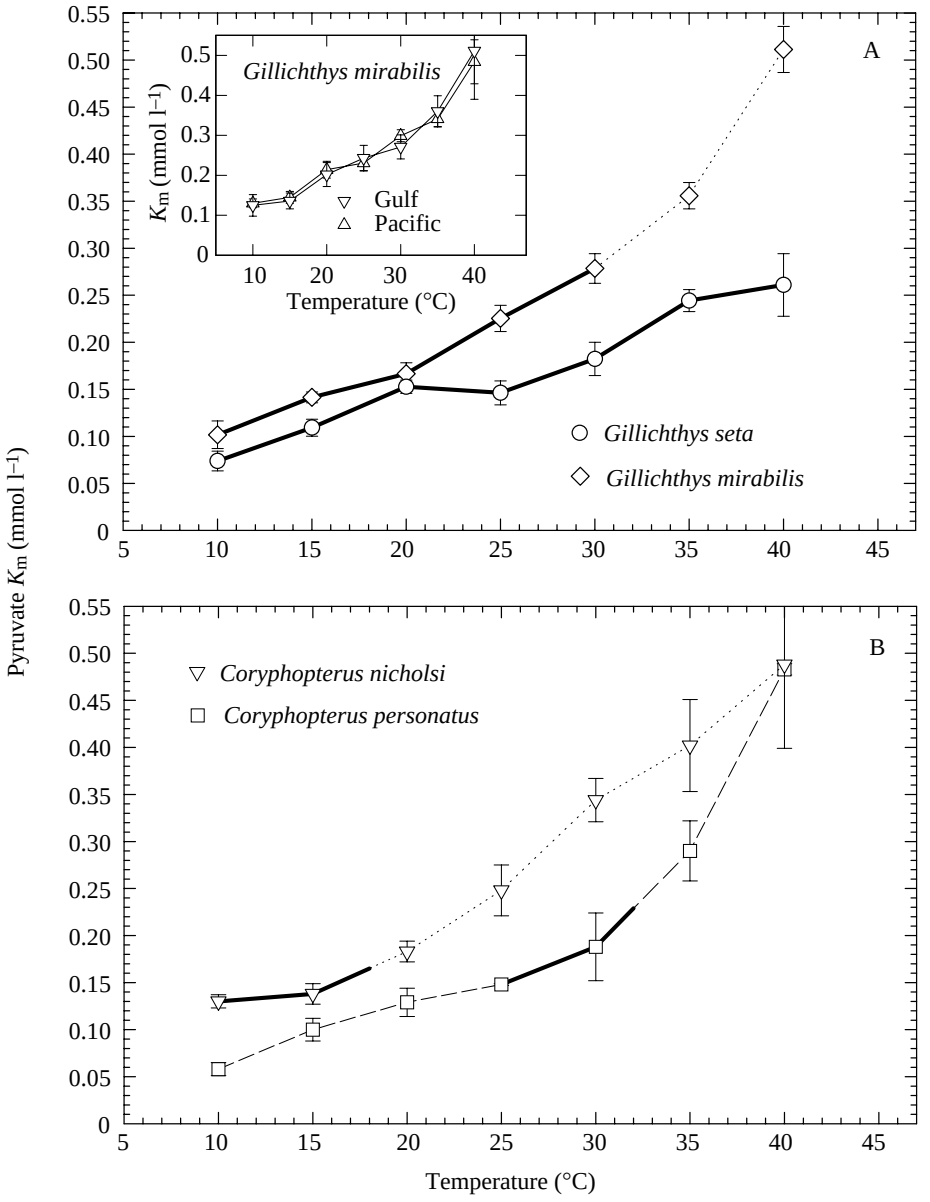

Fig. 1. (A) Apparent Michaelis–Menten constants (K_m values) of pyruvate for A_4-LDH from *G. seta* and the two populations of *G. mirabilis*, measured from 10 to 40 °C. Inset: data for both populations of *G. mirabilis*; axes are identical to the larger graph. Comparisons of *G. mirabilis* (pooled Pacific and Gulf populations) and *G. seta* A_4-LDHs, using paired *t*-tests at each measurement temperature: 10 °C: *G. seta* N=5, *G. mirabilis* N=7, t=–1.419, P>0.10; 15 °C: *G. seta* N=6, *G. mirabilis* N=6, t=–2.984, P<0.05; 20 °C: *G. seta* N=5, *G. mirabilis* N=16, t=–0.655, P>0.50; 25 °C: *G. seta* N=4, *G. mirabilis* N=7, t=–3.784, P<0.005; 30 °C: *G. seta* N=9, *G. mirabilis* N=6, t=–3.887, P<0.005; 35 °C: *G. seta* N=16, *G. mirabilis* N=9, t=–5.921, P<0.001; 40 °C: *G. seta* N=6, *G. mirabilis* N=4, t=–5.450, P<0.001. (B) K_m values of pyruvate for A_4-LDH from *C. nicholsi* (10 °C, N=7; 15 °C, N=8; 20 °C, N=6; 25 °C, N=2; 30 °C, N=5; 35 °C, N=5; 40 °C, N=4) and *C. personatus* (10 °C, N=2; 15 °C, N=3; 20 °C, N=2; 25 °C, N=2; 30 °C, N=2; 35 °C, N=4; 40 °C, N=2) measured from 10 to 40 °C. Error bars represent standard deviations as determined by weighted linear regression (Wilkinson, 1961). Solid lines indicate the normal temperature ranges of the habitat of each species.

there are also no differences in the deduced amino acid sequences of the *G. mirabilis* and *G. seta* orthologues because all of the nucleotide substitutions are for synonymous codons.

This result was unexpected, given the differences in kinetics and thermal stability between the *G. mirabilis* and the *G. seta* A_4-LDHs. To ensure that no contamination had occurred in the handling of the *G. seta* mRNA or cDNA, the full sequencing process was repeated, using new fish and new sequencing reagents. The nucleotide sequence derived from the second *G. seta* preparation was identical to that derived from the first.

