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THERMAL ADAPTATION OF CYTOPLASMIC MALATE DEHYDROGENASES OF EASTERN PACIFIC BARRACUDA (*SPHYRAENA* spp): THE ROLE OF DIFFERENTIAL ISOENZYME EXPRESSION

JEN-JEN LIN* AND GEORGE N. SOMERO†

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0202, USA

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Summary

Kinetic properties, electrophoretic patterns and thermal stabilities of cytoplasmic malate dehydrogenases (cMDHs) were compared in Eastern Pacific barracuda (*Sphyraena* spp) from different latitudes. All tissues of the tropical species *S. ensis* contained only a single, thermostable form of cMDH. Subtropical (*S. lucasana*) as well as north (*S. argentea*) and south (*S. idiastes*) temperate barracuda contained both thermostable and thermolabile cMDHs, the pattern characteristic of most teleosts. Kinetic studies using unfractioned cMDHs showed that the apparent Michaelis–Menten constant (K_m) of cofactor (NADH) increased with temperature, but at the physiological temperatures of the four species, K_m of NADH was conserved within a narrow range (20–23 μmol l^{-1}). Thermostable and thermolabile cMDHs were chromatographically separated and compared. Thermolabile cMDHs had higher K_m values for NADH at all measurement temperatures than did thermostable cMDHs. Thermolabile cMDHs isolated from congeneric barracuda exhibited similar kinetic properties (K_m *versus* temperature, optimal pH, optimal substrate and cofactor concentrations). Thermostable cMDHs, likewise, were similar among the barracuda. Conservation of K_m in the differently thermally adapted barracudas is, therefore, apparently due to adjustments in the ratio of expression of the thermostable and thermolabile isoforms, rather than to temperature-adaptive differences among orthologous homologues, as is commonly found for enzymes encoded by a single gene locus. The effects of temperature on the K_m of NADH for isolated thermostable and thermolabile cMDHs of a eurythermal goby, *Gillichthys mirabilis*, however, were consistent with adaptive change in orthologous homologues of cMDH. The selective basis for the absence of thermolabile cMDH in warm-adapted ectotherms, mammals and birds is discussed.

Key words: adaptation, barracuda, biogeography, isoenzymes, malate dehydrogenase, *Sphyraena*, temperature.

Introduction

Biogeographical patterning, such as changes in species composition with latitude, commonly mirrors gradients or discontinuities in environmental temperature (Fields *et al.* 1993). Physiological and biochemical adaptations to temperature are likely to play important roles in establishing and maintaining these temperature-linked species distribution patterns (Hochachka and Somero, 1984; Somero, 1986; Cossins and Bowler, 1987; Fields *et al.* 1993). Comparisons of ectothermic species from different latitudes have revealed differences in the structural and functional properties of proteins and in mitochondrial thermal resistance that reflect the different adaptation temperatures of the species (Baldwin, 1971; Low *et al.* 1973; Borgmann *et al.* 1975; Borgmann and Moon, 1975; Johnston and Goldspink, 1975; Johnston and Walesby, 1977; Graves and Somero, 1982; Swezey and Somero, 1982; Graves *et al.* 1983; Dahlhoff and Somero, 1993*a*,*b*).

Most studies of temperature adaptation have compared distantly related species belonging to different genera or families. Although such studies have effectively exploited the comparative method to deduce adaptively important changes among species, comparison of distantly related taxa entails the problem of determining whether differences between species are in fact adaptations to the environmental factor in question or merely reflections of phylogenetic history (Huey, 1987). Comparisons of closely related congeneric species provide a means for reducing this type of uncertainty. Congeners adapted to different environments are often so similar in evolutionary

*Present address: Institute of Fisheries Sciences, National Taiwan University, Taipei, Taiwan 10764, Republic of China.
†Present address and author for correspondence: Department of Zoology, 3029 Cordley Hall, Oregon State University, Corvallis, OR 97331-2914, USA.

history, life histories and overall ecological relationships that environmental adaptations appear in sharp relief (Graves and Somero, 1982; Dahlhoff and Somero, 1993*a*,*b*). In a study of A_4-lactate dehydrogenases (A_4-LDHs) of congeneric barracuda (*Sphyraena* spp) from the Eastern Pacific, Graves and Somero (1982) showed that interspecific differences in kinetic properties [Michaelis–Menten constant (K_m) of substrate and catalytic rate constant (k_{cat})] reflected the adaptive patterns observed in comparisons of taxonomically diverse vertebrates with body temperatures spanning a wide temperature range (approximately 50 °C), even though the barracuda differed in average body temperature by only 3–8 °C. These findings showed that differences in average or maximal habitat temperature of the order of a few degrees Celsius appear to be sufficient to favour selection for adaptively different orthologous homologues of enzymes.

We studied a second enzyme, cytosolic malate dehydrogenase (cMDH; EC 1.1.1.37; malate:NAD^+ oxidoreductase), in barracuda congeners to analyze further the biochemical correlates of the thermal distribution patterns of these species. Our studies focused on two aspects of cMDH that distinguish the enzyme from A_4-LDH. First, dimeric cMDH, a close evolutionary relative of LDH, lacks the N-terminal peptide that, in the tetrameric LDH molecule, is important for stabilizing subunit interactions and establishing enzyme thermal stability (Opitz *et al.* 1987). Modifications in the N-terminal region of A_4-LDH may play important roles in adaptation to temperature by this enzyme (L. Holland, M. J. McFall-Ngai and G. N. Somero, in preparation). In view of these differences between LDH and cMDH in quaternary structure and N-terminal sequence, we wished to determine whether cMDH is as thermally sensitive as A_4-LDH and whether orthologous cMDH homologues (homologues encoded by a common gene locus in the different species) of barracuda exhibit interspecific differences in thermal responses similar to those found for orthologous forms of A_4-LDH.

Second, cMDH has the potential for a type of adaptation not available to A_4-LDH. In most vertebrates, including teleost fish, a single gene locus encodes the A-type LDH (Markert *et al.* 1975). Most teleost fishes, however, unlike most higher vertebrates, possess two genes for cMDH. One *cMDH* locus encodes a thermostable isoenzyme and a second encodes a thermolabile cMDH (Schwantes and Schwantes, 1982*a*,*b*). We wished to determine whether these two paralogous isoenzymes (homologues encoded by different gene loci) of cMDH differed in kinetic properties and whether the ratio of thermostable to thermolabile cMDHs varied among species according to their adaptation temperatures. Differential expression of paralogous isoenzymes during thermal acclimation has been reported for some proteins (Baldwin and Hochachka, 1970; Somero, 1975, 1995; Johnston, 1983), but this phenomenon has not been compared in differently thermally adapted species.

