

Phylogenetic Relationships and Biochemical Properties of the Duplicated Cytosolic and Mitochondrial Isoforms of Malate Dehydrogenase from a Teleost Fish, *Sphyraena idiastes*

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Abstract. Unlike birds and mammals, teleost fish express two paralogous isoforms (paralogues) of cytosolic malate dehydrogenase (cMDH; EC 1.1.1.37; NAD⁺: malate oxidoreductase) whose evolutionary relationships to the single cMDH of tetrapods are unknown. We sequenced complementary DNAs for both cMDHs and the mitochondrial isoform (mMDH) of the fish *Sphyraena idiastes* (south temperate barracuda) and compared the sequences, kinetic properties, and thermal stabilities of the three isoforms with those of mammalian orthologues. Both fish cMDHs comprise 333 residues and have subunit masses of approximately 36 kDa. One cytosolic isoform, cMDH-S, was significantly more heat-stable than either the other cMDH (cMDH-L) or mMDH. In contradiction to the generally accepted model of vertebrate cMDH evolution, our phylogenetic analysis indicates that the duplication of the fish cytosolic paralogues occurred after the divergence of the lineages leading to teleosts and tetrapods. cMDH-L and cMDH-S differed in optimal concentrations of substrates and cofactors and apparent Michaelis–Menten constants, suggesting that the two paralogues may play distinct physiological roles. Differences in intrinsic thermal stability among MDH paralogues may reflect different degrees of stabilization in vivo by extrinsic stabilizers, notably protein concen-

tration in the case of mMDH. Thermal stabilities of porcine mMDH and cMDH-L, but not cMDH-S, were significantly increased when denaturation was measured at a high protein (bovine serum albumin; BSA) concentration, but the BSA-induced stabilization reduced the catalytic activity.

Key words: Barracuda — Malate dehydrogenase — Mitochondrial enzymes — Paralogous genes — Protein stabilization — *Sphyraena*

Introduction

Malate dehydrogenase (MDH; EC 1.1.1.37; malate: NAD⁺ oxidoreductase), a dimeric enzyme that catalyzes the interconversion of malate and oxaloacetate (OAA), is involved in several metabolic pathways, including the citric acid cycle, the malate–aspartate shuttle, lipogenesis, and gluconeogenesis. Eukaryotic cells express both mitochondrial (mMDH) and cytosolic (cMDH) isoforms that are encoded by nuclear genes. The two isoforms have similar secondary and tertiary structures (Birktoft et al. 1989; Gleason et al. 1994) yet share only about 20% amino acid sequence identity (Joh et al. 1987a,b). Mitochondrial and cytosolic isoforms also differ in kinetic properties and thermal stability (Gleason et al. 1994; McAlister-Henn 1988). Several primary structures of mammalian cMDH and mMDH have been determined (Birktoft et al. 1989; Joh et al. 1987a, b; Grant et al. 1986), and crystal structures of porcine cMDH and

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mMDH have been measured to a high resolution (Birktoft et al. 1989; Gleason et al. 1994). In addition, the amino acid sequences and crystal structures of MDHs of two prokaryotes, *Escherichia coli* (Hall et al. 1992) and *Thermus flavus* (Nishiyama et al. 1986; Kelly et al. 1993) have been solved. These studies have greatly facilitated understanding of the evolutionary relationships, structure–function linkages, and sources of differences in thermal stability between MDHs from mammals and MDHs from prokaryotes. However, little is known about the evolution and structural and functional characteristics of MDHs in fish.

Unlike mammals and birds, which have only one cMDH, genomes of many fish encode two cMDH isoforms (Bailey et al. 1970; Wheat and Whitt 1971; Wheat et al. 1972; De Luca et al. 1983) whose evolutionary relationships to the single cMDH of tetrapods are unclear. The paralogous homologues (paralogues) of cMDH in fish differ in thermal stability, and it has been proposed that the expression of the thermostable (cMDH-S) and thermolabile (cMDH-L) isoforms may be governed by body temperature (Schwantes and Schwantes 1982; Lin and Somero 1995a, b; Lin et al. 1996). Differential expression of cMDH paralogues in different tissues of fish also has been reported (Bailey et al. 1970; Cashon 1981). These tissue-specific and putative temperature-related patterns of cMDH expression have led to the hypothesis that cMDH-S and cMDH-L may play different metabolic roles reflecting temperature-specific metabolic organization (Coppes et al. 1987).

We have determined the primary structures of the three MDH isoforms present in a teleost fish, the south temperate barracuda (*Sphyræna idiaestes*), in an attempt to establish the phylogenetic relationships of the two fish cMDHs and the single cMDH of tetrapods. In addition, we have characterized the thermal stabilities and some of the kinetic properties of the three MDHs of fish to provide insights into their metabolic roles in the cell.

Materials and Methods

Collection of Specimens and Preparation of Tissues. Specimens of *S. idiaestes* were collected at the Galapagos Islands, Ecuador, and Pisco, Peru, and immediately frozen for shipment to the United States. White epaxial skeletal muscle was removed from frozen specimens and stored at -80°C .

cDNA Library Preparation and MDH Sequencing. mRNA was isolated from white skeletal muscle by the method of Chomczynski and Sacchi (1987). A cDNA library prepared from *S. idiaestes* white skeletal muscle was constructed in λ ZAP II (Stratagene, La Jolla, CA; a gift from Dr. Linda Z. Holland of the Scripps Institution of Oceanography) using the manufacturer's instructions and other standard molecular biology protocols (see Sambrook et al. 1989). PCR amplification of cMDH used a pair of degenerate primers (pair A; see Appendix for all primers used in this study) based on the conserved region of the mammalian cMDH sequences and a partial amino acid sequence of cMDH

from the grass carp, *Ctenopharyngodon idella* (Lin, unpublished data). For mMDH, a second pair of degenerate primers was used (pair B), based on the partial amino acid sequence of mMDH from *S. idiaestes* (forward direction; see below) or on a conserved section of the mammalian mMDH sequences (reverse).

The cDNA library was screened for clones of interest by hybridization with ^{32}P -labeled fragments (random priming kit; Gibco BRL, Grand Island, NY) of the MDH sequences derived from PCR, followed by autoradiography. The plasmids from the λ ZAP II vector were isolated and transformed into XLI-Blue cells and selected using LB/ampicillin–tetracycline plates. The colonies that contained plasmid with MDH cDNA were grown in LB/ampicillin–tetracycline broth for immediate use and for producing long-term stocks.

From this cDNA library we amplified a 420-bp DNA fragment using degenerate primers. The resulting sequence showed approximately 70% identity to the homologous region of mouse cMDH. This DNA fragment was then used to screen the library for cMDH clones. Eighteen positive clones were identified, 14 of which were either full-length (about 1470 bp) or partial DNA clones of one form of cMDH; the other 4 encoded regions of a second isoform of cMDH. Although none of the latter four clones was full-length, two overlapped by ~ 300 bp in the coding region. Together, the two clones encompassed the complete sequence, which is about 1750 bp, including 5'- and 3'-untranslated regions (UTR).