The *ldh-a* nucleotide sequence of *C. nicholsi* does code for one difference in amino acid sequence when compared with the *Gillichthys* orthologue. Although four of the five base differences are silent, the substitution of deoxyguanosine for deoxycytidine at position 233 in the nucleotide sequence results in the replacement of alanine in the *Gillichthys* protein by glycine in that of *C. nicholsi* at position 78 (Fig. 3). The difference in amino acid sequence between the *Gillichthys* spp. and *C. nicholsi* may contribute to the differences in electrophoretic mobility (data not shown). On a non-denaturing polyacrylamide gel stained for LDH activity, the A_4-LDH bands of *G. mirabilis* and *G. seta* were identical, but differed from that of *C. nicholsi*.

Effects of urea and Gdn–HCl on the kinetics and thermal stabilities of A4-LDH

Although treatment with 3 mol l^{-1} urea did not denature any of the orthologues tested, as shown by retention of essentially full activity at the end of the incubation period, we observed significant effects on the kinetics of the enzyme of *G. seta* (Fig. 4). In contrast to the absence of any effect of urea on the K_m *versus* temperature profile for the enzymes of *G. mirabilis* (Fig. 4) and *C. nicholsi* (data not shown), the affinity of *G. seta* A_4-LDH for pyruvate was reduced above 20 °C. At 40 °C, this

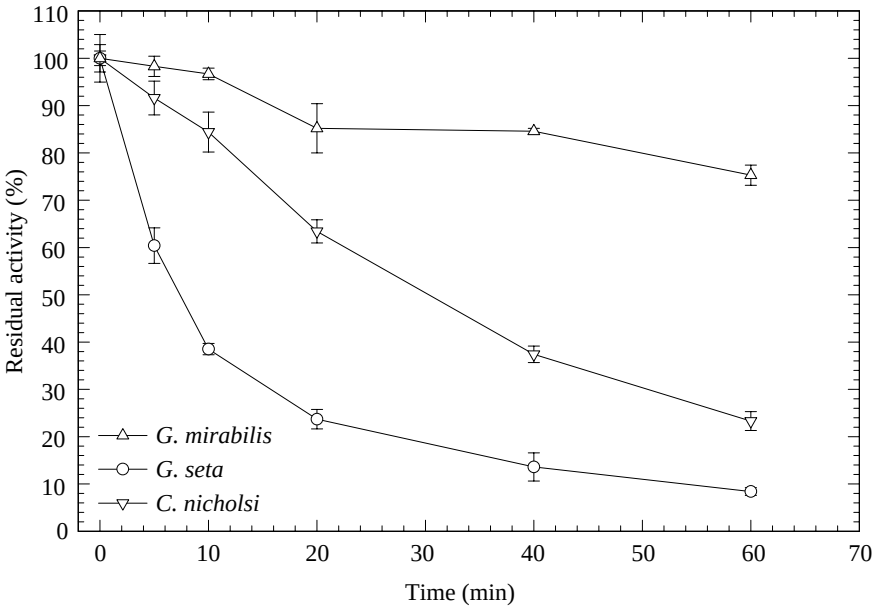

Fig. 2. Residual activity of A_4-LDH from *G. mirabilis*, *G. seta* and *C. nicholsi* after incubation at 50 °C for 0, 5, 10, 20, 40 and 60 min. Means and standard deviations of three replicates are shown.

reduction in affinity was substantial, with the pyruvate K_m of *G. seta* A_4-LDH increasing from 0.261 mmol l^{-1} to 0.568 mmol l^{-1}. Thus, after treatment with 3 mol l^{-1} urea, the kinetics of *G. seta* A_4-LDH became indistinguishable from that of the *G. mirabilis* orthologue. The same effect was observed after treatment of the two orthologues with 6.0 mol l^{-1} Gdn–HCl, a much stronger denaturing regimen than 3.0 mol l^{-1} urea (data not shown).

The effects of treatment with 3 mol l^{-1} urea on the thermal stabilities of *G. mirabilis*, *G. seta* and *C. nicholsi* A_4-LDH from 45 to 55 °C are shown in Fig. 5. These effects were slight and non-uniform, leading in some cases to small increases in retention of activity and, in other cases, to minor decreases. However, despite the ability of urea to convert the kinetics of the *G. seta* A_4-LDH to the pattern seen for *G. mirabilis*, after the same treatment the thermal stabilities of the enzymes remained distinct.

The effect of treatment with 6 mol l^{-1} Gdn–HCl on the stabilities at 50 °C of *G. mirabilis* and *G. seta* A_4-LDH are also shown in Fig. 5 (inset). Despite the strong denaturing properties of Gdn–HCl, there is no apparent alteration in the thermostability of either enzyme.

Molecular mass determinations

Determinations of the subunit mass of LDH-A were made using ESI mass spectrometry on two separate enzyme preparations for each species, yielding values of 36 162.10±2.73 and 36 168.38±2.70 Da for *G. mirabilis* LDH-A and 36 151.03±2.44 and 36 156.82±2.77 Da for *G. seta* LDH-A; each mass value represents the mean ± S.D. of 11–16 peaks in an individual mass-to-charge (*m*/*z*) spectrum. The small difference in the mean masses of the *G. mirabilis* and *G. seta* LDH-A subunits cannot be regarded as significant in view of the resolving power of this technique (Edmonds and Smith, 1990).

