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[†,‡]

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ABSTRACT: Orthologous homologs of lactate dehydrogenase-A (LDH-A) (EC 1.1.1.27; NAD^+:lactate oxidoreductase) of six barracuda species (genus *Sphyraena*) display differences in Michaelis−Menten constants (apparent K_m) for substrate (pyruvate) and cofactor (NADH) that reflect evolution at different habitat temperatures. Significant increases in K_m with increasing measurement temperature occur for all homologs, yet K_m at normal body temperatures is similar among species because of the inverse relationship between adaptation temperature and K_m. Thermal stabilities of the homologs also differ. To determine the amino acid substitutions responsible for differences in K_m and thermal stability, peptide mapping of the LDH-As of all six species was first performed. Then, the amino acid sequences of the three homologs having the most similar peptide maps, those of the north temperate species, *S. argentea*, the subtropical species, *S. lucasana*, and the south temperate species, *S. idiastes*, were deduced from the respective cDNA sequences. At most, there were four amino acid substitutions between any pair of species, none of which occurred in the loop or substrate binding sites of the enzymes. The sequence of LDH-A from *S. lucasana* differs from that of *S. idiastes* only at position 8. The homolog of *S. argentea* differs from the other two sequences at positions 8, 61, 68, and 223. We used a full-length cDNA clone of LDH-A of *S. lucasana* to test, by site-directed mutagenesis, the importance of these sequence changes in establishing the observed differences in kinetics and thermal stability. Differences in sequence at sites 61 and/or 68 appear to account for the differences in K_m between the LDH-As of *S. argentea* and *S. lucasana*. Differences at position 8 appear to account for the difference in thermal stability between the homologs of *S. argentea* and *S. lucasana*. Evolutionary adaptation of proteins to temperature thus may be achieved by minor changes in sequence at locations outside of active sites, and these changes may independently affect kinetic properties and thermal stabilities.

Orthologous homologs of enzymes from organisms adapted to different temperatures typically exhibit variations in thermal stability and kinetic properties that appear temperature-adaptive [reviewed in Jaenicke (1991) and Somero (1995)]. Although kinetic properties like Michaelis−Menten constants (apparent K_m) and catalytic rate constants (k_{cat}) are usually strongly affected by temperature, both K_m and k_{cat} values may be highly conserved among species at their physiological temperatures (Yancey & Somero, 1978; Graves & Somero, 1982; Yancey & Siebenaller, 1987; Dahlhoff & Somero, 1993). Amino acid substitutions responsible for temperature-adaptive differences in kinetic properties and thermal stability of orthologous and paralogous homologs of proteins are not well understood (Powers et al., 1993; Somero, 1995). Elucidating these sequence changes would not only contribute to understanding molecular evolution by revealing the amounts and sites of sequence change needed to modify thermal sensitivities of proteins, but might also provide insights into strategies for rational design of proteins with altered kinetic properties and stabilities.

To investigate sequence changes that affect evolutionary adaptation of proteins to temperature, we selected for study orthologous homologs of lactate dehydrogenase-A (LDH-A,[1] EC 1.1.1.27, NAD^+:lactate oxidoreductase) from fishes of the genus *Sphyraena* (barracudas). We chose LDH-As of these fishes for several reasons. First, LDH-A is a slowly evolving protein for which there are extensive data on amino acid sequence (Li et al., 1983; Crawford et al., 1989; Ishiguro et al., 1990; Tsoi & Li, 1994) and three-dimensional structure

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[‡] DNA sequences have been deposited with GenBank under Accession Numbers U80000 (*Sphyraena argentea*), U80001 (*S. idiastes*), and U80002 (*S. lucasana*).

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[1] Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; LDH, lactate dehydrogenase; LDH-A, A-type isozyme (=M type isozyme) of LDH; NADH, β-nicotinamide adenine dinucleotide (reduced); PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 0.5 × TBE, 0.045 M Tris−borate, 0.001 M EDTA, pH 8.0; TFA, trifluoroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

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(Abad-Zapatero et al., 1987; Gerstein & Chothia, 1991). Second, structure−function analysis of LDH of the bacterium *Bacillus stearothermophilus* has elucidated the roles of numerous amino acid residues in governing the enzyme's kinetic properties (Wigley et al., 1987, 1992; Feeney et al., 1990; Deng et al., 1994; Nicholls et al., 1994). Third, the LDH-A homologs of fishes within the genus *Sphyraena* differ adaptively in kinetic properties (K_m of pyruvate and k_{cat}), although the species have diverged only relatively recently (between approximately 3.5 and 12 million years) and encounter habitat temperatures that differ by only approximately 3−8 °C (Graves & Somero, 1982). We reasoned that there would be relatively few differences in sequence between the LDH-A homologs of these fishes, thus facilitating both the functional interpretations of the observed sequence changes and the testing of the importance of these changes by site-directed mutagenesis.

We initially characterized temperature effects on K_m values of pyruvate and NADH for the LDH-A homologs of six species of barracuda, *S. argentea* (north temperate), *S. idiastes* (south temperate), *S. lucasana* (subtropical), and three tropical species, *S. ensis*, *S. helleri* (*S. acutipinnis*), and *S. barracuda*. We then performed peptide mappings of the six homologs to obtain initial information on overall similarities in sequence. We selected the three most similar homologs, those of eastern Pacific species *S. argentea*, *S. lucasana*, and *S. idiastes*, for cDNA sequencing. The roles of the deduced amino acid substitutions in establishing differences in temperature sensitivity of K_m and thermal stability in two of the homologs (*S. lucasana* and *S. argentea*) were studied by site-directed mutagenesis. Our results suggest that minor changes in sequence at regions outside of the active site are responsible for adaptation to temperature, and that kinetic properties and thermal stability can evolve independently.

EXPERIMENTAL PROCEDURES

Collection and Processing of Specimens. Specimens of the four eastern Pacific species of *Sphyraena* were collected by hook and line or by gill nets at the following locations: *S. argentea* (La Jolla, CA), *S. lucasana* (Guaymas, Mexico), *S. idiastes* (Pisco, Peru, and Galapagos Islands), and *S. ensis* (Ecuador and Panama). Two Hawaiian species, *S. acutipinnis* and *S. barracuda*, were purchased at fish markets in Honolulu, HI.