Plasmids that contained cDNA of interest were extracted either by miniprep (Sambrook et al. 1989) or by using commercial plasmid purification kits (QIAprep Spin Kit for small quantities and Plasmid Midi Kit for large quantities; both from Qiagen, Chatsworth, CA). Forward and reverse strands of DNA were sequenced using Sequenase (DNA Sequencing Kit; USB, Cleveland, OH) or, when strong compression of bands occurred, the femtomole TM DNA sequencing system (Promega Corp., Madison, WI). Sequence data have been submitted to GenBank (accession numbers: *Sphyræna idiaestes* cMDH-S, AF390559; cMDH-L, AF390560; and mMDH, AF390561).

Phylogenetic Analysis. PAUP* 4.0 (Swofford 1998) was used to determine the phylogenetic relationships among the three barracuda MDH isoforms described in this study, as well as all vertebrate MDHs available via a BLAST search for homologous sequences in GenBank (accession numbers: cMDHs—human, U20352; mouse, NM_008618; pig, U44846; and rat, AF093773; mMDHs—human, XM_004905; mouse, NM_008617; pig, M16427; and rat, X04240). *Escherichia coli* MDH was used as an outgroup (accession number AE000403). Both maximum parsimony (MP) and distance were used as the optimality criteria. Alignment was performed using Clustal V (Higgins and Sharp 1988) and checked by eye. Gaps were treated as “missing.” Under MP, amino acid sequences were examined, and of the 362 total characters, 250 were parsimony-informative. A heuristic search using the “random addition of taxa” option and 500 replicates were employed. A number of a priori weighting schemes were used, including unweighted; transversions weighted more than transitions (both 2:1 and 3:1); transversions only; first position, second position, and third position only; and second position transversions only. An exhaustive search of MP trees was also performed to obtain a strict consensus. Using distance as the optimality criterion and Kimura two-parameter weighting, DNA sequences of the MDH open reading frames were also examined.

Recombinant cMDH Expression. For expression of the recombinant cMDHs, the entire coding regions of cMDH-S and cMDH-L were amplified and cloned into a modified pGEMEX vector. For cMDH-S, a full-size clone was PCR-amplified (primer pair C) by Pfu DNA polymerase (Stratagene). Because no full-length clone of cMDH-L was found in our library screening, the two partial clones described above were amplified by Pfu DNA polymerase (primer pairs D and E). To join the DNA fragments, 75 ng of the amplified 5'-end fragment and 90 ng of the 3'-end fragment were mixed with $0.8\ \mu\text{l}$ of dNTP (25 mM), 2 U of Pfu DNA polymerase, the forward primer of pair D, and the

reverse primer of pair E, in a total volume of 50 μ l. The mixture was thermally cycled 30 times (94°C denaturation for 1 min, 64°C annealing for 2 min, and 72°C extension for 3 min) and the ligated cMDH-L cDNA was used as the template in a final PCR amplification.

The amplified full-length cDNAs of cMDH-S and cMDH-L were ligated into the modified pGEMEX vector cut with *Nde*I and *Xho*I restriction enzymes (see Appendix) and transformed into the W945TL-2 strain of *E. coli*, which does not express MDH (Courtright and Henning 1970). The bacteria were lysogenized with a λ -DE3 lysogenization kit (Novagen, Madison, WI) to facilitate overexpression of cMDH. DNA sequencing was performed to confirm the identities of the clones; no mutation was found in either clone. Recombinant cMDHs were expressed using standard microbiology techniques (Sambrook et al. 1989).

Purification of Wild-Type and Recombinant MDHs. The purification of MDHs from white skeletal muscle followed the procedures of Lin and Somero (1995b). Fifty grams of tissue was homogenized in 200 ml of 50 mM potassium phosphate buffer, pH 6.8. The homogenate was centrifuged and the resulting supernatant was poured into an oxamate affinity column to remove lactate dehydrogenase (LDH) (O'Carra and Barry 1972). MDHs in the eluent were precipitated with 80% saturation ammonium sulfate and stored in this form until prepared for chromatography on a Matrex Gel Red A dye affinity column. For the recombinant cMDH-S and cMDH-L (r-cMDH-S, r-cMDH-L), the supernatant of the lysate from a 500-ml culture of bacteria was directly applied to the Matrex Gel Red A dye affinity column. It was not necessary to run the oxamate column for the bacterial lysate because the LDH levels were extremely low in both samples. The running conditions for the dye affinity column were the same as for native MDHs from fish muscle tissue (Lin and Somero 1995b). r-cMDH-L was eluted with 0.4 mM NAD⁺ and 20 mM malate, and r-cMDH-S was eluted with 0.7 mM NAD⁺ and 40 mM malate. To stabilize r-cMDH-L, 20% glycerol and 3 mM 2-mercaptoethanol were added in the washing and elution buffers. For long-term storage, both purified recombinant cMDHs were kept in buffer containing 30% glycerol and 3 mM 2-mercaptoethanol at -20°C.

Peptide Sequencing. Peptide sequencing was necessary to determine which cDNA coded for the heat-stable isoform and which coded for the heat-labile isoform of cMDH. Each MDH purified by dye affinity chromatography was loaded into four lanes of a 6% nondenaturing polyacrylamide gel. Following electrophoresis, one lane was stained with an MDH activity stain and the other lanes stained for protein with Coomassie blue. The bands shown by the protein stain were excised if there was a band at the same distance of migration in the activity stained gel. For peptide sequencing, the excised gel fragment containing MDH was digested with *Staphylococcus aureus* V-8 protease (Sigma, St. Louis, MO) while running in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system and then blotted to a polyvinylidene difluoride (PVDF) membrane for microsequencing (Cleveland 1983).

Enzyme Kinetic Characterization. All chemicals, including porcine cMDH and mMDH, were from Sigma Chemical Co. Imidazole buffer (80 mM, pH 7.5 at 20°C, containing 100 mM KCl) was used for all measurements. A range of substrate and cofactor concentrations was tested to determine the "optimal" concentrations for each isoform, with the optimal substrate and cofactor concentrations defined as the values that gave the highest enzymatic activity under the assay conditions employed. During each test, the concentration of the ligand of interest was varied, while the concentration of the complementary ligand was held constant at one of the following values: 200 μ M NADH, 3 mM NAD⁺, 0.2 mM OAA, or 5 mM malate.

To determine the apparent K_m for NADH and OAA, a series of concentrations from 15 to 80 μ M and from 5 to 200 μ M, respectively, were employed. K_m values were calculated using the weighted linear

regression program of Wilman4 kinetics software for Lineweaver-Burk analysis (Brooks and Suelter 1986).