Discussion

Kinetics of A4-LDH

The effects of temperature on K_m of pyruvate of A_4-LDH in each species reflect the temperatures found in the species' habitats. Although K_m values tend to rise with increasing temperature, numerous studies have shown that A_4-LDH orthologues conserve K_m values within a narrow range when measured at normal body temperatures (Yancey and Somero,

Fig. 3. Nucleotide and deduced amino acid differences compared among *G. mirabilis* (G.m.; sequences for the Gulf and Pacific coast populations are identical), *G. seta* (G.s.) and *C. nicholsi* (C.n.). Differences in nucleotide sequence, relative to the *G. mirabilis* sequence, are indicated by bold and underlined symbols; identical amino acid residues between *C. nicholsi* and the *Gillichthys* spp. are shown by dashes.

		Amino acid number							
		5	10	23	42	65	77	78	216
Gillichthys									
Amino acid		Lys	Val	Thr	Asp	Gln	Val	Ala	Lys
Nucleotide	G.m.	.. AAA ..	GTG ..	ACG ..	GAT ..	CAG ..	GTG	GCT ..	AAA ..
	G.s.	AA**G**	GT**T**	AC**A**	GAT	CAG	GTG	GCT	AA**G**
Coryphopterus									
Amino acid		---	---	---	---	---	---	Gly	---
Nucleotide	C.n.	.. AAA ..	GTG ..	ACG ..	GA**C** ..	CA**A** ..	GT**T**	G**GC** ..	AAA ..

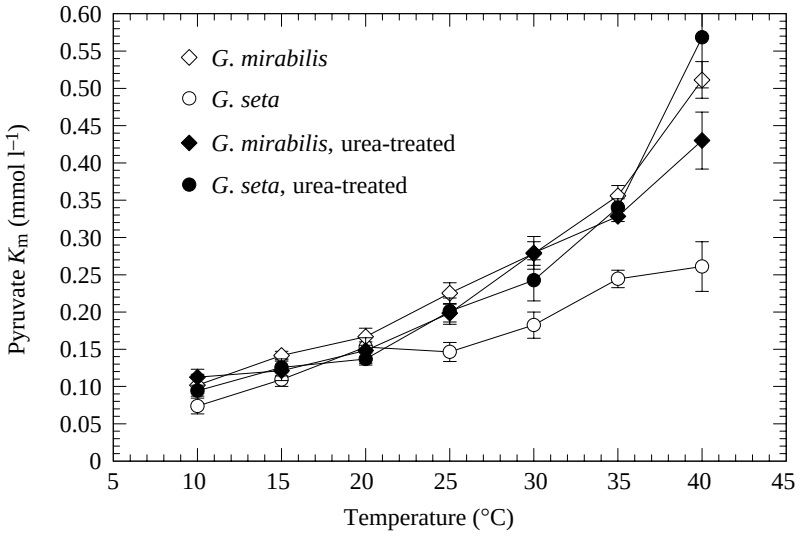

Fig. 4. K_m values of pyruvate for *G. mirabilis* and *G. seta* A_4-LDH before and after treatment with 3 mol l^{-1} urea. Urea-treated *G. seta* A_4-LDH K_m values are significantly different from the other three A_4-LDH samples at 25 °C and higher (Tukey test, $P<0.05$). Sample sizes for non-urea-treated A_4-LDH from *G. mirabilis* and *G. seta* are the same as in Fig. 1. For urea-treated A_4-LDHs at 10 °C: *G. seta* N=4, *G. mirabilis* N=4; 15 °C: *G. seta* N=4, *G. mirabilis* N=6; 20 °C: *G. seta* N=4, *G. mirabilis* N=4; 25 °C: *G. seta* N=7, *G. mirabilis* N=8; 30 °C: *G. seta* N=6, *G. mirabilis* N=11; 35 °C: *G. seta* N=10, *G. mirabilis* N=18; 40 °C: *G. seta* N=7, *G. mirabilis* N=6. Error bars are as in Fig. 1.

1978; Yancey and Siebenaller, 1987; Somero, 1995; Holland *et al.* 1997). Furthermore, these K_m values consistently fall within the range of pyruvate concentrations found in skeletal muscle (Walsh and Somero, 1982). Trends found in these earlier studies are clearly reflected in data from the four goby species. For the two *Coryphopterus* species, the environmental temperature ranges are offset by approximately 15 °C, but have similar breadth (*C. nicholsi* 10–18 °C; *C. personatus* 25–32 °C). However, when K_m values are compared within the normal temperature range of each species, they are found to be similar (*C. nicholsi*, 0.130–0.165 mmol l^{-1} pyruvate; *C. personatus*, 0.148–0.229 mmol l^{-1}; see Fig. 1B).

The two *G. mirabilis* populations show no significant differences in A_4-LDH kinetics across the temperature range tested (inset, Fig. 1A). Although the Gulf of California population probably experiences higher temperatures than the Pacific coast population, their A_4-LDH orthologues are equally sensitive to temperature. However, the differences between the thermal habitats of the two *G. mirabilis* populations are not as extreme as those between the *Coryphopterus* species, and there is evidence that *G. mirabilis* behaviorally thermoregulates by burrowing (Barlow, 1961). Thus, the high surface-water temperatures of habitats in the Gulf of California may not be an adequate measure of the actual thermal conditions