Purification of LDH-A. LDH-A was extracted from white skeletal muscle and purified by oxamate affinity chromatography by the method of O'Carra and Barry (1972), as modified by Yancey and Somero (1978). Silver-stained SDS−PAGE gels yielded a single band representing LDH-A.

Peptide Mapping of LDH-A. Purified LDH-A was reduced and alkylated as follows: To a 10 mg/mL solution of LDH-A in 10 mM Tris-HCl buffer (pH 8.3 at 20 °C) containing 5 mM EDTA and 61 μg of dithiothreitol/mg of protein was added urea to a concentration of 8 M. After 2 h at room temperature, 25 μl/mL of 0.1 M Tris-HCl buffer (pH 8.3) containing 420 μg of iodoacetamide/mg of protein was added. Alkylation proceeded in the dark for 4 h. After dialysis against 50 mM NH_4HCO_3 (pH 8.0), a protein precipitate usually formed. This precipitate was not removed before digestion with trypsin.

Tryptic digests of reduced and alkylated LDH-A were prepared by incubating 3 mg of protein in 1 mL of 50 mM NH_4HCO_3 buffer (pH 8.0) containing 30 μg of TPCK-treated trypsin, for 3−24 h. Because all digestion times gave identical peptide maps, except for some differences in relative peak heights, the results shown are for a 6 h digestion time, unless otherwise noted. Any precipitate remaining after digestion was removed, either by centrifugation at 12000*g* for 5 min or by filtration through a 0.2 μm filter. Peptide maps were obtained by high-performance liquid chromatography of the tryptic digests on a Vydac wide-pore C-18 column (The Separations Group, Hesperia, CA) equilibrated in 0.1% TFA in water. Elution at 1 mL/min was isocratic for 10 min, followed by a gradient up to 0.1% TFA in 60% acetonitrile over 90 min. Detection of peaks was at 219 nm.

Protein Sequencing. For sequencing, 1 mL of a tryptic digest was chromatographed in 150 μL aliquots. Corresponding peaks were pooled from each run and evaporated to dryness. Sequencing was performed at the University of California, Los Angeles, protein sequencing facility on an Applied Biosystems (Foster City, CA) gas phase sequencer.

Synthesis of cDNA. Total RNA was purified by the acid guanidinium thiocyanate method of Chomczynski and Sacchi (1987), from approximately 20 g of white skeletal muscle dissected from fish that were freshly killed or frozen on dry ice and stored for several months at −80 °C. Total RNA was stored as a precipitate in 75% ethanol at −20 °C. mRNA was purified from total RNA with Dynabeads Oligo $(dT)_{25}$ (Dynal, A. S., Oslo, Norway) according to the manufacturer's protocol. First-strand cDNA was reverse-transcribed from approximately 250 ng of mRNA with the SuperScript Preamplification System (BRL Life Technologies, Inc., Gaithersburg, MD); 1.5 μL of the resulting 10 μL of first-strand cDNA was used in each PCR reaction.

PCR Amplification. PCR amplification was by the RACE method of Frohman et al. (1988). The entire cDNA (2214 bp), except for the extreme 3′ untranslated end (3′UTR), was amplified with three pairs of primers based on the cDNA sequence of the scorpaenid fish *Sebastolobus alascanus* (unpublished data of the authors). These primer pairs were: (−98)5′GGAAAAGCTGCACTCAGGAG3′(−79) plus (640)5′GGCTCTGCAGAGAAACTCCGGCA3′(617); (583)-5′ACTCCAGTGTGCCTGTGTGG3′(603) plus (948)-5′CCAGCTGCTTCTCCTCTTCGGGC3′(927); and (890)-5′ACAGCGGCCTGACAGACGTGA3′(910) plus (2238)5′-TATCATTATCCTGGCAGATT3′(2219). A primer (1970)-5′TGTCTGCGCATCCTAAATACCC3′(1992), based on the sequence of the 3′UTR of the *S. idiastes* LDH-A, and a primer for the poly(A) tail (5′GCGGCCGCT_{17}3′) were used to amplify the extreme 3′UTR. Additional primers were synthesized as needed for sequencing.

DNA Sequencing. Double-stranded sequencing was by the fmol DNA Sequencing system (Promega Corp., Madison, WI). Most PCR products were sequenced directly. When multiple bands were present, the PCR products were electrophoresed on low gelling-temperature agarose in 0.5× TBE, and the appropriate band was excised and purified on PCR preps resin (Promega Corp.). Before sequencing, all PCR products were purified with the Magic PCR Preps DNA purification system (Promega). The PCR products amplified with 890/910 and 2219/2238 were not ample, and were, therefore, cloned for sequencing into the pCR-Script plasmid with the pCR-Script SK(+) cloning system (Stratagene, La Jolla, CA). Because of possible PCR errors, six clones were sequenced for each gene.