Thermal Stability Measurements. BSA (1 mg/ml) was added to MDH samples for measurements of intrinsic stability, or, where the effects of protein (BSA) concentration on stability were examined, levels as high as 150 mg/ml were added. Aliquots (100 μ l) were dispensed in microcentrifuge tubes and incubated in a water bath at 42 \pm 0.1°C for r-cMDH-L or porcine mMDH or at 48 \pm 0.1°C for cMDH-S or porcine cMDH. The higher incubation temperature for the latter isoforms was used because their greater inherent stability made incubation at the lower temperature inconvenient for the determination of half-lives. Samples were removed from the water bath at predetermined times, chilled on ice for 5 min, and then centrifuged at 15,000g and 4°C. MDH activity in the supernatant was assayed immediately. The residual activity was calculated as the percentage of MDH activity in the heated sample compared to the activity in an unheated control held in an ice-water bath. The time required for loss of 50% of the initial activity (half-life) was estimated by interpolation.

Results

Deduced Amino Acid Sequences of Barracuda cMDHs and mMDH. The deduced amino acid sequences of the two paralogues of barracuda cMDH are shown in Fig. 1 along with the sequences of the porcine and mouse orthologues. Both teleost cMDHs contain 333 residues and are 1 residue shorter than the mammalian cMDHs, which have an additional residue at their C termini. To determine which deduced sequences corresponded to the thermally stable and thermally labile paralogues, we used peptide sequencing to obtain a partial sequence of MDHs purified from *S. idiaestes* muscle. Only two bands of MDH activity, one of which was heat stable and one thermally labile, could be identified by activity-staining of nondenaturing gels on which either crude muscle homogenates or purified MDHs were electrophoresed (data not shown). The partial sequence of one peptide from the heat-stable isoform was determined and it matched perfectly (underlined sequence in Fig. 1) the deduced amino acid sequence determined from the cDNA sequences of the more common cMDH-encoding clone identified in screening. Thus, this protein was designated cMDH-S. Peptide sequencing of the heat-labile isoform seen on nondenaturing gels indicated that this protein was mMDH (underlined sequences in Fig. 2). A peptide sequence matching the cMDH-L cDNA was not obtained.

In our previous studies of cMDH in barracuda, we concluded that the major heat-labile band detected in native gel electrophoresis was cMDH-L. This conclusion was based in part on the fact that this band was very prominent in anaerobically poised white skeletal muscle and stained only weakly when homogenates of liver, a more aerobic tissue, or mitochondrial extracts were electrophoresed (Lin and Somero 1995b; Lin et al. 1996). Furthermore, the banding patterns detected in four species of barracudas appeared consistent with the heat-labile band being cMDH-L (Lin and Somero 1995b). The

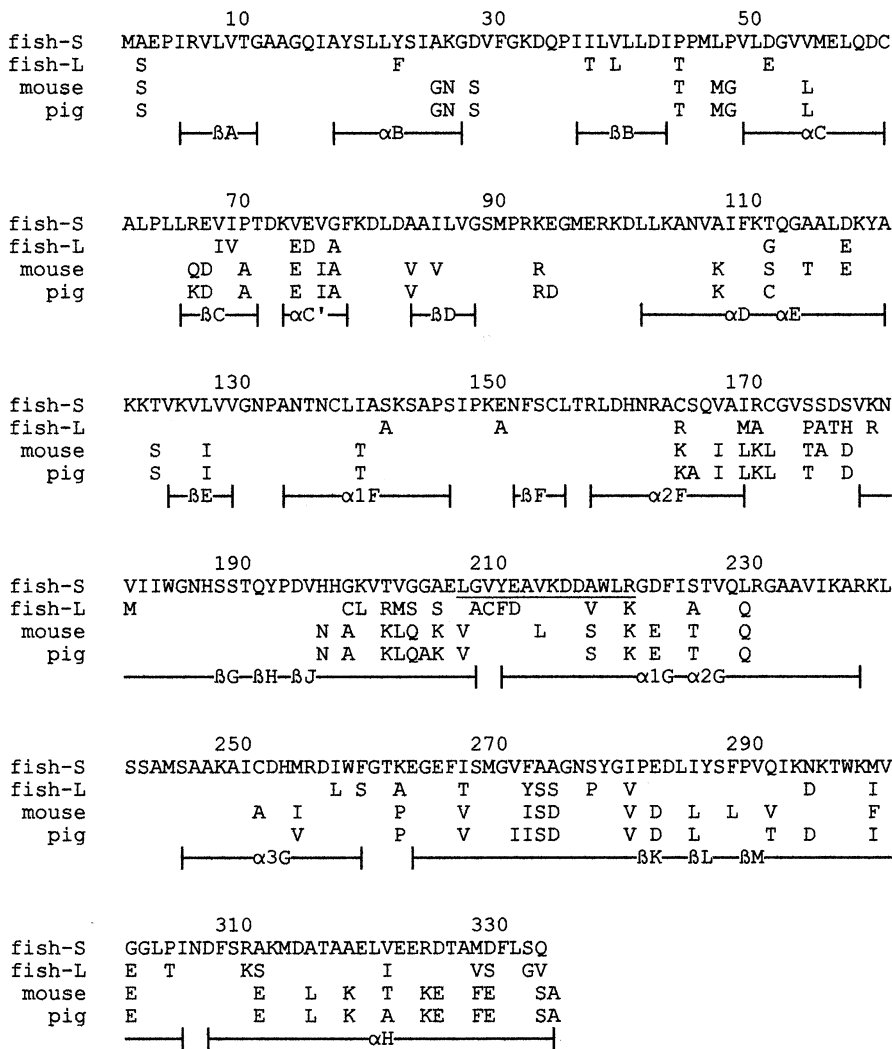


Fig. 1. A comparison of amino acid sequences of cMDH of barracuda [stable form, cMDH-S (fish-S); and labile form, cMDH-L (fish-L)], mouse (Joh et al. 1987b), and pig (Birktoft et al. 1989). Except for the porcine sequence, whose N-terminal methionine residue was absent as determined by X-ray analysis and chemical methods, all sequences were deduced from cDNA sequences. The underlined portion of the barracuda cMDH-S sequence was the segment that was confirmed by the peptide sequencing. Amino acids are shown only when differences occur from the sequence of cMDH-S. Residue numbering follows the porcine sequence of Birktoft et al. (1989). At the *bottom* of each segment of sequence is the designation for the secondary structure, which is based on the model of Gleason et al. (1994). The GenBank accession numbers for barracuda cMDH-S and cMDH-L are AF390559 and AF390560, respectively. Mouse and pig cMDH accession numbers are given in the text.

fact that our sequencing studies showed that the major heat-labile band seen on native gels is mMDH, not cMDH-L, indicates that our initial interpretation of the banding patterns on native gels (Lin and Somero 1995b) was incorrect.