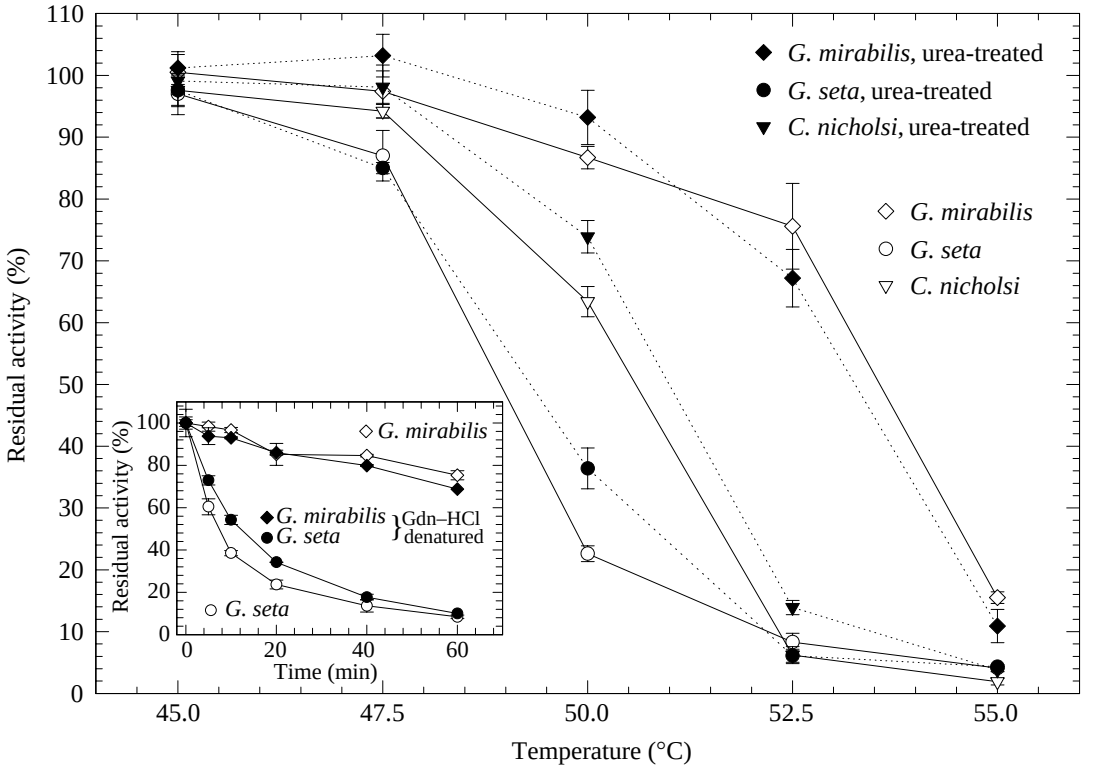

Fig. 5. Residual activity of 3 mol l^{-1} urea-treated and non-urea-treated A_4-LDH from *G. mirabilis*, *G. seta* and *C. nicholsi* after incubation for 20 min at temperatures from 45 to 55 °C. Means and standard deviations of three replicates are shown. Inset: effect of denaturation with 6 mol l^{-1} Gdn–HCl on thermostability at 50 °C of *G. mirabilis* and *G. seta* A_4-LDH. Means and standard deviations of three replicates are shown; axes are as in Fig. 2.

encountered by *G. mirabilis*. The lack of divergence in the A_4-LDH kinetics of the two *G. mirabilis* populations may therefore be due to a number of factors, including the inherent eurythermy of the species and an ability to ameliorate environmental temperature fluctuations through behavioral thermoregulation. In addition, the lack of differences in A_4-LDH sequence and function between the populations could reflect either ongoing gene flow between the populations sampled or relatively recent geographical separation (not more than 10 000–15 000 years bp, the approximate time at which waters warmed after the last glaciation and coastal marine populations migrated northward).

In comparison with the other gobies examined, the kinetic parameters of *G. seta* A_4-LDH are remarkable in their insensitivity to temperature. *G. seta* A_4-LDH maintains a relatively constant affinity for pyruvate at temperatures where the K_m values of its confamilials rise steeply and reach values far above physiological pyruvate concentrations. In agreement with the extreme eurythermy of *G. seta*, its A_4-LDH has the most temperature-insensitive K_m of any A_4-LDH yet examined (Yancey and Somero, 1978; Somero, 1995).

Although the time at which *G. seta* and *G. mirabilis* last shared a common ancestor cannot be accurately determined, certain factors suggest that divergence was relatively recent. These congeners are nearly indistinguishable morphologically as juveniles and occur in the same habitats (Barlow, 1961). Barlow suggests that *G. seta* is a paedomorphic form of *G. mirabilis*, adapted to life in an extreme and unstable environment. If this hypothesis is correct, it supports the view of Gould (1977, 1988) that paedomorphosis through progenesis can be the result of rapid adaptation to a variable environment.

Thermal stability of A_4-LDH

Although no difference was noted in A_4-LDH kinetics between the *G. mirabilis* populations and *C. nicholsi*, the A_4-LDH of the latter species loses activity more rapidly than *G. mirabilis* A_4-LDH when incubated at temperatures between 47.5 and 55 °C (Figs 2, 5). The only difference in the amino acid sequences of these orthologues is at position 78, where the alanine found in the orthologue of *G. mirabilis* is replaced by a glycine in the A_4-LDH of *C. nicholsi*. This location is contained within a six-stranded parallel sheet that contributes to the stability of the LDH-A subunit (Abad-Zapatero *et al.* 1987). Although replacement of an alanyl residue by a glycyl residue represents only a minor change in the overall structure of a protein, this type of substitution has been shown to effect shifts in thermal stability of other enzymes. Work by Matthews *et al.* (1987) involving site-directed mutations in bacteriophage T4 lysozyme showed that a glycine-to-alanine shift increased the thermal stability of the enzyme. These authors argued that the replacement of a glycyl residue by an alanyl residue should enhance protein thermal stability by reducing the configurational entropy of the unfolded protein. Imanaka *et al.* (1986) likewise found that glycine-to-alanine substitutions enhanced thermal stability, and attributed this effect in part to an enhanced potential for hydrophobic interactions in the alanine-containing protein. Thus, there is a strong basis for concluding that the change in amino acid sequence at position 78 accounts for the difference in thermal stability between the A_4-LDH orthologues of *G. mirabilis* and *C. nicholsi*.

The large difference in thermal stability between *G. mirabilis* and *G. seta* A_4-LDHs is more enigmatic, because it cannot be explained by an underlying difference in amino acid sequence. Further, the thermal stabilities of the enzymes of these species are reversed with respect to the thermal sensitivity of their kinetics. Relative to the A_4-LDH of *G. mirabilis*, the orthologue of *G. seta* denatures at a lower temperature despite having a more thermally stable K_m of pyruvate.