To address these questions, we studied cMDHs of four Eastern Pacific barracuda: *Sphyraena argentea* (north temperate; habitat temperature range approximately 15–22 °C); *Sphyraena idiastes* (south temperate; habitat temperature range 15–22 °C); *Sphyraena lucasana* (subtropical; Sea of Cortez; habitat temperature range 17–28 °C); and *Sphyraena ensis* (tropical equatorial; habitat temperature range 22–30 °C). We compared electrophoretic patterns, the effects of temperature on the apparent Michaelis–Menten constant (K_m) of NADH and the thermal stabilities of both unfractionated cMDH isoenzymes and chromatographically separated thermostable and thermolabile isoenzymes. We combined chromatographically separated thermostable and thermolabile cMDH isoenzymes in different ratios to study the additivity of kinetic properties in mixed isoenzyme systems. We purified cMDH isoenzymes from a fish belonging to a different family to determine whether orthologous forms of the two cMDH isoenzymes differed among species from different families. We conclude that variation in the ratio of paralogous thermostable to thermolabile cMDHs is important in adaptation to temperature. The absence of thermolabile cMDH in warm-adapted species may be a specific adaptation to high temperature rather than merely a reflection of phylogeny.

Materials and methods

Animals

Barracuda were caught by hook-and-line fishing. *S. argentea* (Girard) were caught offshore from San Diego, California; *S. idiastes* (Heller and Snodgrass) were caught in cold upwelling regions near the Galapagos Islands and off the coast of Peru; *S. lucasana* (Gill) were caught at Guaymas, Mexico; and *S. ensis* (Jordan and Gilbert) were collected in Panama. Long jawed mudsuckers, *Gillichthys mirabilis*, were captured in baited traps in a slough near San Felipe, Baja California. Fish were transported on dry ice and stored at −20 or −80 °C until used.

Preparation of unfractionated cMDH

White muscle was homogenized in 4 vols of ice-cold 50 mmol l^{-1} sodium phosphate buffer (pH 6.8) using a Waring blender. The crude homogenate was centrifuged at 27 000 ***g*** for 30 min at 4 °C. The supernatant was collected and filtered through glass wool to remove unhomogenized tissue and suspended lipid. An oxamate affinity column was used to remove LDH from the homogenate because of potential interference with the cMDH assay (degradation of oxaloacetate to pyruvate would lead to activity of any LDH present) (O'Carra and Barry, 1972; Yancey and Somero, 1978). The presence of MDH was determined by activity assay (see below). Fractions containing MDH activity were pooled and solid ammonium sulphate was added to give a final concentration of 0.56 g l^{-1} (approximately 80 % saturation). The enzyme precipitated in ammonium sulphate was stored at 4 °C. Prior to use in assays, samples of the ammonium sulphate suspension were centrifuged at 27 000 ***g*** for 30 min at 4 °C. The pellet was dialyzed against 20 mmol l^{-1} imidazole chloride buffer (pH 7.0 at 20 °C) for approximately 12 h at 4 °C.

Dialyzed enzyme was centrifuged as before and the supernatant was retained for assaying enzymatic activity.

Assay of MDH activity

Total cMDH activity in chromatographically separated fractions and thermal stability assays was determined using an assay buffer (80 mmol l^{-1} imidazole chloride, pH 7.0 at 20 °C) containing 100 mmol l^{-1} KCl, 0.3 mmol l^{-1} oxaloacetate and 0.12 mmol l^{-1} NADH. The reaction was initiated by adding 10 μl of enzyme solution to 2.0 ml of assay medium. Oxidation of NADH was monitored by the decrease in absorbance at 340 nm in a Perkin-Elmer Lambda 3B recording spectrophotometer. Cuvette temperature was maintained at 20±0.1 °C.

Measurement of K_m *of NADH*

The apparent K_m of NADH was measured in an assay medium (2.0 ml) containing 80 mmol l^{-1} imidazole chloride buffer (pH 7.0 at 20 °C), 100 mmol l^{-1} KCl, 0.3 mmol l^{-1} oxaloacetate and different concentrations of NADH (15, 20, 25, 30, 40, 60 or 80 μmol l^{-1}). Enzyme was added to yield an absorbance change of 0.095–0.105 absorbance units per minute at the temperature of measurement, at the highest concentration of NADH. Triplicate assays were run for each NADH concentration. K_m of NADH was calculated by Lineweaver–Burk analysis using the weighted linear regression method of Wilkinson (1961) and Wilman software (Brooks and Suelter, 1986).

For experiments in which different proportions of thermostable and thermolabile cMDHs of *S. argentea* were combined, the amount of each isoenzyme was quantified by measuring MDH activity in a reaction mixture containing 80 mmol l^{-1} imidazole chloride buffer (pH 7.5 at 30 °C), 100 mmol l^{-1} KCl, 0.2 mmol l^{-1} oxaloacetate and 0.2 mmol l^{-1} NADH.

Thermostability of MDH

Unfractioned mixtures

Samples (200 μl) of dialyzed unfractioned cMDH (homogenates from which LDH had been removed; see above) in 20 mmol l^{-1} imidazole chloride buffer (pH 7.0 at 20 °C) were placed in 1.75 ml microcentrifuge tubes and incubated in a water bath at 40 or 45 °C. After 10, 20 and 30 min, sample tubes were removed and placed in an ice bath for 5 min. Samples were then centrifuged in a microcentrifuge at 14 000 ***g*** for 10 min. MDH activity in the supernatant was assayed immediately at 20 °C. Residual activity was calculated as the ratio of MDH activity in the heated sample to that in an unheated control held in an iced-water bath. No activity was lost in the control samples during the course of the experiment.