The deduced amino acid sequences of the cMDHs of *S. idiaestes* share 82.3% identity (Table 1). When compared to the cMDHs of mammals, cMDH-S is 79.3 and 79.9% identical to cMDH of mouse and pig, respectively, and cMDH-L is 75.4 and 75.7% identical to the mouse and pig cMDHs, respectively (Table 1). The underlying cDNA sequences show similar patterns—cMDH-S and cMDH-L are 80.2% identical, while the former is 69.6 and 70.6% identical to the pig and mouse cMDH cDNAs, respectively, and the latter is 66.8 and 67.8% identical to the two mammalian paralogues. Percentage identities of both amino acid and nucleotide sequences between the barracuda paralogues and those of other species are also provided in Table 1 for comparison. Sequence identity among the barracuda and mammalian cMDHs is greater in the N-terminal half of the enzyme, which includes the nucleotide binding domain,

than in the C-terminal half of the molecule (Fig. 1), a result consistent with other analyses of MDH sequences.

The deduced amino acid sequence for mMDH is shown in Fig. 2. By comparing the deduced amino acid sequence of barracuda mMDH to the N-terminal sequence that was determined chemically, we found that a 23-residue peptide is cleaved from the initial precursor molecule during processing. This peptide is thought to function in targeting of mMDH to the mitochondrion and, possibly, in its translocation into this organelle (Gleason et al. 1994). This N-terminal peptide in barracuda is one residue shorter than the sequence of mouse, however, there is still ~50% homology between the two peptides (Fig. 2). Thus, the mature barracuda mMDH, like the mouse orthologue, contains 314 residues. The barracuda mMDH is 81.4% identical in sequence to mouse mMDH, and 16.2 and 14.7% identical to cMDH-S and cMDH-L, respectively (Table 1). Thus, regardless of their similarity in biochemical function and 3-D structure (Gleason et al. 1994), the cMDHs and mMDH exhibit a low similarity in their protein sequences.

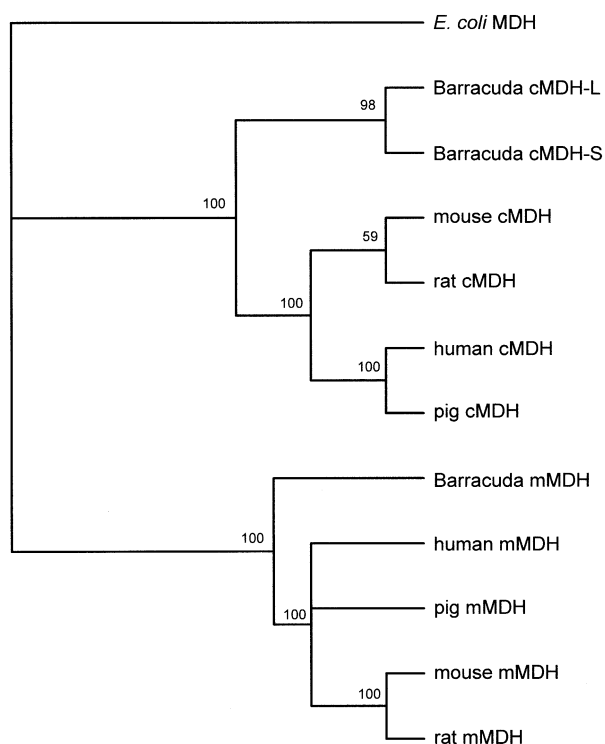


Fig. 3. A representative tree of vertebrate MDH relationships, based on amino acid sequences. This 50% majority rule consensus maximum parsimony bootstrap tree was produced using PAUP* 4.0 (Swofford 1998) with all characters unweighted. Numbers indicate bootstrap values. A variety of weighting and exclusion schemes all supported the monophyly of barracuda cMDHs with respect to mammalian cMDHs.

Thermal Stability of MDHs. Intrinsic thermal stabilities of MDH isoforms were compared by determining residual activities after heat treatment at 42°C for different times (Fig. 4). The calculated half-lives of barracuda muscle cMDH-S and r-cMDH-S differed somewhat (480 and 810 min, respectively), but the shapes of the denaturation curves were very similar and distinctly different from those found for the other MDHs. Porcine cMDH was most stable and lost less than 50% of its activity during 50 h of incubation. The mMDHs of fish and pig had similar and relatively short half-lives, ~5 min. The most labile MDH was r-cMDH-L, which lost half its activity within 3 min.

Measurements of thermal stability in varying concentrations of BSA were performed to determine the effects of macromolecular crowding on MDH stability. Figure 5 shows that as BSA concentrations are raised from 1 to 40 or 100 mg/ml, there is no effect on the residual activity of cMDH-S. cMDH-L, however, shows a marked increase in stability at these higher BSA concentrations. Interestingly, porcine MDH isoforms show a similar pattern—porcine cMDH stability is relatively unaffected by increased levels of BSA (data not shown), but porcine mMDH half-life at 42°C increases 28-fold as BSA concentration is increased from 1 to 100 mg/ml (Fig. 5, inset). Maximal stabilization of both porcine

mMDH and barracuda cMDH-L was achieved at approximately 100 mg/ml BSA. The enhanced stability of porcine mMDH caused by increasing BSA concentrations is accompanied by a steady reduction in catalytic activity (Fig. 5, inset).

Optimal Concentrations of Substrates and Cofactors. To characterize further the catalytic properties of the MDH isoforms, we measured optimal substrate and cofactor concentrations (Table 2), which are defined as those that produce the highest activities under given assay conditions. MDH activity rises with substrate concentration, but after reaching a maximum activity may decrease with further increases in substrate, because of substrate inhibition. The K_m 's of OAA and NADH were also measured for the barracuda and porcine MDHs. Only minor differences were noted between cMDH-S purified from muscle and r-cMDH-S, suggesting that the native and cloned enzymes were similar in functional properties.

One consistent difference between the cMDH paralogues was the concentration of substrate—malate or OAA—that yielded maximal activity. The cMDH-S purified from fish muscle had optimal concentrations of OAA and malate of 0.5 and 10 mM (0.6 and 8 mM for r-cMDH-S). In contrast, r-cMDH-L had much lower optima: 0.1 mM for OAA and 4 mM for malate. The optimal OAA concentration for porcine cMDH was similar to the optimum of barracuda cMDH-S, but the optimal malate concentration was approximately threefold higher in the porcine isoform. The optimal malate concentrations of all cMDHs were lower than those of mMDHs. mMDHs of pig and barracuda had identical optimal malate concentrations, but the porcine mMDH had a sixfold higher optimal OAA concentration than that of barracuda. The optimal concentrations of cofactors (not shown in Table 2) were similar for all the fish MDHs in both catalytic directions, with the optimal [NADH] falling between 0.16 and 0.3 μ M and optimal [NAD⁺] between 2 and 4 μ M (optimal cofactor concentrations were not measured for the porcine isoforms).

The K_m values measured for the barracuda and porcine MDHs fell into three groups (Table 2). Barracuda cMDH-S was similar to porcine mMDH in that the K_m of OAA in each isoform was greater than the K_m of NADH; for barracuda mMDH and porcine cMDH this relationship was reversed, the K_m of OAA was less than the K_m of NADH. r-cMDH-L was unique in that its K_m values for OAA and for NADH were similar; in each case these K_m values were lower in r-cMDH-L than in the stable barracuda isoform, cMDH-S.