Site-Directed Mutagenesis. For site-directed mutagenesis, the entire coding region of the LDH-A genes of *S. lucasana* and *S. argentea* was amplified and cloned into a pGEMEX vector modified to remove the *Nde*I site at position 3259 by the methods of Pauls and Berchtold (1993). The primers used for PCR amplification were reverse 5′TTCAG(G\A)G-(C\T)GG(C\A)(C\G)TGGAG(A\G)A(A\T)TCAGAGG3′ and forward 5′CCATATGTCCACCAAGGAGAAGCTCATCG-GCCACGTGATG3′ for *S. argentea* LDH-A or 5′CCATAT-G T C C A C C A A G G A G A A G C T C A T C G A C C A - CGTGATG3′ for *S. lucasana* LDH-A. The PCR products were first blunt-end-ligated into the pCR-Script vector (Stratagene) according to the manufacturer's instructions. The inserts were excised with *Nde*I and *Eco*RI and ligated into the modified pGEMEX vector cut with the same enzymes. The identity of clones was confirmed by sequencing. Expression was induced in *E. coli* strain BL21 with 93 mg/L IPTG. To release the expressed enzyme, bacteria were resuspended in 50 mL of 50 mM KPO_4 buffer (pH 6.8) and sonicated. The cloned enzyme from *S. lucasana* was active. However, all of the approximately 12 clones of the *S. argentea* enzyme failed to yield active enzyme when expressed. Sequencing revealed a mistake in each one due to misincorporation of nucleotides by Taq polymerase. However, one inactive clone with an extra C at position 966, resulting in a frame-shift, was useful for creating mutant enzymes. To create such mutants, portions of this clone were excised with restriction enzymes and exchanged with the corresponding fragments of the *S. lucasana* LDH-A clone. Restriction digests were electrophoresed on a 1% agarose gel in 0.5× TBE, the appropriate bands were excised, and the DNA was extracted by repeated freezing and thawing of the gel slice. The appropriate restriction fragments were combined, ligated, and expressed in *E. coli*. The identity of the mutants was confirmed by sequencing. Amino acid 8 was mutated by cutting out a fragment of the inactive *S. argentea* enzyme (bases 1−127, corresponding to amino acid residues 1−41) with *Nde*I and *Eco*0109 and exchanging it for the corresponding fragment of the active *S. lucasana* cDNA. The reciprocal hybrid enzyme, which was inactive, was also constructed and used to mutate amino acids 61 and 68, by cutting with *Ppu*M1 and *Eco*RI to excise a fragment consisting of the 800 most 3′ bp and exchanging it with the corresponding fragment of the *S. lucasana* clone.

Apparent Michaelis−Menten Constants. K_m values for pyruvate and NADH were determined from the relationship between initial velocity and substrate concentration, using the weighted linear regression method of Wilkinson (1961) and Wilman4 Software (Brooks & Suelter, 1986), which yields an estimate of K_m and the standard deviation of this estimate. For the K_m of pyruvate, the assay medium contained 80 mM imidazole hydrochloride buffer (pH 6.98 at 20 °C), 150 μM NADH, and pyruvate concentrations ranging between 0.01 and 2.0 mM. For the K_m of NADH, the assay medium contained 80 mM imidazole hydrochloride buffer, 2 mM pyruvate, and NADH concentrations ranging from 7.5 to 150 μM. Typically, duplicate measurements at seven concentrations of substrate which spanned the value of K_m were used to determine a K_m value.

Isoelectric Points. Precast gels (Pharmacia, Inc., Piscataway, NJ) with a pH range of 3.5−9.0 were used according to the manufacturer's instructions. Isoelectric points were determined by interpolation from a regression curve of protein standards of known p*I*. When purified LDH-A was focused, staining was with Coomassie Brilliant Blue. For crude muscle homogenates or bacterial lysates containing cloned LDH-A, staining was with an LDH activity stain: 300 μM β-NAD^+, 85 μM phenazine methosulfate, 0.2 mg/mL nitro blue tetrazolium, and 25 mM lactic acid.

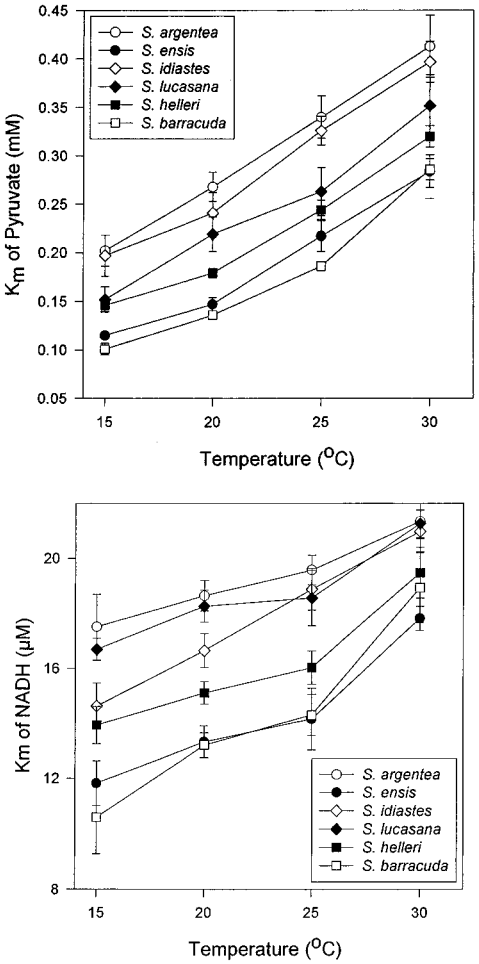

FIGURE 1: Effects of temperature on the apparent Michaelis−Menten constants (K_m) of pyruvate (top) and NADH (bottom) for LDH-As of six species of barracuda. Error bars designate standard deviations. Data for K_m of pyruvate for *S. argentea*, *S. lucasana*, *S. idiastes*, and *S. ensis* are from Graves and Somero (1982).

Thermal Stability Measurements. Thermal stability was measured by incubating purified LDH-A at 45 °C in a 10 mM potassium phosphate buffer (pH 7.5 at room temperature) containing 0.15 M KCl and 0.1% bovine serum albumin (Place & Powers, 1984). Between 0.5 and 1 mg of LDH-A was used in each incubation, in a total volume of 10 mL; 75 μL aliquots were removed at different times, placed on ice, and assayed for LDH activity at the end of the experiment. No renaturation of the enzyme occurred during the period of holding on ice prior to conducting activity assays. LDH activity was analyzed at 20 °C in 80 mM imidazole buffer (pH 7.0 at 20 °C) containing 0.4 mM pyruvate and 150 μM NADH.

Statistical Analysis. Tests for significant differences between K_m values for the native and cloned enzymes were performed by analysis of variance (ANOVA) (Systat for the MacIntosh), followed by post hoc comparisons of individual means using Tukey's test (Zar, 1984).