Chromatographically separated cMDHs

To measure the thermostabilities of the thermostable and thermolabile cMDHs of *S. argentea*, the separated isoenzymes were first washed in a microconcentrator (Centricon 30; Amicon, Inc.) with 20 mmol l^{-1} imidazole chloride buffer (pH 7.0) to remove 2-mercaptoethanol. Bovine serum albumin was then added, to a concentration of 1 mg ml^{-1}. The initial activity of the enzyme was measured. Samples (50 μl) were dispensed in microcentrifuge tubes and incubated in a water bath at 42±0.1 °C. At different times up to approximately 9 h, samples were removed and placed on ice for 5 min. Samples were then centrifuged at 14 000 ***g*** for 10 min, and MDH activity in the supernatant was assayed immediately. The residual activity was calculated as the percentage of MDH activity remaining after heat treatment.

Polyacrylamide gel electrophoresis

To determine isoenzyme banding patterns and to confirm separation of thermostable and thermolabile cMDH, we performed non-denaturing polyacrylamide gel electrophoresis (native PAGE). Samples were prepared as follows. Homogenates from different tissues and fractions from chromatographic columns were first adjusted for MDH content by measuring MDH activity. Samples were diluted so that 10 μl of sample contained enough MDH activity to give an absorbance change of approximately 0.2 units min^{-1}. The gel system consisted of a 3 % acrylamide stacking gel, a 7.5 % acrylamide running gel and Tris–glycine electrode buffer (pH 8.3). After electrophoresis at 4 °C, the gel was stained for MDH activity following the protocol of Richardson *et al.* (1986). The staining solution contained 168 mmol l^{-1} Tris chloride buffer (pH 8.0 at 20 °C), 210 mmol l^{-1} D,L-malate, 0.30 mmol l^{-1} NAD^+, 26 μmol l^{-1} Nitroblue Tetrazolium and 86 μmol l^{-1} phenazine methosulphate.

Ion-exchange and affinity chromatography

Partial purification and complete separation of cMDH isoenzymes were performed using ion-exchange chromatography (*S. lucasana*) or affinity chromatography (*S. argentea*, *S. ensis*, *G. mirabilis*). For ion-exchange chromatography, the starting material was unfractionated cMDH isolated from white muscle of *S. lucasana*, as described previously. This unfractionated cMDH was dialyzed against 20 mmol l^{-1} imidazole chloride buffer (pH 8.0 at 20 °C), centrifuged (14 000 ***g***, 10 min) to remove denaturated protein and loaded onto a column of DEAE–Sephacel (Sigma Chemical Co.) equilibrated at 4 °C with dialysis buffer. The column was washed exhaustively with this buffer until no protein could be detected (by absorbance at 280 nm) in the eluted fractions. Thermostable cMDH was eluted with buffer containing 17.5 mmol l^{-1} KCl. After all thermostable cMDH had been eluted, the KCl concentration in the buffer was increased to 40 mmol l^{-1} to elute thermolabile cMDH. Affinity chromatography with triazinyl dye (Matrex Gel Red A; Amicon, Inc.) was conducted following a modification of the procedure of Smith *et al.* (1982). Samples applied to the column were from white muscle of *S. argentea*, *S. ensis* or *G. mirabilis* and had been treated in the same way as the sample used for DEAE–Sephacel chromatography, except that they were dialysed against 10 mmol l^{-1} sodium phosphate buffer

(pH 7.2) containing 1 mmol l^{-1} 2-mercaptoethanol. After sample addition, the column was washed serially with 20 mmol l^{-1} KCl, 20 mmol l^{-1} D,L-malate, 20 mmol l^{-1} KCl, 0.35 mmol l^{-1} NAD^+ (all in 10 mmol l^{-1} phosphate buffer) and buffer alone. Thermostable cMDH was eluted with 20 mmol l^{-1} D,L-malate and 0.4 mmol l^{-1} NAD^+ in buffer. Thermolabile cMDH was then eluted using a continuous gradient of NAD^+ and D,L-malate (buffer A, 0.4 mmol l^{-1} NAD^+ and 20 mmol l^{-1} D,L-malate; buffer B, 6.0 mmol l^{-1} NAD^+ and 200 mmol l^{-1} D,L-malate). Fractions containing MDH activity were pooled in groups, washed with dialysis buffer to remove substrates, and concentrated by ultrafiltration (Centricon 30). These samples were loaded onto native PAGE to test for isoenzyme separation. For storage of separated isoenzymes, 1 mmol l^{-1} 2-mercaptoethanol was added to the enzyme solution.

Optimal pH and substrate and cofactor concentrations

The optimal pH and concentrations of substrate and cofactor were defined as the values that gave the highest MDH activity under the assay conditions employed. Imidazole chloride buffer (80 mmol l^{-1}, pH 7.5 at 20 °C; 100 mmol l^{-1} KCl) was used in all measurements. For optimal pH determination, concentrated HCl or KOH was used to adjust the buffer pH. For the measurements of optimal concentrations of oxaloacetate, malate, NADH and NAD^+, reaction mixtures contained concentrations of the cosubstrate that yielded optimal activity: 200 μmol l^{-1} NADH or 3 mmol l^{-1} NAD^+, and 0.2 mmol l^{-1} oxaloacetate or 5 mmol l^{-1} malate, respectively.

Results

Unfractioned cMDH isoenzymes: electrophoretic patterns, thermal stabilities and kinetics

Two types of patterns were observed when tissues of the four species were examined using native PAGE (Fig. 1). For the temperate (*S. argentea* and *S. idiastes*) and subtropical (*S. lucasana*) species, two major bands were observed in white muscle, red muscle, brain, liver and heart (only white muscle is shown in Fig. 1). The fast- and slow-running bands, which we interpret as homodimeric enzymes, each showed similar migration rates in these three species. Analysis of eleven individuals of *S. ensis*, the tropical species, revealed only a single major cMDH band in these tissues in all specimens. In some specimens of *S. ensis*, there were also two minor bands, which migrated faster than the dominant band and, like the dominant band, were thermally stable (see below). The intensity of the two minor bands varied with the freshness of the sample and conditions of electrophoresis. These bands were not interpreted as evidence for allozymic variants of the faster-migrating cMDH. We did not detect the mitochondrial isoenzyme, mMDH, which usually migrates much more slowly than cMDHs and which is not often detected in fish muscle (Basaglia, 1989).