Discussion

Sequence Homologies of MDHs. Because tetrapods express only one cMDH, it has been hypothesized that

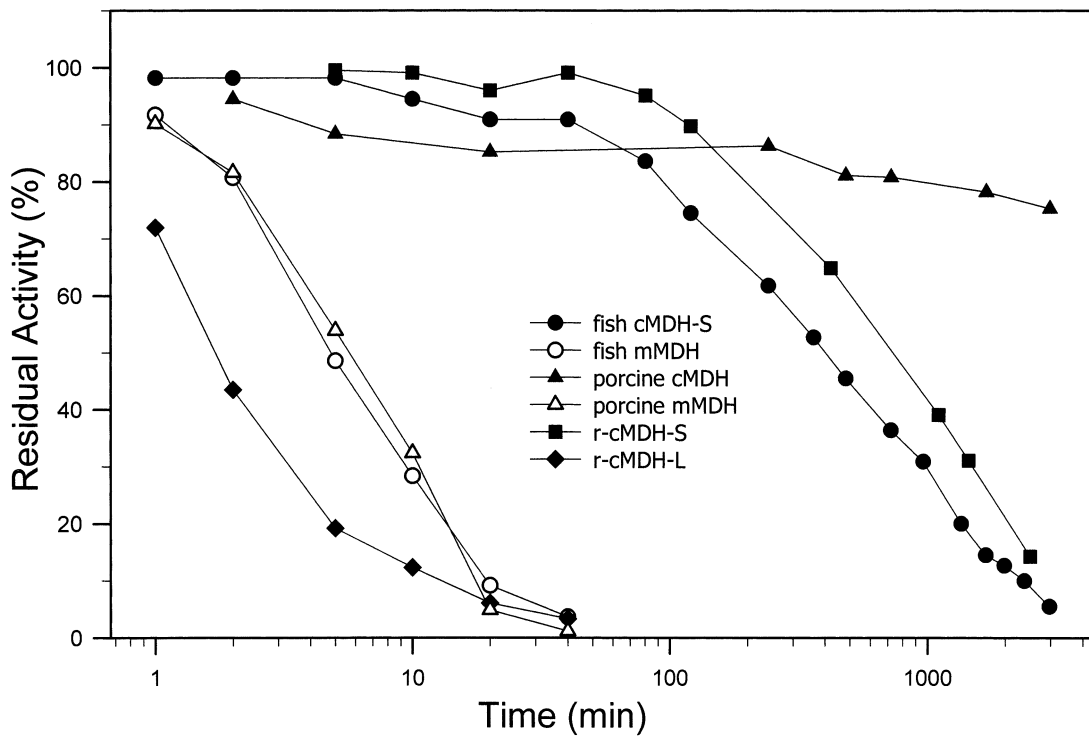


Fig. 4. Residual activities of purified native porcine and *S. idiestes* MDHs and recombinant cMDHs of *S. idiestes* after heat treatment. Samples in 100- μ l aliquots were incubated at 42°C (r-cMDH-L and porcine mMDH) or 48°C (cMDH-S and porcine cMDH). At specific time intervals, a sample was removed, put on ice, spun briefly, and

assayed immediately at 20°C. The residual activity is expressed as the percentage of enzyme activity remaining in the heated sample relative to the activity in a control (sample held on ice for the entire experimental period).

either the cMDH gene locus has undergone duplication in fish after the separation of the tetrapod lineage from other vertebrates or one of the cMDH gene loci in the ancestral line of tetrapods was silenced or lost during evolution (Fisher et al. 1980). The latter hypothesis has been favored because almost all groups of living fish, including members of the jawless fish (agnatha) and elasmobranchs, are reported to express two cMDHs based on electrophoretic evidence (Fisher et al. 1980; Basaglia 1989; Coppes 1990; Coppes et al. 1990). Furthermore, the isozyme pattern of cephalochordates, e.g., *Amphioxus*, reflects the presence of two cMDH loci (Fisher et al. 1980; Basaglia 1989). Because fish cMDH-S and mammalian cMDHs share a high thermal stability, we predicted a higher sequence homology between these cMDH isoforms than between fish cMDH-L and mammalian cMDHs. Indeed, the amino acid sequence of mammalian cMDHs are ~80% identical to cMDH-S and only ~75% identical to cMDH-L (Table 1). However, comparison of the sequences of the two cMDH paralogues of barracuda show them to be 82.3% identical in amino acid sequence and 80.2% identical in the DNA sequence of the coding region. The amino acid sequence identity between cMDH-S and cMDH-L thus is higher than the sequence identity between either fish cMDH and the mammalian cMDHs, suggesting that the duplication event leading to the two cMDH paralogues in *S. idiestes* occurred after the divergence of the lineage leading to

the tetrapods. In fact, our phylogenetic analysis corroborates these conclusions under all the phylogenetic reconstruction methods examined. Regardless of the weighting scheme used in MP, or in minimum evolution analyses, the barracuda cMDHs and the mammalian cMDHs appear as distinct clades with strong bootstrap support (Fig. 3). This is in contrast to the pattern determined for the A and B paralogues of lactate dehydrogenase (LDH), which we had considered as a model for the evolution of MDH isoforms. In LDH, there is a greater sequence identity between teleost and mammalian orthologues than between paralogues of a single species (Crawford et al. 1989). Resolution of the conflict between the cMDH sequences presented here and the LDH model, as well as the MDH electrophoretic evidence from fish described above, must await the sequencing of cMDH paralogues from additional vertebrate taxa such as elasmobranchs and agnathans, as well as from cephalochordates.

Functional Differences Among MDH Isoforms. Interpreting the functions of different isoforms of MDH is complicated by the fact that MDH contributes to several metabolic pathways, including the citric acid cycle (conventionally, this means the generation of OAA from malate to support the production of citrate); fatty acid synthesis, whereby acetyl CoA groups are transferred from the mitochondria to the cytosol; gluconeogenesis (the intramitochondrial reduction of OAA to malate, which is