These findings, notably, the difference in sequence between the orthologues of *C. nicholsi* and *G. mirabilis*, suggest that the kinetics and thermal stability of an enzyme can evolve independently and that different regions of a protein may be involved in effecting these two types of change. This conclusion is supported by the findings of Powers *et al.* (1993), who showed that the thermal stability of B_4-LDH of the killifish (*Fundulus heteroclitus*) could be modified without any effect on the kinetic properties of the enzyme. Recently, Holland *et al.* (1997) showed that some single amino acid substitutions in A_4-LDHs of barracuda congeners modify only K_m, others modify only thermal stability and still other substitutions lead to changes in both characteristics.

Potential mechanisms accounting for differences in kinetics and thermal stability between A_4-LDHs of G. mirabilis *and* G. seta

The discovery that orthologues of a protein with identical deduced amino acid sequences are characterized by different heat stabilities and temperature effects on K_m suggests that the maturation processes that lead to the physiologically active form of A_4-LDH may differ between the *Gillichthys* congeners. Potential differences between the mature A_4-LDHs of *G. mirabilis* and *G. seta* include (1) covalent modification of the proteins, (2) non-covalent binding of a modulator of enzyme function and stability, and (3) the assumption of different conformational states in the two proteins, as a result of different pathways of folding, subunit assembly, or both.

In order to differentiate among these possibilities, we began a series of experiments that involved the treatment of A_4-LDH with the denaturants urea and Gdn–HCl. Our initial method for incubating enzymes in $3\,mol\,l^{-1}$ urea and subsequently removing the urea by dialysis was based on the discovery by Ozernyuk *et al.* (1994) that such urea treatment could modify the kinetic properties of A_4-LDH of the loach (*Misgurnus fossilis*). In this fish, A_4-LDH from specimens acclimated to different temperatures had different kinetic properties, with the K_m *versus* temperature curve for the cold-acclimated form being left-shifted with respect to that of the warm-acclimated form. This acclimation-induced difference in kinetic properties disappeared after treatment of the enzyme with $3\,mol\,l^{-1}$ urea, such that the kinetics of the two forms became identical and

intermediate. Although the work of Ozernyuk *et al.* (1994) focused on thermal acclimation in one species, as opposed to adaptation in two related species, the lack of difference between the *Gillichthys* LDH-A amino acid sequences suggested that a similar approach might provide evidence regarding the mechanism underlying the kinetic and thermal stability differences noted in the present study.

The treatments with urea and Gdn–HCl did not affect thermal stability, possibly because the conditions used were insufficient either to change the conformation or to remove a non-covalently bound factor. Attempts to denature the enzymes more fully with combinations of Gdn–HCl and high temperature failed to yield an active enzyme upon renaturation (data not shown). Consequently, data from the thermal stability experiments do not allow distinction among the hypotheses listed above. However, the results of kinetics studies (Fig. 4) do provide evidence in support of a mechanism involving either conformational differences or a non-covalently bound modulator. We have shown that the K_m of pyruvate of *G. seta* A_4-LDH can be modified to become indistinguishable from that of the *G. mirabilis* enzyme, and we believe that the ability of urea to alter the K_m of pyruvate of *G. seta* A_4-LDH is evidence against differential covalent modification as a mechanism for the interspecific differences observed, because washing with $3\,mol\,l^{-1}$ urea is unlikely to affect covalent bonds.

The finding that the molecular masses of the A_4-LDHs of the *Gillichthys* congeners cannot be discriminated by the sensitive technique of electrospray ionization mass spectrometry is further evidence that the differences between the enzymes are due either to alternative conformations or to binding of a modulator to the enzyme of *G. seta*. While the molecular mass data do not allow us to exclude the possibility that identical covalent modifications at different sites on the molecules may be responsible for the variations in K_m and thermal stability, we feel that such an explanation for the identical masses is unlikely.

Our data do not allow us to distinguish whether the differences in K_m *versus* temperature relationships and thermal stability between the A_4-LDHs of the *Gillichthys* congeners are due to different conformational states or to non-covalent binding of a modulator to the LDH-A from *G. seta*. However, a potential role for the former mechanism is supported by results from several other studies of LDHs and additional enzymes, in which evidence for multiple conformations of a single protein is presented. In addition to the work of Ozernyuk *et al.* (1994), there are several other reports of conformers of A_4-LDH that differ in kinetic or structural properties. Xue and Yeung (1995) used a laser-based methodology that allowed replicate measurements of the activities of single A_4-LDH molecules. They found that the activities of individual enzyme molecules from an electrophoretically pure preparation differed by up to fourfold, a result that they attribute to the occurrence of several stable conformers of the enzyme. Levi and Kaplan (1971), through experiments involving denaturation and renaturation of A_4-LDH of dogfish, showed that LDH can exist in multiple catalytically active conformations. The conformation of the renatured, active enzyme in this study was different from that of the native form, as measured by protein fluorescence, optical rotary properties and the reactivity of sulfhydryl groups. King and Weber (1986*a*,*b*) used high pressure and low temperature to dissociate LDH, and subsequently followed the time course of reassociation. They provided evidence that LDH subunits can exist in multiple conformations that differ both in their ability to assemble into tetramers and in their enzymatic activity. They further showed that the conformational changes responsible for the loss of activity were independent of those that affected tetramer reassembly. Several others enzymes have been shown to exist in multiple conformational states, including alcohol dehydrogenase (Jacobson *et al.* 1970), glyceraldehyde-3-phosphate dehydrogenase (Marangos and Constantinides, 1974) and pyruvate kinase (Susor and Rutter, 1968; Somero, 1969). Interestingly, the temperature-dependent conformers of pyruvate kinase of the king crab (*Paralithodes camtschatica*) differed in their kinetic properties in an apparently adaptive manner (Somero, 1969). Nickerson (1973) has presented a theoretical analysis of what he terms 'multistable' proteins, emphasizing that a single primary structure may have multiple folded states with different free energies, and that the conformation assumed by a protein under physiological conditions need not be the one that is thermodynamically most stable. He argues that multiple conformers of enzymes can be generated under different environmental conditions and that these may have adaptive significance.