To determine which bands corresponded with the thermostable and thermolabile cMDH isoenzymes reported by

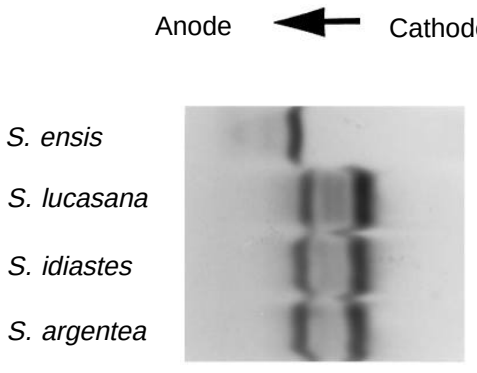

Fig. 1. Native PAGE of cytoplasmic malate dehydrogenases (cMDHs) from white skeletal muscle of four barracuda species. cMDH bands are visualized by activity staining.

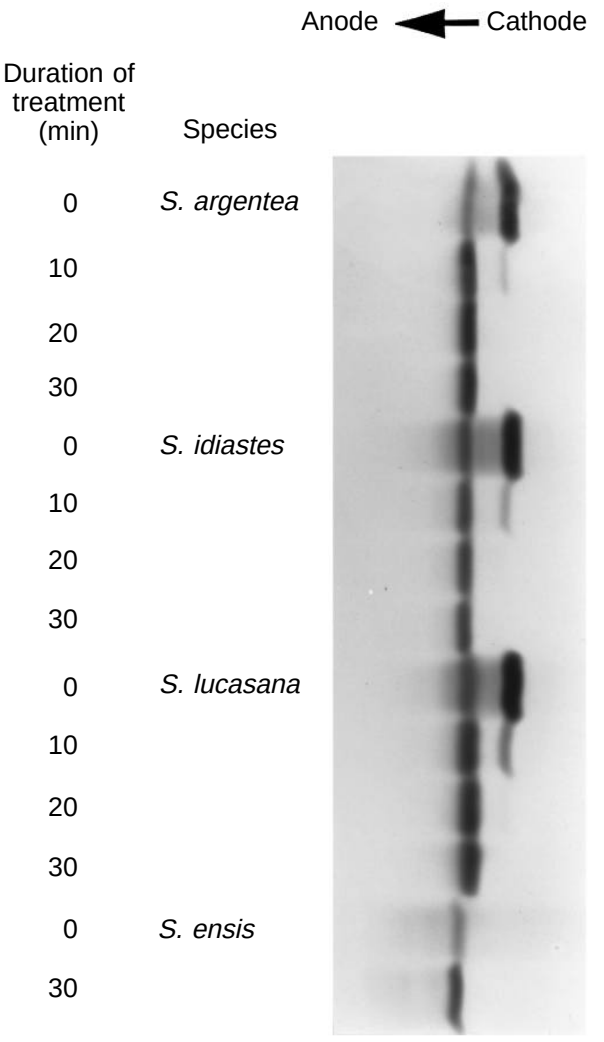

Fig. 2. The effects of heat treatment on electrophoretic patterns of cMDHs from white skeletal muscle of four barracuda species. Samples of unfractionated cMDH were incubated at 45 °C for 10, 20 or 30 min, electrophoresed on native PAGE and stained for residual activity.

Schwantes and Schwantes (1982*a*,*b*), we used native PAGE to examine cMDHs from homogenates previously heated to 45 °C for 10, 20 or 30 min (Fig. 2). The faster-migrating cMDH was the thermostable isoenzyme, as shown by retention of activity after 30 min of heat treatment. The slower-migrating (more cathodal) cMDH lost activity rapidly at 45 °C. Most activity disappeared after 10 min of heat treatment, and all activity was lost after 20 min of heating.

Interspecific differences in thermal stability were noted when unfractionated cMDH was incubated at different temperatures for various times and assayed for residual MDH activity (Fig. 3). At 40 °C, cMDH of all species lost approximately 15–30 % of activity during 30 min of incubation. Some of the apparent heat denaturation of cMDH was probably due to sedimentation of active cMDH with denatured protein when the heat-treated homogenates were centrifuged prior to activity assay. At 45 °C, a difference was noted between *S. ensis* and the other species, consistent with the differences shown in Fig. 2. For all species except *S. ensis*, approximately half of the cMDH activity was lost during 30 min of incubation. The enzyme of *S. ensis* lost only approximately 20 % of its activity.

To compare the effects of temperature on cMDH function in the four species, we first examined the response of the apparent K_m of cofactor (NADH) to measurement temperature using cMDHs that were not fractionated into separate isoenzymes (unfractionated cMDH; see above). K_m estimates made using unheated enzyme revealed that the cMDH of *S. ensis* had a lower K_m of NADH at all temperatures than did the cMDHs of the other species, which contained both thermostable and thermolabile isoforms ($P<0.01$; analysis of covariance and *post hoc* contrast analysis). After heat treatment of the enzyme preparations, the K_m of NADH for the *S. ensis* enzyme was unchanged, but the K_m values for cMDHs of the other species were lowered to values that were not significantly different ($P=0.728$, analysis of covariance) from those for cMDH of *S. ensis* (Fig. 4). The K_m values for cMDHs of *S. argentea*, *S. lucasana* and *S. idiastes* were significantly lower in the heated preparations compared with unheated preparations ($P<0.01$, analysis of covariance). For control and heated enzymes we observed linear Lineweaver–Burk plots for all species, despite the occurrence of two cMDHs with different K_m values in muscle of three species (see below).

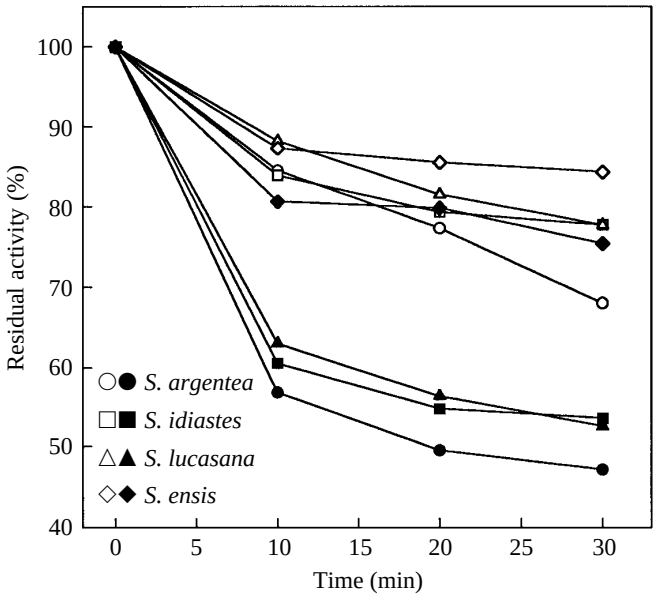

Fig. 3. Residual activity of cMDH in unfractionated cMDH preparations from white skeletal muscle following incubation at 40 °C (open symbols) or 45 °C (filled symbols) for 10, 20 or 30 min.