RESULTS

Enzyme Kinetics. The K_m values for substrate (pyruvate) (Figure 1, top) and cofactor (NADH) (Figure 1, bottom)

Table 1: Habitat Temperature Ranges, Midrange Body Temperature (MRT), K_m Values at 25 °C and at MRT, and Isoelectric Points (p*I*) for LDH-As of Congeneric Barracuda Fishes

species	K_m[a] of pyr at 25 °C [at MRT] (mM)	K_m[a] of NADH at 25 °C [at MRT] (μM)	p*I*
S. argentea	0.34 ± 0.02 [0.24] (13−22 °C, [18 °C])	19.6 ± 0.54 [18]	8.65
S. lucasana	0.26 ± 0.02 [0.24] (16−28 °C, [23 °C]	18.6 ± 1.02 [18]	8.17
S. idiastes	0.33 ± 0.02 [0.23] (13−22 °C, [18 °C]	18.9 ± 0.77 [16]	8.65
S. ensis	0.22 ± 0.02 [0.20] (22−30 °C, [26 °C]	14.2 ± 1.12 [15]	7.70
S. helleri	0.24 ± 0.01 [0.22] (22−30 °C, [26 °C]	16.0 ± 0.60 [17]	8.45
S. barracuda	0.19 ± 0.01 [0.20] (22−30 °C, [26 °C]	14.3 ± 0.75 [15]	7.40

[a] K_m values (±SD) determined using duplicate activity measurements at 7 concentrations of substrate. K_m values of pyruvate values for *S. argentea*, *S. lucasana*, and *S. ensis* are from Graves and Somero (1982), and from Graves (1981) for *S. idiastes*. K_m values for midrange of body temperatures are interpolated from data in Figure 1 and from data in Graves and Somero (1982).

reflect the adaptation temperatures of the six species. At most measurement temperatures, values for the apparent K_m of pyruvate and NADH are highest for the most cold-adapted species, *S. argentea* and *S. idiastes*, intermediate for the subtropical species, *S. lucasana*, and lowest for the three tropical species, *S. ensis*, *S. helleri*, and *S. barracuda*. As a consequence of these differences, K_m is highly conserved among the six species at their midrange body temperatures: K_m of pyruvate varies between approximately 0.20 and 0.24 mM pyruvate, and K_m of NADH ranges between approximately 15 and 18 μM NADH (Figure 1; Table 1).

Peptide Mapping. The first step taken to determine the structural bases of these interspecific differences in kinetic properties was to obtain peptide maps of the LDH-A homologs of the six species. Figure 2 shows these maps ordered top to bottom in approximate ranking of overall similarity. Ten peaks are labeled 1−10 based on similarities in elution times and peak heights. Although LDH-As typically have 30−35 lysines and arginines, theoretically resulting in about 30 peaks on a chromatogram of a tryptic digest, sequencing of these 10 peaks for the LDH-A of *S. argentea* showed that there is not always a one-to-one correlation between peaks and peptides. That is, although most peaks contain only a single peptide, some include several. For example, peak 1, which has an obvious shoulder, contains three peptides. Furthermore, all two- or three-amino acid peptides probably eluted together in one peak just after the injection artifact. Conversely, sometimes two peaks represent but a single peptide as a result of incomplete cleavage. For example, peak 4, which elutes either as a doublet or as a somewhat larger peak with a shoulder (*S. idiastes*), includes two peptides with the same amino terminus, one of which is longer than the other by three amino acids due to incomplete cleavage at Lys-72. In addition, the longest and most hydrophobic peptides are not represented at all on the chromatograms because of their insolubility in aqueous media. The deduced amino acid sequences (see below) show that there are 5 tryptic peptides over 15 amino acids in length, which should have given large peaks. However, only two were present in the peptide maps, peak 7 (residues 177−216) and peak 10 (residues 245−264). Some of the other long peptides were obtained from extraction of the aqueous-insoluble material with 0.1% TFA in 20% acetonitrile and chromatography with a gradient of 0.1% TFA in 20% acetonitrile to 0.1% TFA in 100% acetonitrile. Thus, the peptide maps do not accurately represent the number of sequence differences among the LDH-As of the six species. However, they do provide an approximation of similarity among the homologs useful for selecting the most similar homologs for sequencing.

The three most similar peptide maps are for *S. argentea*, *S. lucasana*, and *S. idiastes*. The maps for *S. idiastes* and *S. lucasana* are identical and differ from that for *S. argentea* only in the elution time of peak 6, which is earlier for *S. argentea*. Additional tryptic peptide mapping with cleavage at lysines blocked by citraconylation (data not shown) revealed another difference, not apparent in the maps in Figure 2, among these three species. One peptide, identified by amino acid sequencing as the amino-terminal 17 amino acids, had a different elution time for each of the 3 species, suggesting additional amino acid differences. This peptide is N-terminally blocked, but cleavage with trypsin after deblocking the lysines liberated a peptide (residues 12−17) confirming its identity. The deduced amino acid sequences (see below) show that within the amino-terminal 17 residues each of the 3 species has a different amino acid at position 8.

Tryptic maps for LDH-As of *S. ensis* and *S. barracuda* are quite similar to each other, but differ considerably from those of the other four species. Peaks 1 and 2 may not represent the same peptides for *S. barracuda* and *S. ensis* as in the other species because, although their elution times are similar to those of the respective peaks for the other species, the relative peak heights are different. Peak 1 is larger than peak 2 for *S. argentea*, *S. lucasana*, *S. idiastes*, and *S. helleri*, while the reverse is true for *S. ensis* and *S. barracuda*. Peak 6 elutes at about the same time in the maps for *S. ensis*, *S. barracuda*, *S. lucasana*, and *S. idiastes*. However, peak 7 elutes later for *S. ensis* and *S. barracuda* (and slightly later for the latter than the former species) than for the other four species. Finally, peak 9 elutes at the same time for both the *S. barracuda* and *S. argentea* enzymes.

Deduced Amino Acid Sequences. To investigate the differences in amino acid sequence responsible for the interspecific differences in K_m and thermal stability (see below), we chose to sequence the LDH-A cDNAs of the species with the most similar LDH-A peptide maps, *S. argentea*, *S. idiastes*, and *S. lucasana*. The deduced amino acid sequences for these three enzymes are shown in Figure 3. All 3 LDH-A homologs of the barracudas are 331 residues in length. The amino acid sequence of dogfish LDH-A, which has one extra amino acid residue relative to the barracuda sequences (Stock & Powers, 1995), plus sequences for the teleost fish *Fundulus heteroclitus*, chicken, and cow are shown for comparison.