A further example of conformational variation in polypeptides sharing a single amino acid sequence is provided by the prion phenomenon. A protein normally found in the brain cells of humans and other mammals, prion protein (PrP^C) has been shown to undergo a conformational shift to a form termed PrP^{Sc} for the disease scrapie (Prusiner, 1991). This form shows extensive β-sheets in place of the α-helices of PrP^C (Pan *et al.* 1993) and differs from the native form in important biochemical properties, including a tendency to aggregate and resistance to proteolysis (Prusiner, 1994). Interestingly, PrP^C can convert irreversibly to PrP^{Sc} in the presence of other PrP^{Sc} molecules, resulting in intracellular protein aggregations that cause a number of diseases including scrapie, bovine spongiform encephalopathy and Creutzfeldt–Jakob disease (Telling *et al.* 1996). Each of these is infectious through contact with diseased tissue, but has no apparent nucleic acid component. These findings imply that PrP^{Sc} acts as a template that converts PrP^C molecules to the PrP^{Sc} form (Prusiner, 1994).

A fascinating prion analog in yeast is the [*psi*$^+$] trait, involving the aggregation of the Sup35 protein, normally involved in translation termination (Ter-Avanesyan *et al.* 1994). Sup35 polypeptides in a particular conformation, [*psi*$^+$], appear to act as templates that cause a shift in conformation of [*psi*$^-$] proteins to the [*psi*$^+$] form. The heat shock protein Hsp104 appears to mediate this conversion, such that when Hsp104 levels are transiently increased, Sup35 in [*psi*$^+$] cells

begins to disaggregate and revert to the native [*psi*$^-$] conformation. If Hsp104 remains at high levels, the yeast cell can be 'cured' of the [*psi*$^+$] condition and shows no more aggregates (Patino *et al.* 1996). These results indicate that intracellular proteins can exhibit alternative conformers depending on the chaperone proteins present; furthermore, such conformational shifts can be due to changes in Hsp levels, as opposed to changes in the Hsp structures themselves (Chernoff *et al.* 1995; Lindquist and Kim, 1996).

The diverse studies described above show that conformers of proteins can exhibit differences in functional characteristics and thermal stability, despite the absence of differences in sequence and covalent modification. These results thus provide support for our hypothesis that conformational variants can account for the differences in K_m *versus* temperature relationships of the A_4-LDHs of the *Gillichthys* congeners. However, the present study is unprecedented in suggesting that conformers may be responsible for adaptive differences between species.

The mechanisms that could generate these hypothesized conformers of A_4-LDH in the *Gillichthys* congeners remain unknown. However, at least one potential mechanism can be excluded: temperature-dependent folding. Because specimens of *G. seta* and *G. mirabilis* were acclimated to a common temperature (approximately 15 °C), the alternative conformations proposed for their A_4-LDHs could not simply be a direct effect of temperature on the processes of folding and subunit assembly. Rather, unidentified cellular factors are hypothesized to direct folding along different maturation pathways in the two species. These factors might include low-molecular-mass constituents of the cell that bind to the nascent LDH-A polypeptides and affect folding or assembly. Possibly, these low-molecular-mass factors remain bound non-covalently to the protein and affect its stability and kinetics. Because the kinetic properties of LDHs are influenced by the energy changes that result from conformational alterations during binding and release of substrates and cofactors (see Holland *et al.* 1997), bound solutes that affect conformational mobility could have significant effects on kinetic constants such as K_m. Thus, non-covalent binding of low-molecular-mass solutes to A_4-LDH, while seemingly an alternative explanation for the differences between the enzymes of *G. seta* and *G. mirabilis*, may also exert its effects by influencing the conformation of the enzyme or, more precisely, the energy changes that occur during functionally important shifts in conformation.

The involvement of molecular chaperones is hypothesized to be another potential mechanism for generating conformers. Although chaperones are generally regarded as facilitating folding without determining the final folded state of the protein (Hartl, 1996), the discovery that Hsp104 can affect the conformation of Sup35 protein suggests that differences in chaperoning activity could potentially lead to alternative folding of A_4-LDH in the two *Gillichthys* species.

Whatever the mechanism is that underlies the differences in kinetics and stability between the mature A_4-LDHs of the *Gillichthys* congeners, our results suggest that differences in amino acid sequence are not always necessary for adapting proteins to temperature. If the kinetics and stabilities of enzymes can be modified through the production of stable alternative conformers during the maturation of a newly synthesized protein, then another mechanism for the adaptation of enzymes, in addition to changes in their amino acid sequence, may exist.

We thank Ms L. Z. Holland for her help in preparation and sequencing of the goby cDNA. We thank Mr R. McConnaughey and Dr M. E. Clarke for their assistance in collecting specimens. P.A.F. would also like to express his gratitude to Dr J. B. Graham for helpful advice and guidance during much of this work. The work described in this paper was funded in part by NSF grant IBN92-06660, in part by a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, US Department of Commerce, under grant number NA89AA-D-SG138, project number 72-C-N, through the California Sea Grant College, and in part by the G. Unger Vetlesen Foundation. The views expressed herein are those of the authors and do not necessarily reflect the views of NOAA or any of its sub-agencies. The US Government is authorized to reproduce and distribute for governmental purposes.

References

ABAD-ZAPATERO, C., GRIFFITH, J. P., SUSSMAN, J. L. AND ROSSMANN, M. G. (1987). Refined crystal structure of dogfish M_4 apo-lactate dehydrogenase. *J. molec. Biol.* **198**, 445–467.