Chromatographically fractionated cMDH isoenzymes: thermal stabilities and kinetics

To characterize more precisely the temperature sensitivities of thermostable and thermolabile cMDH isoenzymes, we partially purified the isoforms using ion-exchange (*S. lucasana*; Fig. 5A) or affinity chromatography (other species; e.g. *S. argentea*, Fig. 5B). The latter method was more convenient because it allowed isoenzyme separation to be achieved using the same elution conditions for all species. Electrophoretic analysis of the fractions using native PAGE showed full separation of thermostable and thermolabile cMDHs by both methods (Fig. 5). In fractions from the affinity column that contained high concentrations of substrate and cofactor, the bands were smeared, but complete isoenzyme separation was evident (Fig. 5B).

Isolated thermostable and thermolabile cMDHs exhibited

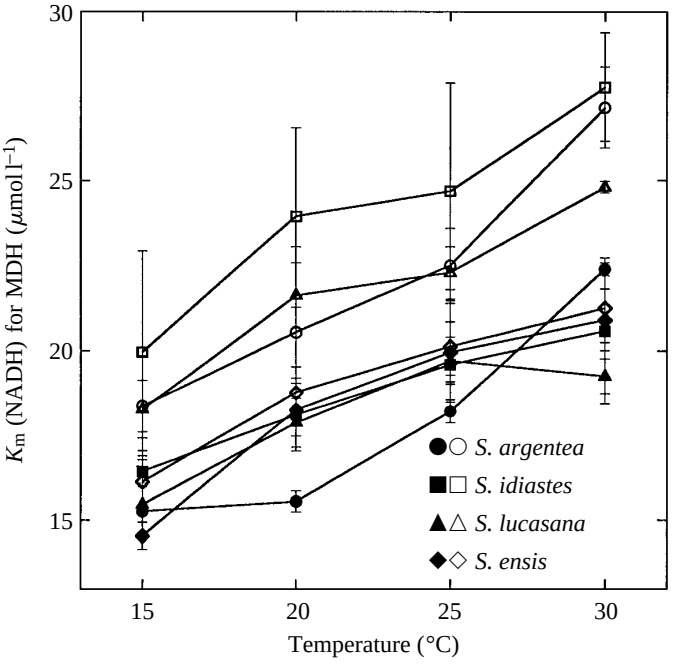

Fig. 4. The effect of heat treatment (45 °C for 30 min) on Michaelis–Menten constants (K_m) of NADH for unfractionated cMDHs (unheated preparations, open symbols; heated preparations, filled symbols). Error bars are standard errors of the mean for single K_m values determined using triplicate assays of activities at seven concentrations of NADH (see Materials and methods).

differences in thermal stability consistent with the differences observed using unfractionated isoenzymes (Fig. 6). At an incubation temperature of 42 °C, the half-life of thermolabile cMDH was only 2 min, but that of thermostable cMDH was longer than 9 h. Both isoenzymes appeared to be less thermally stable after partial purification. This loss in thermal stability is probably a result of the lower total protein concentration in the chromatographically separated samples compared with that of unfractionated samples.

The effects of temperature on the K_m of NADH for thermostable and thermolabile cMDHs of *S. argentea* and *S. lucasana* are shown in Fig. 7. Consistent with the data in Fig. 4, thermostable cMDH had a lower K_m at all temperatures than did the thermolabile isoenzyme. Orthologous cMDHs exhibited similar K_m *versus* temperature profiles in comparisons involving *S. lucasana* and *S. argentea* (Fig. 7). This observation suggests that, unlike the A_4-LDHs of these two species (Graves and Somero, 1982), the cMDHs do not differ between the temperate and subtropical barracuda (see Discussion).

We combined different ratios of the chromatographically separated cMDH isoforms of *S. argentea* and measured the K_m of NADH (at 30 °C) as a function of the percentage of thermolabile cMDH (Fig. 8). The contribution of the high-K_m thermolabile isoenzyme predominated when this isoenzyme was present in approximately twofold excess to the thermostable isoenzyme. To estimate the ratio of thermolabile to thermostable isoenzymes in muscle, we placed on the graph the K_m data obtained using unfractionated muscle homogenates. For the three specimens of *S. argentea* so examined, between 60 and 70 % of the cMDH was the thermolabile isoenzyme. A similar ratio of thermostable to thermolabile cMDH appears to exist in the subtropical species, based on the similar K_m values of NADH found for the two species (Fig. 4). The south temperate species *S. idiastes* appears to have a slightly higher percentage of thermolabile cMDH than do *S. argentea* and *S. lucasana*, as judged by higher K_m of NADH at all temperatures (Fig. 4).

Studies of several classes of enzyme have shown that, despite a strong effect of temperature on K_m values, at physiological temperatures the K_m values for orthologous homologues are very similar among species (see Discussion). For cMDHs, the conservation of K_m could be achieved by two different means: either the set points of K_m could be adjusted to achieve conservation, the commonly noted pattern in studies of orthologous homologues; or the ratio of thermostable to thermolabile cMDHs could be adjusted. In the barracuda, regulation of the ratio at which the two paralogous isoenzymes are expressed appears to be the mechanism used to conserve K_m of NADH.

To determine whether the K_m values of the thermolabile and thermostable isoenzymes of cMDH differed among species from different families, we chromatographically separated and studied kinetically the cMDH isoenzymes from a goby, *Gillichthys mirabilis* (Fig. 9). This is a highly eurythermal estuarine species that encounters habitat temperatures ranging from approximately 9 to 38 °C (Dietz and Somero, 1992); i.e.