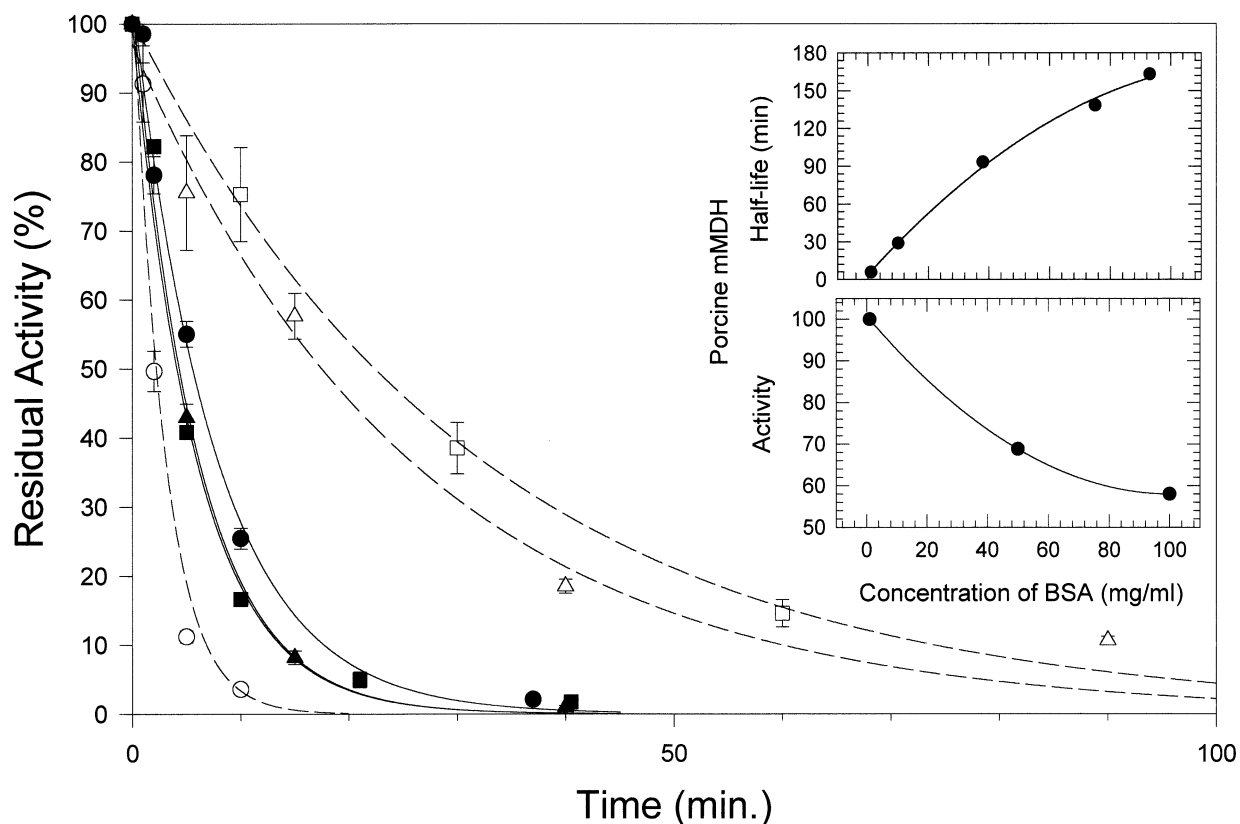


Fig. 5. Effects of BSA concentration on the thermal stability of r-cMDH-L (at 42°C) and cMDH-S (at 48°C). r-cMDH-L at 1 (○), 40 (△), and 100 (□) mg/ml BSA. cMDH-S at 1 (●), 40 (▲), and 100 (■) mg/ml BSA. **Inset:** The effect of increasing BSA concentration on porcine mMDH catalytic activity and thermal stability.

Table 2. Comparison of purified native and recombinant cMDHs and mMDHs of *S. diasties* and cMDH and mMDH of pig

	<i>Sphyraena diasties</i>				Pig	
	cMDH-S	mMDH	r-cMDH-S	r-cMDH-L	cMDH	mMDH
Optimal [substrate] (mM)						
[OAA]	0.5	0.1	0.6	0.1	0.6–0.8	0.6
[L-Malate]	10	100	8	4	30	100
K_m (μ M) at 20°C, pH 7.5 (\pm SE)						
OAA	30.2 \pm 1.8	13.5 \pm 0.6	39.0 \pm 1.3	11.3 \pm 0.4	8.3 \pm 0.6	34.8 \pm 1.5
NADH	21.4 \pm 1.3	24.4 \pm 1.3	20.7 \pm 1.0	13.4 \pm 0.5	20.5 \pm 1.3	14.5 \pm 0.8

then transferred to the cytosol, where it is reoxidized to OAA); and the malate–aspartate shuttle for transferring reducing equivalents from cytosol to mitochondria. The latter two processes involve both cMDH and mMDH.

The kinetic properties of the different MDH isoforms presented in Table 2 offer some clues about the different functional roles that might be played by mMDH, cMDH-S, and cMDH-L. Because mMDH appears to function preferentially in the direction of malate to OAA in the citric acid cycle and in the malate–aspartate shuttle, we compared the kinetic properties of mMDH to those of the two fish cMDH paralogues to determine if one cMDH paralogue more closely resembled mMDH and, therefore, appeared to be adapted for function in the oxidation of malate to OAA. Porcine mMDH is characterized by a

higher optimal concentration of malate and a higher K_m of OAA than cMDH. cMDH-S has higher values for both characteristics than cMDH-L (Table 2), which suggests that cMDH-S, like porcine mMDH, is poised to function in the production of OAA from malate. Interestingly, barracuda mMDH shows a ratio of K_m^{OAA} to K_m^{NADH} opposite that of porcine mMDH. This suggests that barracuda mMDH and cMDH-S both may be poised for the efficient production of cytosolic OAA necessary for high levels of intramuscular gluconeogenesis. This fits well with the findings of Milligan and Wood (1986), who determined that the Cori cycle is relatively unimportant in teleosts and that a large fraction of the lactate generated in fish white locomotor muscle during high-speed swimming is converted to glycogen within the muscle

cells. cMDH-L, with its lower optimal concentration of malate and lower K_m for OAA, may be poised for conversion of OAA to malate, as needed for the malate–aspartate shuttle. We emphasize that these differences in physiological roles between isoforms remain conjectural.

Adaptation Temperature and Molecular Crowding May Influence Intrinsic Protein Stability. A common trend found in comparisons of orthologous proteins from species adapted to different temperatures is an increase in intrinsic thermal stability with rising adaptation temperature (Jaenicke 1991; Somero 1995). This temperature-related variation in thermal stability is seen in our comparison of barracuda cMDH-S with the cMDH of a mammal (Fig. 4). If modification of intrinsic stability is an important element in adaptation of proteins to temperature, one might predict that isoforms of enzymes of a single species would have similar thermal stabilities because all function at a common temperature. However, this prediction is not fulfilled in the case of MDH. In barracuda, the three isoforms of MDH are significantly different in thermal stability, in the order cMDH-S > mMDH > cMDH-L; in mammals, cMDH is much more stable than mMDH (Fig. 4).

These differences in intrinsic stability between MDH isoforms of a single species may reflect differences in the amount of extrinsic stabilization that is afforded by the microenvironments within the cell in which the different isoforms occur. For instance, the mitochondrial matrix may be nearly 50% protein, by mass (Srere 1981), and this high concentration of protein would exert a strong stabilizing effect on all matrix proteins due to molecular crowding effects (Garner and Burg 1994). Proteins found in this highly stabilizing microenvironment might require low intrinsic stabilities to ensure that the conformational flexibility that is required for enzymatic function is conserved. We suggest that the low intrinsic stability of mMDH and the high degree of stabilization caused by elevated BSA concentrations reflect this evolutionary relationship, namely, that the evolution of intrinsic protein stability is influenced by the amount of extrinsic stabilization provided by the milieu in which the protein occurs. The inhibition of mMDH activity by rising concentrations of BSA is a reflection of the tight linkage between conformational flexibility and rate of catalytic function that has been seen repeatedly in studies of enzymes (see Dunn et al. 1991).