The amino acid sequences of the LDH-As of *S. idiastes* and *S. lucasana* are more similar to one another than either is to that of the LDH-A of *S. argentea* (Figure 3), consistent with the estimated divergence times of the three species (Graves & Somero, 1982). The only difference in sequence between the enzymes of *S. lucasana* and *S. idiastes* is at position 8. This substitution, Asp-8 in *S. lucasana versus* Asn-8 in *S. idiastes*, is in a region outside the active site. Based on the sequence and three-dimensional structure of

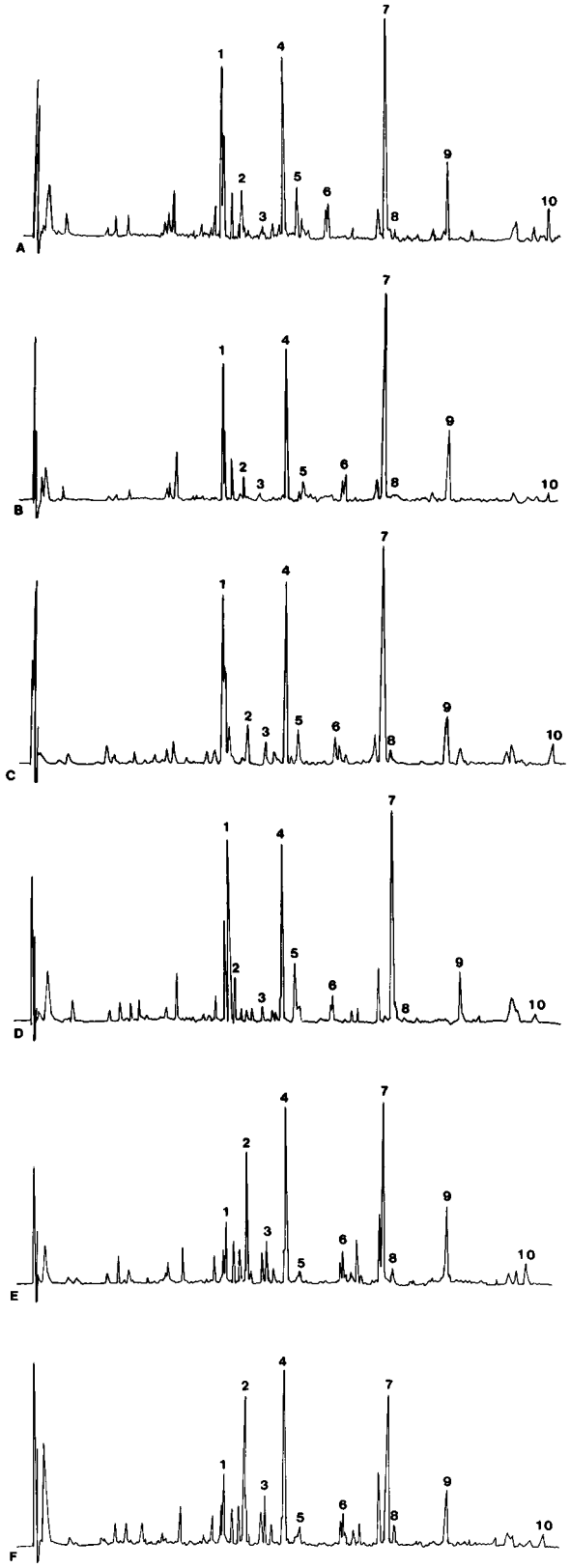

FIGURE 2: Peptide maps of LDH-As of six species of barracuda: A (*S. argentea*), B (*S. lucasana*), C (*S. idiastes*), D (*S. helleri*), E (*S. ensis*), and F (*S. barracuda*). Peak 1 = residues 76−80, 157−168, 217−227; peak 2 = 106−111; peak 3 = amino-terminal peptide; peak 4 = 318−327; peak 5 = 232−242; peak 6 = 59−72, 59−75; peak 7 = 177−216; peak 8 = 42−56; peak 9 = 278−283; peak 10 = 245−264. The identities of numbered peaks were confirmed by amino acid sequencing. Peak 3 is assumed to be the amino terminal peptide because it was N-terminal-blocked.

dogfish LDH-A (Holbrook et al., 1975; Abad-Zapatero et al., 1987; Stock & Powers, 1995), position 8 in the barracuda sequence probably is the first residue C-terminal to a short α-helix (helix A) near the N-terminus (Figure 3). The differences among the barracuda species at position 8 are responsible for the differences in elution time of the amino-terminal peptide on tryptic peptide maps done after blocking of lysine residues (data not shown). However, this difference in elution time is not seen on maps in Figure 2, presumably because the peptide is only seven residues long and would be expected to be a small peak.

The sequence of LDH-A of *S. argentea* differs from the other two sequences by four amino acids, those at positions 8, 61, 68, and 223. Sites 61 and 68 are in helix C (Abad-Zapatero et al., 1987) and occur in tryptic peptide 6 (Figure 2). The Ala (61) and Ser (68) residues present in *S. argentea*, rather than the Val (61) and Gly (68) residues present in *S. idiastes* and *S. lucasana*, are responsible for the earlier elution of the peptide of *S. argentea*. The final difference is at position 223, where the enzymes of *S. lucasana* and *S. idiastes* contain a Thr and *S. argentea* LDH-A contains a Ser. Residue 223 is near the amino terminus of α-helix 1-G (Figure 3; Abad-Zapatero et al., 1987). Because the substitution at position 223 occurs in one of the three peptides included in tryptic peak 1, we conclude that this conservative substitution does not result in a change in elution time (Figure 2).