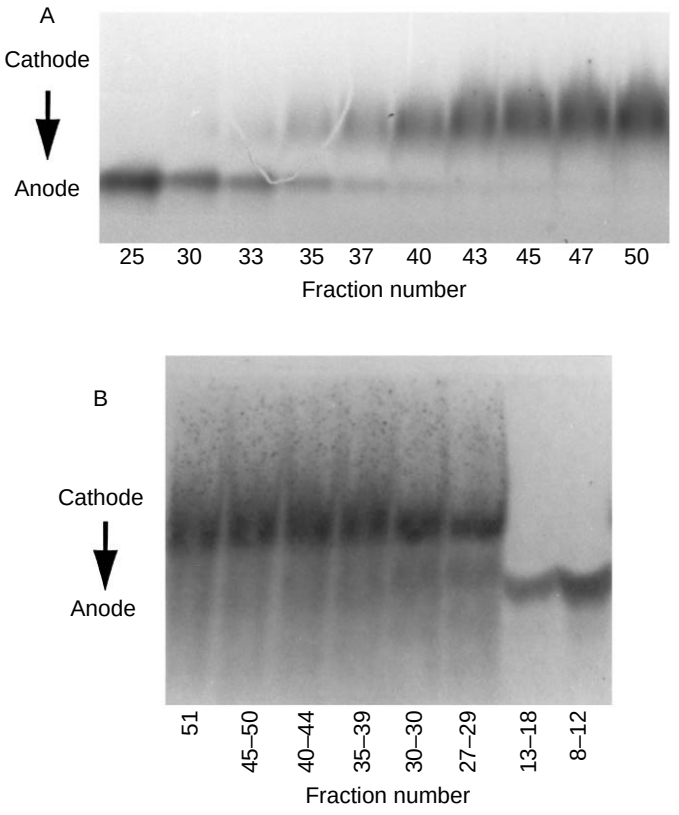

Fig. 5. Separation of thermostable and thermolabile isoforms of cMDH by ion-exchange chromatography (A, *Sphyraena lucasana*) or affinity chromatography (B, *S. argentea*). Fractions from the chromatographic columns were run on native PAGE and stained for MDH activity. The more anodal band is thermostable cMDH.

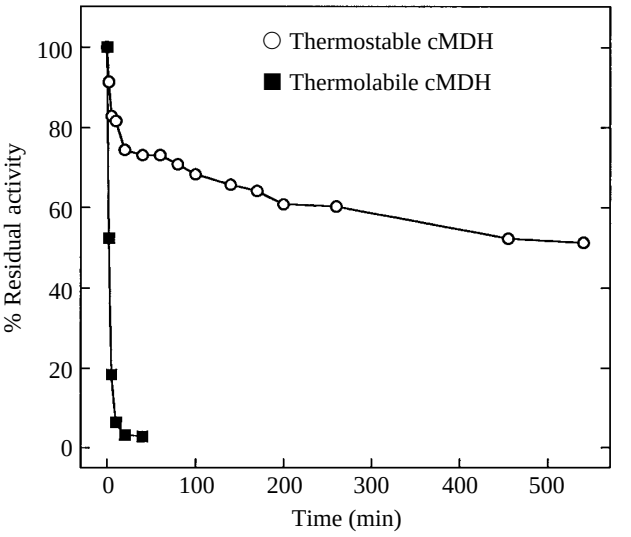

Fig. 6. Decrease in activity of chromatographically separated thermostable and thermolabile isoforms of cMDH from *S. argentea* as a function of incubation time at 42 °C. Each point is the mean of three determinations.

G. mirabilis encounters temperatures several degrees lower and higher than all of the barracuda combined. The thermolabile cMDH of *G. mirabilis* had a higher K_m of NADH at most assay temperatures, and the thermostable cMDH had a lower K_m value at all assay temperatures. These differences between the goby and barracuda cMDHs are consistent with adaptive change in both pairs of orthologous homologues and reflect the differences noted between the orthologous A_4-LDHs of these species (Yancey and Somero, 1978; Graves and Somero, 1982).

Other kinetic characteristics of the thermolabile and thermostable cMDHs of barracuda

Comparisons of thermostable and thermolabile cMDHs of *S. argentea* and *G. mirabilis* and the thermostable isoenzyme of *S. ensis* revealed similar kinetic properties for orthologous homologues and differences between the paralogous homologues. For all species, the two isoforms had similar pH optima for both directions of the reaction (pH 7.6–7.7 for oxaloacetate reduction and pH 10.4–11.0 for malate oxidation). Optimal substrate and cofactor concentrations differed between isoenzymes: thermostable cMDH had a higher optimum concentration for oxaloacetate (0.2–0.3 mmol l^{-1} *versus* 0.1 mmol l^{-1}), but a lower optimum for malate (10–20 mmol l^{-1} *versus* 100 mmol l^{-1}). K_m of oxaloacetate (20 °C) was higher for the thermostable isoenzyme (18–33 μmol l^{-1} *versus* 10–15 μmol l^{-1}). Optimal concentrations for cofactor were similar for both isoenzymes (0.2–0.4 mmol l^{-1} for NADH and 2–6 mmol l^{-1} for NAD^+).

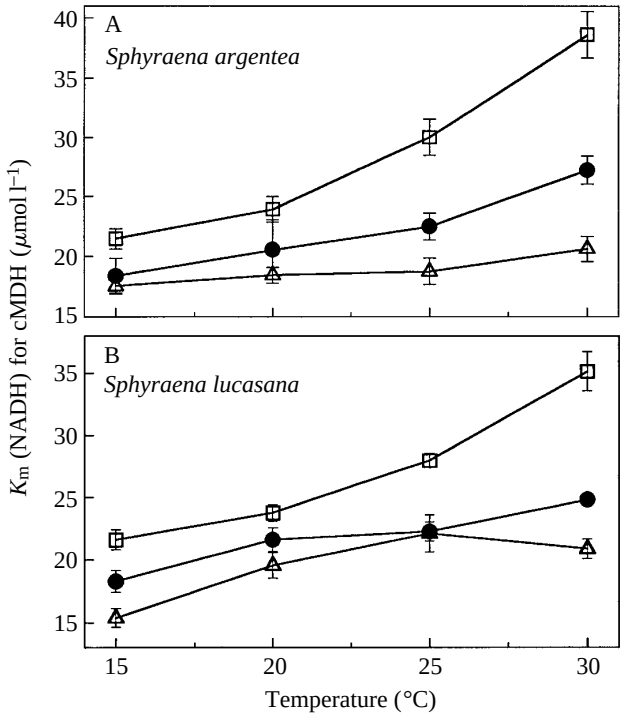

Fig. 7. Michaelis–Menten constants of NADH for unfractionated cMDH and chromatographically separated thermolabile and thermostable cMDHs of *S. argentea* (A) and *S. lucasana* (B). Error bars indicate standard errors of the mean for single K_m values determined using triplicate assays of activity at seven concentrations of NADH. Filled circles, unfractionated cMDH; open squares, thermolabile cMDH; open triangles, thermostable cMDH.