BARLOW, G. W. (1961). Gobies of the genus *Gillichthys* with comments on the sensory canals as a taxonomic tool. *Copeia* **1961**, 423–437.

BROOKS, S. P. J. AND SUELTER, C. H. (1986). Estimating enzyme kinetic parameters: A computer program for linear regression and nonparametric analysis. *Int. J. Bio-med. Comput.* **19**, 89–99.

CHERNOFF, Y. O., LINDQUIST, S. L., ONO, B.-I., INGE-VECHTOMOV, S. G. AND LIEBMAN, S. W. (1995). Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [*psi*$^+$]. *Science* **268**, 880–883.

CHOMCZYNSKI, P. AND SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Analyt. Biochem.* **162**, 156–159.

COATES, A. G., JACKSON, J. B. C., COLLINS, L. S., CRONIN, T. M., DOWSETT, H. J., BYBEL, L. M., JUNG, P. AND OBANDO, J. A. (1993). Closure of the isthmus of Panama: The near-shore marine record of Costa Rica and western Panama. *Geol. Soc. Am. Bull.* **104**, 814–828.

DAHLHOFF, E. AND SOMERO, G. N. (1993). Kinetic and structural adaptations of cytoplasmic malate dehydrogenases of Eastern Pacific abalone (genus *Haliotis*) from different thermal habitats: biochemical correlates of biogeographical patterning. *J. exp. Biol.* **185**, 137–150.

EDMONDS, C. G. AND SMITH, R. D. (1990). Electrospray ionization mass spectrometry. In *Methods in Enzymology*, vol. 193, *Mass Spectrometry* (ed. J. A. McCloskey), pp. 412–431. New York: Academic Press.

EDWARDS, J. B. D. M., DELORT, J. AND MALLET, J. (1991). Oligodeoxyribonucleotide ligation to single-stranded cDNAs – a new tool for cloning 5′-ends of messenger RNAs and for constructing cDNA libraries by *in vitro* amplification. *Nucleic Acids Res.* **19**, 5227–5232.

FIELDS, P. A., GRAHAM, J. B., ROSENBLATT, R. H. AND SOMERO, G. N. (1993). Effects of expected global climate change on marine faunas. *Trends Ecol. Evol.* **8**, 361–365.

FROHMAN, M. A., DUSH, M. K. AND MARTIN, G. R. (1988). Rapid production of full-length cDNA's from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8998–9002.

GOULD, S. J. (1977). *Ontogeny and Phylogeny*. Cambridge: Belknap Press.

GOULD, S. J. (1988). The uses of heterochrony. In *Heterochrony in Evolution* (ed. M. L. McKinney), pp. 1–13. New York: Plenum Press.

GRAVES, J. E. AND SOMERO, G. N. (1982). Electrophoretic and functional enzymic evolution in four species of eastern Pacific barracudas from different thermal environments. *Evolution* **36**, 97–106.

HARTL, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature* **381**, 571–580.

HOCHACHKA, P. W. AND SOMERO, G. N. (1984). *Biochemical Adaptation*. Princeton: Princeton University Press.

HOLLAND, L. Z., MCFALL-NGAI, M. AND SOMERO, G. (1997). Evolution of lactate dehydrogenase-A homologs of barracuda fishes (Genus *Sphyraena*) 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.

HORNES, E. AND KORSNES, L. (1990). Magnetic DNA hybridization properties of oligonucleotide probes attached to superparamagnetic beads and their use in the isolation of poly(A) mRNA from eukaryotic cells. *Genet. analyt. Tech. Appl.* **7**, 145–150.

HUBBS, C. L. (1948). Changes in the fish fauna of Western North America correlated with changes in ocean temperature. *J. mar. Res.* **7**, 459–482.

IMANAKA, T., SHIBAZAKI, M. AND TAKAGI, M. (1986). A new way of enhancing the thermostability of proteases. *Nature* **324**, 695–697.

JACOBSON, K. B., MURPHY, J. B. AND HARTMAN, F. C. (1970). Isoenzymes of *Drosophila* alcohol dehydrogenase. *J. biol. Chem.* **245**, 1075–1083.

JAENICKE, R. (1991). Protein stability and molecular adaptation to extreme conditions. *Eur. J. Biochem.* **202**, 715–728.

JOHNSTON, I. A. AND WALESBY, N. J. (1977). Molecular mechanisms of temperature adaptation in fish myofibrillar adenosine triphosphatases. *J. comp. Physiol.* **119**, 195–206.

KING, L. AND WEBER, G. (1986*a*). Conformational drift of dissociated lactate dehydrogenases. *Biochemistry* **25**, 3632–3637.

KING, L. AND WEBER, G. (1986*b*). Conformational drift and cryoinactivation of lactate dehydrogenase. *Biochemistry* **25**, 3637–3640.

KNOWLTON, N., WEIGT, L. A., SOLORZANO, L. A., MILLS, D. K. AND BERMINGHAM, E. (1993). Divergence in proteins, mitochondrial DNA and reproductive compatibility across the isthmus of Panama. *Science* **260**, 1629–1632.

LEVI, A. S. AND KAPLAN, N. O. (1971). Physical and chemical properties of reversibly inactivated lactate dehydrogenases. *J. biol. Chem.* **246**, 6409–6417.

LINDQUIST, S. AND KIM, G. (1996). Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc. natn. Acad. Sci. U.S.A.* **93**, 5301–5306.

MARANGOS, P. J. AND CONSTANTINIDES, S. M. (1974). Multiple forms of flounder muscle glyceraldehyde-3-phosphate dehydrogenase. *J. biol. Chem.* **249**, 951–958.

MATTHEWS, B. W., NICHOLSON, H. AND BECKTEL, W. J. (1987). Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc. natn. Acad. Sci. U.S.A.* **84**, 6663–6667.

MCFALL-NGAI, M. AND HORWITZ, J. (1990). A comparative study of the thermal stability of the vertebrate eye lens: Antarctic fish to the desert iguana. *Exp. Eye Res.* **50**, 703–709.