Site-Directed Mutagenesis of S. lucasana LDH-A. Because there were few nucleotide differences between the coding regions of the LDH-A genes of *S. argentea* and *S. lucasana*, we were able to take advantage of common restriction sites to create chimeric enzymes by site-directed mutagenesis, to test for the effects of amino acid substitutions on K_m (Table 2) and thermal stability (Figure 4). Two chimeric LDH-As were generated: C-8, in which the Asp at position 8 in the native LDH-A of *S. lucasana* was replaced with a Gly, as found in native LDH-A of *S. argentea*, and C-61−68, in which the Val and Gly residues, respectively, of the *S. lucasana* enzyme were replaced with Ala and Ser residues found in the *S. argentea* homolog. We did not alter site 223.

Table 2 shows the effects of these substitutions on the K_m of pyruvate at 25 °C, together with the K_m values for both the cloned−unmodified and native (purified from muscle) LDH-As of *S. lucasana* and the native LDH-A of *S. argentea*. There was no significant difference between the K_m of pyruvate of the native LDH-A of *S. lucasana* (K_m = 0.258 ± 0.021) and the cloned−unmodified enzyme (0.260 ± 0.008)(Table 2; $p > 0.999$). Thus, it does not appear that any posttranslational changes in the native enzyme affect the K_m of pyruvate, so that K_m values for the cloned enzymes can be compared directly to those for the native enzymes.

Because residue 8 is the only one that differs between the LDH-As of *S. lucasana* and *S. idiastes* (Figure 3), we predicted that differences at position 8 also would be responsible for the differences in K_m of pyruvate between the *S. argentea* and *S. lucasana* enzymes. However, a substitution of Asp-8 in the *S. lucasana* enzyme by Gly-8, as in the native *S. argentea* enzyme, had no measurable effect on the K_m of pyruvate (Table 2). The K_m of pyruvate for the chimeric *S. lucasana* LDH-A with Val-61 and Gly-68 mutated to Ala-61 and Ser-68, as in the native LDH-A of *S. argentea*, was significantly higher than the K_m of the native *S. lucasana* enzyme (0.370 ± 0.020 *versus* 0.258 ± 0.021; $p = 0.00015$) and statistically indistinguishable from the K_m

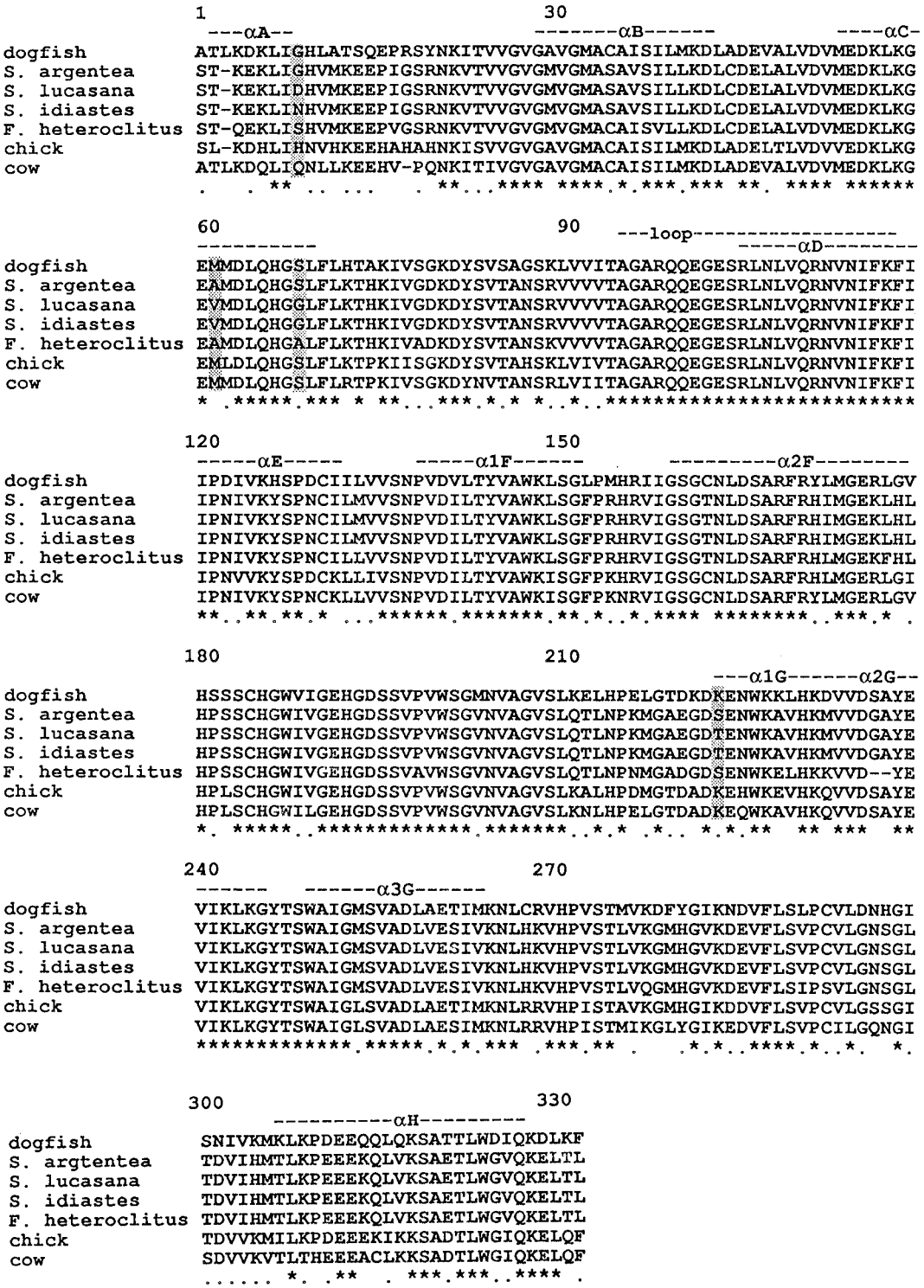

FIGURE 3: A comparison of the amino acid sequences of LDH-A homologs of the spiny dogfish (*Squalus acanthias*), three barracudas (*S. argentea*, *S. lucasana*, and *S. idiastes*), the killifish (*Fundulus heteroclitus*), the chicken, and cow. Residue numbering follows the revised and corrected dogfish sequence of Stock and Powers (1995), and reflects removal of the amino-terminal methionine residue. Because residue 3 in the dogfish sequence is absent in the barracuda sequences, residues 3 through 331 in the barracuda sequence correspond to residues 4 through 332 in the dogfish homolog. Positions 8 (9), 61 (62), 68 (69), and 223 (224), numbered according to the amino acid sequence of the barracuda LDH-A homologs, with dogfish LDH-A residues in parentheses, are shaded to denote sequence changes in the barracuda homologs. Dashed lines indicate the positions of the α-helices and the loop. Asterisks indicate identities; dots indicate similarities. Killifish sequence is from Quattro et al. (1995); chicken sequence is from Hirota et al. (1990); and cow sequence is from Ishiguro et al. (1990).