The low intrinsic stability of cMDH-L and the strong stabilization of this isoform by rising concentrations of BSA suggest that this isoform of MDH, too, may occur in a strongly stabilizing intracellular microenvironment. Although found in the cytosol, cMDH-L may be localized to a relatively protein-rich environment or function within a multiprotein complex (e.g., with aspartate aminotransferase, which “feeds” cMDH OAA within the malate–aspartate shuttle), such that its stability *in vivo* is enhanced substantially over its stability *in vitro*. If

cMDH-L can be localized intracellularly, then it may be possible to interpret more clearly its thermal stability and its kinetic properties.

Alternatively, it is possible that the duplication of the cMDH gene will ultimately result in the silencing of one paralogue, cMDH-L, through random drift toward a non-functional peptide, an untranslated message, or an untranscribed pseudogene. It is argued that this has been the fate of 80–90% of the duplicated loci resulting from the postulated tetraploidization of the vertebrate genome ~500 Mya (Fisher et al. 1980). In this scenario, cMDH-L is currently under no selective pressure, and so its kinetic properties have no adaptive significance. We view this possibility as unlikely, however, because despite nearly 20% divergence in the amino acid sequence between the two cytosolic paralogues, cMDH-L expression and catalytic function have been maintained.

Thermal Stability Differences and Amino Acid Sequence. It is difficult to predict which amino acid replacements among the 57 differences between cMDH-S and cMDH-L are responsible for the markedly lower stability of cMDH-L. By comparing the crystal structures of MDH from the thermophilic bacterium *Thermus flavus* and porcine cMDH, Kelly et al. (1993) showed that more ion pairs, additional helix dipole charge stabilization, enhanced stabilization of helices by incorporation of alanine, and increased hydrophobicity and decreased flexibility in α -helical and interdomain regions all appeared to contribute to the stability of the thermophilic bacterium’s MDH. Among these factors, the additional ion pairs found in the subunit and domain interfaces of *T. flavus* MDH seemed to be the most significant. The amino acid residues involved in hydrogen bonding between the subunits of porcine cMDH (Birktoft et al. 1989) all are conserved in both fish cMDHs. Therefore, to determine whether any other electrostatic interactions could explain the different stabilities found for the fish cMDH paralogues, we examined all the residue replacements between the two fish cMDHs that resulted in charge differences. There are six positions where cMDH-L (and porcine and mouse cMDHs) carry charges lacking in cMDH-S. However, except for 176His (Ser for cMDH-S and Asp for mouse and pig), all these replacements are within α -helical or β -sheet structures. On the other hand, among the five charged amino acids that are found in cMDH-S (and in mammalian cMDH in most cases) but are replaced by noncharged residues in cMDH-L, four of them, Glu149, Arg169, Asp175, and Lys261, occur at loop regions and should be accessible to solvent according to the known MDH structure. However, whether these charged residues could form ion pairs with other charged residues and the resulting importance of these charged amino acids in stabilizing the quaternary protein remain to be tested.

Another interesting substitution involved in subunit interactions is found at position 27. In pig and mouse

cMDHs, the amino acid at this position is Ser. In *Thermus flavus* MDH, this Ser is replaced by Glu, which can form an intersubunit ion pair with Lys31, thus enhancing the thermal stability (Kelly et al. 1993). In fish cMDH-L and cMDH-S, an Asp that potentially could form an ion pair with Lys31 and enhance thermal stability replaces the Ser. While this particular ion pair appears adaptive for increasing the stability of *Thermus flavus* MDH, the occurrence of a negatively charged residue at this site in both labile and thermally stable fish cMDHs, but not in the more thermally stable porcine cMDH, shows the potential danger of assigning major importance to single substitutions in proteins with substantial differences in sequence. As shown in recent comparisons of proteins from differently thermally adapted organisms, substitutions at many sites throughout a protein can effect adaptive differences in kinetic properties and stability (Jaenicke 1991; Fields and Somero 1998; Holland et al. 1997).