MILLER, D. J. AND LEA, R. N. (1972). Guide to the coastal marine fishes of California. *Cal. Fish Bull.* **157**.

MULLIS, K. B. AND FALOONA, F. A. (1987). Specific synthesis of DNA *in vitro via* a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155**, 335–350.

NICKERSON, K. W. (1973). Biological functions of multistable proteins. *J. theor. Biol.* **40**, 507–515.

OZERNYUK, O. D., KLYACHKO, O. S. AND POLOSUKHINA, E. S. (1994). Acclimation temperature affects the functional and structural properties of lactate dehydrogenase from fish (*Misgurnus fossilis*) skeletal muscles. *Comp. Biochem. Physiol.* **107**B, 141–145.

PAN, K.-M., BALDWIN, M., NGUYEN, J., GASSET, M., SERBAN, A., GROTH, D., MEHLHORN, I., HUANG, Z. AND PRUSINER, S. B. (1993). Conversion of α-helices into β-sheets features in the formation of the scrapie prion proteins. *Proc. natn. Acad. Sci. U.S.A.* **90**, 10962–10966.

PATINO, M. M., LIU, J.-J., GLOVER, J. R. AND LINDQUIST, S. (1996). Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. *Science* **273**, 622–626.

PLACE, A. R. AND POWERS, D. A. (1984). Purification and characterization of the lactate dehydrogenase (LDH-B_4) allozymes of *Fundulus heteroclitus*. *J. biol. Chem.* **259**, 1309–1318.

POWERS, D. A., SMITH, M., GONZALEZ-VILLASENOR, I., DIMICHELLE, L., CRAWFORD, D., BERNARDI, G. AND LAUERMAN, T. (1993). A multidisciplinary approach to the selectionist/neutralist controversy using the model teleost, *Fundulus heteroclitus*. In *Oxford Surveys in Evolutionary Biology*, vol. 9 (ed. D. Futuyama and J. Antonovics), pp. 43–107. Oxford: Oxford University Press.

PRUSINER, S. B. (1991). Molecular biology of prion diseases. *Science* **252**, 1515–1522.

PRUSINER, S. B. (1994). Biology and genetics of prion diseases. *A. Rev. Microbiol.* **48**, 655–686.

SMITH, L. M., KAISER, R. J., SANDERS, J. Z. AND HOOD, L. E. (1987*a*). The synthesis and use of fluorescent oligonucleotides in DNA sequence analysis. *Methods Enzymol.* **155**, 260–301.

SMITH, L. M., SANDERS, J. Z., KAISER, R. J., HUGHES, P., DODD, C., CONNELL, C. R., HEINER, C., KENT, S. B. H. AND HOOD, L. E. (1987*b*). Fluorescence detection in automated DNA sequence analysis. *Nature* **321**, 674–679.

SOMERO, G. N. (1969). Pyruvate kinase variants of the Alaskan king-crab. *Biochem. J.* **114**, 237–241.

SOMERO, G. N. (1981). pH–temperature interactions on proteins: principles of optimal pH and buffer system design. *Mar. Biol. Lett.* **2**, 163–178.

SOMERO, G. N. (1995). Proteins and temperature. *A. Rev. Physiol.* **57**, 43–68.

SUSOR, W. A. AND RUTTER, W. J. (1968). Some distinctive properties of pyruvate kinase purified from rat liver. *Biochem. biophys. Res. Commun.* **30**, 14–20.

TELLING, G. C., PARCHI, P., DEARMOND, S. J., CORTELLI, P., MONTAGNA, P., GABIZON, R., MASTRIANNI, J., LUGARESI, E., GAMBETTI, P. AND PRUSINER, S. B. (1996). Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. *Science* **274**, 2079–2082.

TER-AVANESYAN, M. D., DAGKESAMANSKAYA, A. R., KUSHNIROV, V. V. AND SMIRNOV, V. N. (1994). The *SUP35* omnipotent suppressor gene is involved in the maintenance of the non-Mendelian determinant [*psi*$^+$] in the yeast *Saccharomyces cerevisiae*. *Genetics* **137**, 671–676.

TESSIER, D. C., BROUSSEAU, R. AND VERNET, T. (1986). Ligation of single-stranded oligodeoxyribonucleotides by T4 RNA ligase. *Analyt. Biochem.* **158**, 171–178.

THOMSON, D. A., FINDLEY, L. T. AND KERSTITCH, A. N. (1979). *Reef Fishes of the Sea of Cortez*. New York: John Wiley and Sons.

WALSH, P. J. AND SOMERO, G. N. (1982). Interactions among pyruvate concentration, pH and K_m of pyruvate in determining *in vivo* Q_{10} values of the lactate dehydrogenase reaction. *Can. J. Zool.* **60**, 1293–1299.

WILKINSON, G. N. (1961). Statistical estimations in enzyme kinetics. *Biochem. J.* **80**, 323–332.

XUE, Q. AND YEUNG, E. S. (1995). Differences in the chemical reactivity of individual molecules of an enzyme. *Nature* **373**, 681–683.

YANCEY, P. H. AND SIEBENALLER, J. F. (1987). Coenzyme binding ability of homologues of M_4-lactate dehydrogenase in temperature adaptation. *Biochim. biophys. Acta* **924**, 483–491.

YANCEY, P. H. AND SOMERO, G. N. (1978). Temperature dependence of intracellular pH: its role in the conservation of pyruvate apparent K_m values of vertebrate lactate dehydrogenases. *J. comp. Physiol.* **125**B, 129–134.

ZANEVELD, J. S. (1983). *Caribbean Fish Life: Index to the Local and Scientific Names of the Marine Fishes and Fishlike Invertebrates of the Caribbean Area (Tropical Western Central Atlantic Ocean)*. Leiden: E. J. Brill.