of pyruvate of the native LDH-A of *S. argentea* (0.369 ± 0.022; $p > 0.999$). We conclude, therefore, that the higher K_m of pyruvate for *S. argentea* LDH-A relative to the homolog of *S. lucasana* is due to substitutions either at site 61, at site 68, or both. In addition, we conclude that the substitution of Thr-223 (*S. idiastes* and *S. lucasana*) for Ser-223 (*S. argentea*) does not affect the K_m of pyruvate. Although the amino acid difference at position 8 between the *S. argentea* and *S. lucasana* homologs does not appear to contribute to the observed differences in the K_m of pyruvate, for the *S. idiastes* and *S. lucasana* enzymes amino acid 8 is the only position that differs. Therefore, we propose that the differences in K_m between the LDH-As of these two species are due to substitutions at position 8.

Table 2: K_m of Pyruvate at 25 °C of Native and Cloned LDH-As[a]

enzyme	K_m of pyruvate at 25 °C [mean ± SD (*n*)] (mM)
S. argentea, native	0.369 ± 0.022 (3)*
S. lucasana, native	0.258 ± 0.021 (3)**
S. lucasana, cloned	0.260 ± 0.008 (4)**
C-8	0.283 ± 0.021 (5)**
C-61−68	0.370 ± 0.020 (5)*

[a] K_m values ± SD (*n*) determined using 3−5 individual estimates of K_m, each of which was based on duplicate assays at 7 concentrations of pyruvate. Native refers to enzymes isolated from fish muscle. See text for definitions of cloned enzymes C-8 and C-61−68. K_m values denoted by a common symbol, * or **, are not significantly different from each other (analysis of variance and post hoc comparison of means by Tukey's test). See text for probability values.

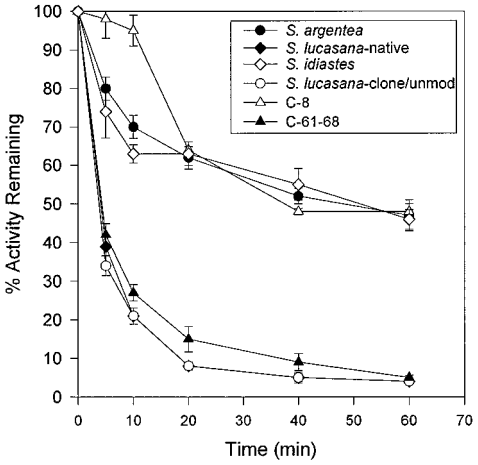

FIGURE 4: Heat denaturation at 45 °C of native (*S. argentea*, *S. idiastes*, and *S. lucasana*) and cloned LDH-As. Cloned LDH-As C-8 and C-61−68 are defined in the text. Error bars designate standard deviations ($n = 3$).

Position 8 differences may contribute to differences in thermal stability (Figure 4) as well as in K_m. The native LDH-As of *S. argentea* and *S. idiastes* are more resistant to heat denaturation than the homolog of *S. lucasana*, despite the higher average body temperature of the subtropical species (Table 1). Comparisons of heat denaturation of the native and cloned enzymes revealed the following. First, the cloned−unmodified *S. lucasana* LDH-A and native enzyme had indistinguishable denaturation profiles. Second, the thermal stability of the chimeric enzyme C-61−68 did not differ significantly from that of native or cloned−unmodified *S. lucasana* enzymes. Third, despite not influencing the K_m of pyruvate, substitution of a Gly for an Asp at position 8 in the *S. lucasana* sequence (C-8 enzyme) increased thermal stability. Therefore, we conclude that modifications in the K_m of pyruvate and thermal stability can occur independently.

Isoelectric Points. The p*I* values for all six LDH-A homologs are given in Table 1. Differences in p*I* between LDH-As of the subtropical species, *S. lucasana*, and the LDH-As of the two temperate species, *S. argentea* and *S. idiastes*, were consistent with the differences found in the deduced amino acid sequences (Table 2; Figure 3). Consistent with the sequence differences, the p*I* value of the LDH-A of *S. lucasana*, which has an Asp residue at position 8, is lower than that of the LDH-As of *S. argentea* and *S. idiastes*, which have Gly and Asn residues, respectively, at this position. There was no correlation between adaptation temperature and p*I*. Furthermore, as shown by Graves et al. (1983) in a comparison of several groups of teleost fishes, differences in electrophoretic mobility do not necessarily denote differences in kinetic properties among LDH-A homologs.

DISCUSSION

We used orthologous homologs of LDH-A from six species of barracuda fishes whose midrange body temperatures differ by approximately 5−8 °C, as well as two chimeric LDH-As constructed by site-directed mutagenesis, to investigate temperature adaptation of kinetic properties and thermal stability and their bases in the amino acid sequence. We found that species differences in the relationship of K_m *versus* temperature for LDH-As favor conservation of K_m for substrate and cofactor at physiological temperatures. These results agree with previous findings for dehydrogenase enzymes of other vertebrates and invertebrates (Graves & Somero, 1982; Coppes & Somero, 1990; Dahlhoff & Somero, 1993), and show that even relatively minor changes in temperature, such as those predicted by some models of global warming, appear adequate to favor selection for adaptive change in proteins.