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References

- Bailey GS, Wilson AC, Halver JE, Johnson CL (1970) Multiple forms of supernatant malate dehydrogenase in salmonid fishes: Biochemical, immunological and genetic studies. *J Biol Chem* 245:5927–5940
- Basaglia F (1989) Some aspects of isozymes of lactate-dehydrogenase, malate-dehydrogenase and glucosephosphate isomerase in fish. *Comp Biochem Physiol* 92B:213–226
- Birktoft JJ, Rhodes G, Banaszak LJ (1989) Refined crystal-structure of cytoplasmic malate-dehydrogenase at 2.5-Å resolution. *Biochemistry* 28:6065–6081
- Brooks SPJ, Suelter CH (1986) Estimating enzyme kinetic-parameters: A computer program for linear regression and nonparametric analysis. *Int J Biomed Comput* 19:89–99
- Cashon RE (1981) The malate dehydrogenase isozymes and allozymes of *Fundulus heteroclitus*. PhD thesis, The Johns Hopkins University, Baltimore
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal Biochem* 162:156–159
- Cleveland DW (1983) Peptide-mapping in one dimension by limited proteolysis of sodium dodecyl sulfate-solubilized proteins. *Methods Enzymol* 96:222–229
- Coppes ZL (1990) Isozymes of soluble malate-dehydrogenase (s-MDH) in fishes of the subclasses Chondrichthyes and Actinopterygii. *Comp Biochem Physiol* 96B:23–31
- Coppes ZL, Schwantes MLB, Schwantes AR (1987) Adaptive features of enzymes from family Sciaenidae (Perciformes). I. Studies on soluble malate-dehydrogenase (s-MDH) and creatine-kinase (CK) of fishes from the south coast of Uruguay. *Comp Biochem Physiol* 88B:203–209
- Coppes ZL, De Vecchi S, Ferreira E, Hirshhorn M (1990) Multilocus isozyme systems in fishes. *Comp Biochem Physiol* 96B:1–13
- Courtright JB, Henning U (1970) Malate dehydrogenase mutants in *Escherichia coli* K-12. *J Bacteriol* 102:722–728
- Crawford DL, Constantino HR, Powers DA (1989) Lactate dehydrogenase-b cDNA from the teleost *Fundulus heteroclitus*: Evolutionary implications. *Mol Biol Evol* 6:369–383
- De Luca PH, Schwantes MLB, Schwantes AR (1983) Adaptive features of ectothermic enzymes. 4. Studies on malate-dehydrogenase of *Astyanax fasciatus* (Characidae) from lobo reservoir (Sao Carlos, Sao Paulo, Brasil). *Comp Biochem Physiol* 47B:315–324
- Dunn CR, Wilks HM, Halsall DJ, Atkinson T, Clarke AR, Muirhead H, Holbrook JJ (1991) Design and synthesis of new enzymes based on the lactate dehydrogenase framework. *Phil Trans R Soc Lond B* 332:177–184
- Fields PA, Somero GN (1998) Hot spots in cold adaptation: Localized increases in conformational flexibility in lactate dehydrogenase A₄ orthologs of Antarctic notothenioid fishes. *Proc Natl Acad Sci USA* 95:11476–11481
- Fisher SE, Shaklee JB, Ferris SD, Whitt GS (1980) Evolution of 5 multilocus isoenzyme systems in the chordates. *Genetica* 52:73–85
- Garner MM, Burg MB (1994) Macromolecular crowding and confinement in cells exposed to hypertonicity. *Am J Physiol* 266:C877–C892
- Gleason WB, Fu Z, Birktoft JJ, Banaszak LJ (1994) Refined crystal structure of mitochondrial malate dehydrogenase from porcine heart and the consensus structure for dicarboxylic-acid oxidoreductases. *Biochemistry* 33:2078–2088
- Grant PM, Tellam J, May VL, Strauss AW (1986) Isolation and nucleotide sequence of a cDNA clone encoding rat mitochondrial malate dehydrogenase. *Nucleic Acids Res* 14:6053–6066
- Hall MD, Levitt DG, Banaszak LJ (1992) Crystal structure of *Escherichia coli* malate dehydrogenase: A complex of the apoenzyme and citrate at 1.87 angstrom resolution. *J Mol Biol* 226:867–882
- Higgins DG, Sharp PM (1988) Clustal: A package for performing multiple sequence alignment on a microcomputer. *Gene* 73:237–244
- Holland LZ, McFall-Ngai M, Somero GN (1997) Evolution of lactate dehydrogenase-A homologs of barracuda fishes (genus *Sphyræna*) from different thermal environments: Differences in kinetic properties and thermal stability are due to amino acid substitutions outside the active site. *Biochemistry* 36:3207–3215
- Jaenicke R (1991) Protein stability and molecular adaptation to extreme conditions. *Eur J Biochem* 202:715–728
- Joh T, Takeshima H, Tsuzuki T, Shimada K, Tanase T, Morino Y (1987a) Cloning and sequence analysis of cDNAs encoding mammalian mitochondrial malate dehydrogenase. *Biochemistry* 26:2515–2520
- Joh T, Takeshima H, Tsuzuki T, Setoyama C, Shimada K, Tanase S, Kuramitsu S, Kagamiyama H, Morino Y (1987b) Cloning and sequence analysis of cDNAs encoding mammalian cytosolic malate dehydrogenase: Comparison of the amino acid sequences of mammalian and bacterial malate dehydrogenase. *J Biol Chem* 262:15127–15131
- Kelly CA, Nishiyama M, Ohnishi Y, Beppu T, Birktoft JJ (1993) Determinants of protein thermostability observed in the 1.9-angstrom crystal structure of malate dehydrogenase from the thermophilic bacterium *Thermus flavus*. *Biochemistry* 32:3913–3922
- Lin J-J, Somero GN (1995a) Temperature-dependent changes in expression of thermostable and thermolabile isozymes of cytosolic malate dehydrogenase in the eurythermal goby fish *Gillichthys mirabilis*. *Physiol Zool* 68:114–128
- Lin J-J, Somero GN (1995b) Thermal adaptation of cytoplasmic malate dehydrogenases of eastern pacific barracuda (*Sphyræna* spp): The role of differential isoenzyme expression. *J Exp Biol* 198:551–560
- Lin J-J, Macleod AS, Kuo C-M (1996) Qualitative and quantitative strategies of thermal adaptation of grass carp (*Ctenopharyngodon idella*) cytoplasmic malate dehydrogenases. *Fish Physiol Biochem* 15:71–81

- McAlister-Henn L (1988) Evolutionary relationships among the malate dehydrogenases. *Trends Biochem Sci* 13:178–181
- Milligan CL, Wood CM (1986) Tissue intracellular acid-base status and the fate of lactate after exhaustive exercise in the rainbow trout. *J Exp Biol* 123:123–144
- Nishiyama M, Matsubara N, Yamamoto K, Iijima S, Uozumi T, Beppu T (1986) Nucleotide sequence of the malate dehydrogenase gene of *Thermus flavus* and its mutation directing an increase in enzyme activity. *J Biol Chem* 261:14178–14183
- O'Carra P, Barry S (1972) Affinity chromatography of lactate dehydrogenase. *FEBS Lett* 21:281–285
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Schwantes MLB, Schwantes AR (1982) Adaptative features of ectothermic enzymes. 2. The effects of acclimation temperature on the malate dehydrogenase of the spot, *Leiostomus xanthurus*. *Comp Biochem Physiol* 72B:59–64
- Somero GN (1995) Proteins and temperature. *Annu Rev Physiol* 57:43–68
- Srere PA (1981) Protein crystals as a model for mitochondrial matrix proteins. *Trends Biochem Sci* 6:4–7
- Swofford DL (1998) PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Sunderland, MA
- Wheat TE, Whitt GS (1971) *In-vivo* and *in-vitro* molecular hybridization of malate dehydrogenase isozymes. *Experientia* 27:647–648
- Wheat TE, Whitt GS, Childers WF (1972) Linkage relationships be-

tween the homologous malate dehydrogenase loci in teleosts. *Genetics* 70:337–340

Appendix

Primers used for the molecular studies described in this paper are as follows. For amplification of cMDHs: primer pair A—forward, 5'-(157)GTGCTGATGGARYTSCARGAYTG(179)-3'; and reverse, 5'-(578)GGGTACTGRGTRGWNGWRTGRTT(556)-3'.

For amplification of mMDH: primer pair B—forward, 5'-(465)GAAGMGVGGVGTSTACAACC(484)-3'; and reverse, 5'-(986)CCTTCTTRATDSWBGCTTSAG(964)-3'.

For amplification of cMDH-S from the pGEMEX vector: primer pair C—forward, 5'-(−13)CTCCGCGACCCATATGGCTGAACC-GATCCG(17)-3' (containing an *NdeI* site); and reverse, 5'-(1091)GGTCTTCAGACTCGAGAGCCTGGAGCCTTCAG(1060)-3' (containing an *XhoI* site).

For ligation of cMDH-L partial clones: for the 5'-fragment, primer pair D—forward, 5'-(−12)CTCCGTGTGCATATGTCTGAGC-CCATTCG(17)-3' (containing an *NdeI* site); and reverse, 5'-(540)GTTCCCTCACGTGGGTTGCAGG(520)-3'; and for the 3'-end, primer pair E—forward, 5'-(354)GTACGCCAAGAAGACCGT-CAAGG(386)-3'; and reverse, 5'-(1056)GTCTGGAGTTCTCGAGC-TGTCTAAGTCTGGC(1025)-3' (containing an *XhoI* site). For amplification of the resultant full-length clone, the forward primer was the same as that used in amplification of the 5'-end fragment, while the reverse primer was the same as that for 3'-end amplification.