Discussion

Comparative studies of proteins from species adapted to different temperatures have generally focused on orthologous homologues of proteins, i.e. protein variants encoded by a single gene locus common to all the species. Orthologous homologues of several enzymatic and structural proteins have been shown to differ in thermal stability (collagen, Bailey, 1968; actin, Swezey and Somero, 1982; eye lens proteins, McFall-Ngai and Horwitz, 1990; pyruvate kinase, Low and Somero, 1976; myofibrillar ATPase, Johnston and Walesby, 1977) and in the temperature range over which temperature-sensitive kinetic properties such as K_m values are conserved among all species (Hochachka and Somero, 1984; Yancey and Siebenaller, 1987; Jaenicke, 1991; Somero, 1995). In eurythermal ectotherms, especially species that encounter high temperatures, the effects of temperature on K_m values are generally much smaller than those noted for homologous enzymes of stenothermal species (Baldwin, 1971; Yancey and Somero, 1978; Coppes and Somero, 1990; Somero, 1991, 1995; Dahlhoff and Somero, 1993*a*).

A second mechanism by which a eurythermal species might

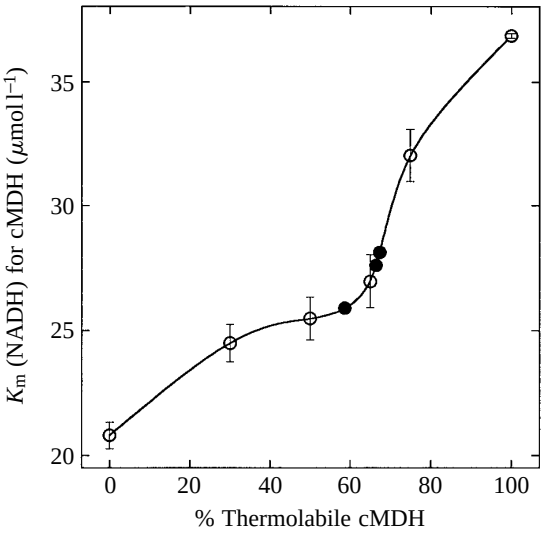

Fig. 8. Michaelis–Menten constant of NADH at 30 °C for known mixtures (open circles) of thermostable and thermolabile cMDHs isolated from white skeletal muscle of *S. argentea*. The K_m values for unfractionated cMDH from white muscle of three specimens of *S. argentea* (filled circles) were placed on the curve to yield estimates of the ratio of thermostable to thermolabile cMDH isoforms in the muscle of the three specimens. Error bars are standard errors of the mean for single K_m values determined using triplicate assays of activities at seven concentrations of NADH.

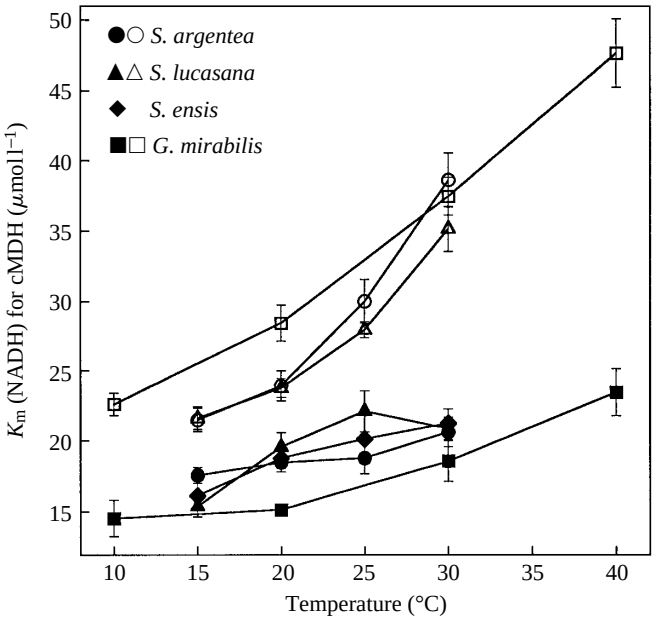

Fig. 9. Comparisons of the Michaelis–Menten constants of NADH for chromatographically separated thermolabile and thermostable cMDHs of *S. argentea, S. lucasana, S. ensis* and *Gillichthys mirabilis.* Error bars represent standard errors of the mean for single determinations of K_m made using triplicate assays of activity at seven concentrations of NADH. Open symbols designate thermolabile cMDHs; filled symbols designate thermostable cMDHs.

conserve enzymatic function over a wide temperature range is potentially available in the case of enzymes such as cMDH that are encoded by duplicated gene loci. Not only do the two paralogous cMDHs of teleost fish differ in thermal stability, as first shown by Schwantes and Schwantes (1982*a*,*b*), but the effects of temperature on K_m values differ between isoforms (Figs 4, 9). As a result, variation in the ratio of expression of the thermostable and thermolabile cMDHs leads to conservation in the value of K_m of NADH between approximately 20 and 23 μmol l^{-1} at physiological temperatures. This is the approximate range in which the K_m of NADH has been found to be conserved in cMDHs of a variety of vertebrates (Grimm and Doherty, 1961; Englard and Breiger, 1962; Yancey and Siebenaller, 1987) and invertebrates (McReynolds and Kitto, 1970; Dahlhoff and Somero, 1993*a*). Note that K_m values, including those for NADH, are generally influenced by the composition of the assay medium (e.g. salt composition and concentration), so comparing data sets gathered using different protocols may lead to underestimates of the extent of K_m conservation (Greaney and Somero, 1980). Adaptation to temperature by the cytosolic form of MDH in teleosts therefore differs from the pattern noted for A_4-LDH in barracuda. For both classes of enzyme, there is a conservation of K_m at physiological temperatures, but this is achieved in A_4-LDH by an evolutionary change in the amino acid sequence of the orthologous homologues (L. Holland, M. J. McFall-Ngai and G. N. Somero, in preparation), whereas for cMDH, conservation of K_m may also be achieved by altering the ratio of thermostable and thermolabile paralogous isoenzymes, as seen in the barracuda congeners.

Combining chromatographically separated thermostable and thermolabile cMDHs in different ratios had a complex effect on K_m of NADH (Fig. 8). The much lower K_m of NADH for the thermostable isoform played the dominant role in establishing the K_m of the mixture until approximately 70% of the total cMDH was in the thermolabile isoform. The K_m values of NADH determined for unfractionated and unheated homogenates from three specimens of *S. argentea* were similar and, when these values were placed on the graph of the relationship between K_m of NADH and percentage thermolabile cMDH, we estimated that between 60 and 70% of cMDH was thermolabile cMDH. For the three species in which both thermostable and thermolabile cMDHs occur, it appears that *S. idiastes* may have the highest percentage of thermolabile cMDH, because unfractioned muscle homogenates yielded the highest K_m of NADH values (Fig. 4).

The similar values for K_m of NADH for the orthologous thermostable and thermolabile cMDHs of the barracuda species suggest that temperature-adaptive differences among orthologous homologues of cMDH have not occurred in this genus, despite divergence times of up to approximately 1.5×10^7 years between the most divergent pair of species (*S. ensis* and *S. lucasana*; Graves and Somero, 1982). The importance of adaptive change in orthologous forms of cMDH among more distantly related taxa remains to be firmly established. The thermostable and thermolabile cMDHs of the eurythermal goby *G. mirabilis* differed somewhat from their counterparts in barracuda. Compared with the barracuda cMDHs, thermolabile cMDH of *G. mirabilis* had higher values for K_m of NADH at most measurement temperatures, and thermostable cMDH had lower values (Fig. 9). Although these differences were generally small, they appear to be consistent with the different thermal ranges of the five species: the barracuda live at temperatures between approximately 15 and 30 °C, but *G. mirabilis* encounters temperatures between approximately 9 and 38 °C (Dietz and Somero, 1992). In *G. mirabilis*, therefore, one would predict that cMDHs adapted to both colder and warmer temperatures, relative to the barracuda, would be advantageous. Thus, the differences found between the orthologous forms of the two cMDHs in the goby and barracuda are consistent with trends noted in other comparisons of orthologous homologues, e.g. A_4-LDH (Somero, 1991, 1995), acetylcholinesterases (Baldwin, 1971) and pyruvate kinases (Low and Somero, 1976).

Our inability to detect thermolabile cMDH in any tissue of the eleven *S. ensis* specimens analyzed is not likely to have been a consequence of loss of enzymatic activity due to freezing or other experimental manipulations. Thermolabile cMDH was stable under freezing conditions in the other three species and could readily be detected even in specimens held frozen for periods of approximately 2 years. The absence of thermolabile cMDH in the tropical barracuda could be the

result of either (a) loss of the gene (or a functional form of the gene) encoding this isoform, or (b) the repression of gene transcription at high temperatures. Our data do not allow a test of these two possibilities, but it would be revealing to acclimate *S. ensis* to the lower temperatures characteristic of, for example, *S. argentea*, to determine whether expression of the thermolabile cMDH isoform could be induced. The two cMDHs of *G. mirabilis* vary in ratio seasonally and as a result of laboratory acclimation (Lin and Somero, 1995). These findings suggest that temperature can affect one or more of the processes that establish cMDH isoenzyme ratios; for example, gene transcription or protein degradation. In a relatively stenothermal tropical species such as *S. ensis*, however, if thermolabile cMDH is not required physiologically (see below), the gene encoding this isoform may be lost or silenced.

One implication of the absence of thermolabile cMDH in *S. ensis* is that kinetic differences between the thermolabile and thermostable isoforms related to differences in metabolic function may be minor, such that both isoforms are not needed to ensure the metabolic integrity of the organism. Alternatively, if thermolabile cMDH has a distinct physiological role, this function may not be required at higher body temperatures. Available kinetic data do not clearly establish what the different metabolic roles for the two teleost cMDHs might be. Coppes *et al.* (1987) have postulated that the thermostable and thermolabile cMDH isoforms may influence the direction of carbon flow between glycolysis and gluconeogenesis. Until physiological studies are carried out to complement *in vitro* biochemistry, this hypothesis cannot be evaluated. Thermostable cMDH differs from thermolabile cMDH in having a lower optimal pH for oxidation of malate, a slightly lower sensitivity to inhibition by oxaloacetate, a higher sensitivity to inhibition by high malate concentrations and a higher K_m of oxaloacetate (this study; Bailey *et al.* 1970; De Luca *et al.* 1983). These characteristics are conserved among orthologous forms of each cMDH. Thus, as in the case of A_4-LDH, orthologous forms are more similar kinetically than are paralogous forms (Markert *et al.* 1975).

An additional form of variation in cMDHs has been reported in bovine heart (Cassman and Vetterlein, 1974). Two cMDHs have been characterized, one with non-cooperative binding of NADH, as found for teleost cMDHs, and a second with cooperative binding of NADH and allosteric regulation by fructose 1,6-bisphosphate. The phosphorylation states of the two cMDHs differed. Kinetic differences between the two cMDHs of barracuda appear not be the result of post-translational modification, because NADH binding was always non-cooperative and neither teleost isoenzyme was affected by the addition of fructose 1,6-bisphosphate (J. J. Lin and G. N. Somero, unpublished observations).

The kinetic properties of cMDHs of birds and mammals resemble those of teleost thermostable cMDH more than those of thermolabile cMDH, which implies that the thermostable enzyme of teleosts is ancestral to the single cMDH of birds and mammals (Schwantes and Schwantes, 1982*a*). The absence of thermolabile cMDH in birds and mammals, as well as in a warm-adapted teleost fish (*S. ensis*), is consistent with the hypothesis that the occurrence of this isoform of cMDH may be related to adaptation temperature and may not be strictly a consequence of phylogeny.

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