Peptide Mapping as a Basis for Phylogenetic Inference. Although peptide mapping of the six LDH-A homologs was done principally to identify the homologs most appropriate for sequencing and site-directed mutagenesis experiments, the peptide maps shown in Figure 2 also are useful in the context of phylogenetic analysis. The use of tryptic maps of LDH-A homologs to infer phylogenetic relations of closely related fishes has been shown to be a more sensitive technique than conventional native gel electrophoresis of the enzymes (Wilson et al., 1990, 1991). Similar results have been obtained with peptide maps of avian myosin light chain kinases (Dalla Libera, 1993). Our peptide mapping results, in combination with amino acid sequencing, have shown that although such maps do not provide a precise measure of the number of amino acid differences between LDH-A homologs, they do provide an approximation of the phylogenetic relationships among congeners. The peptide maps shown in Figure 2 suggest that the two temperate and one subtropical barracudas are more closely related to each other than to any of the three tropical species, and that the subtropical species is most closely related to the south temperate species. This result, which is confirmed by cDNA sequencing of the LDH-As of the subtropical and temperate species, is in agreement with the relationships for four barracuda species previously determined using native enzyme electrophoresis (Graves & Somero, 1982).

Sequence Differences, Kinetic Properties, and Thermal Stabilities. Our results show that posttranslational modifications of the barracuda LDH-As are not responsible for the interspecific differences noted in K_m or thermal stability. Because the K_m of pyruvate and the thermal stability of the cloned−unmodified LDH-A of *S. lucasana* did not differ from those of the native enzyme isolated from skeletal muscle, the differences in kinetic properties and thermal stability among the barracuda LDH-A homologs seem likely to be due to differences in amino acid sequence.

A comparison of the sequences of the native and chimeric LDH-As of barracudas with those of other species (Figure 3) shows that amino acid residues in the active site cannot be responsible for the differences in K_m of pyruvate or NADH and thermal stability. LDH-As from a wide variety

of organisms have been sequenced, and crystallographic data for several species have clearly identified the regions of the enzyme involved in the catalytic process (Abad-Zapatero et al., 1987). These regions include both the residues involved in substrate and cofactor binding and the loop region which undergoes a large change in conformation during ligand binding/release. Our finding that the loop regions (residues 95−119; Gerstein & Chothia, 1991) of the three barracuda LDH-As are identical to each other and to those of LDH-As from other vertebrates, including the dogfish (Abad-Zapatero et al., 1987; Stock & Powers, 1995), the teleost fishes *F. heteroclitus* (Quattro et al., 1995) and *Sebastolobus alascanus* (Holland and Somero, unpublished data), chicken, and mammals (Ishiguro et al., 1990; Gerstein & Chothia, 1991), shows that the residues responsible for temperature adaptation of LDH-As do not reside in the loop region. Furthermore, the region involved in substrate binding (residues 157−171; Ishiguro et al., 1990) is also highly, although not totally, conserved among all vertebrate LDH-As (Figure 3; Crawford et al., 1989). The identity of this region among the three barracuda LDH-As shows that differences in the K_m of pyruvate and NADH cannot be due to differences in the types of residues that directly interact with substrate and cofactor. Therefore, differences in the K_m among the barracuda homologs must be due to substitutions that occur outside of the loop and ligand binding regions that are able to transmit their effects to the catalytic function of the enzyme.

Our ability to alter the K_m of pyruvate and thermal stability using site-directed mutagenesis to substitute one or two amino acids at a time in the *S. lucasana* LDH-A provides a strong basis for concluding that very minor changes in sequence at sites remote from the catalytic site or loop region can effect significant changes in kinetic properties and structural stability. We show that only one or two changes in sequence are adequate to modify either the K_m of pyruvate or the thermal stability. Our results further show that while in some instances the changes that alter thermal stability can also modify K_m, in others a substitution can modify one trait and not the other. For example, because the only difference in amino acid sequence between the LDH-As of the two most closely-related species, *S. lucasana* and *S. idiastes*, is at position 8—an Asp occurs in the former species and an Asn in the latter—this substitution must be responsible for the differences both in K_m and in thermal stability between these two homologs. Our site-directed mutagenesis experiments also point to position 8 as being primarily responsible for differences in thermal stability between the homologs of *S. argentea* and *S. lucasana* because the mutation of residue 8 from Asp to Gly in the C-8 clone of the *S. lucasana* enzyme increased thermal stability. In contrast, the differences in the K_m of pyruvate between the *S. argentea* and *S. lucasana* homologs cannot be explained solely on the basis of differences in sequence at position 8. The K_m of pyruvate of the C-8 clone did not differ from that of the native LDH-A of *S. lucasana* (Table 2). However, when Ala-61 and Ser-68 were replaced by Val-61 and Gly-68 (clone C-61−68), the K_m of pyruvate increased to a value indistinguishable from that of the LDH-A of *S. argentea*. Note, however, that values of the K_m of pyruvate for the homologs of *S. argentea* and *S. idiastes* are the same despite differences at sites 61 and 68. Therefore, we conclude that the differences in the K_m of pyruvate between the LDH-As of *S. argentea* and *S. lucasana* must involve effects at sites 8 plus 61 and/or 68.

Some insights into the mechanisms by which amino acid substitutions at sites remote from the catalytic site and loop region can modify kinetic properties and thermal stabilities can be gleaned from work with other LDH homologs of animals and bacteria. These studies suggest two possible mechanisms whereby changes in amino acids not directly involved in substrate or cofactor binding could alter kinetic properties and/or thermal stability. First, as shown by studies of Nobbs et al. (1994) with LDH of *Bacillus stearothermophilus*, amino acid substitutions distant from the catalytic site can change both thermal stability and catalytic efficiency by modifying the steric or charge properties of the catalytic site. Second, amino acid substitutions distant from the catalytic site can alter the energy changes associated with movement of the loop, which can involve displacements of amino acid residues by as much as 15 Å (Gerstein & Chothia, 1991). These energy changes determine the size of the catalytic rate constant, k_{cat} (Holbrook & Gutfreund, 1973). Because the LDH-A homologs of *S. argentea*, *S. lucasana*, and *S. ensis* differ in k_{cat} as well as K_m (Graves & Somero, 1982), we hypothesize that some or all of the amino acid substitutions that distinguish the LDH-As of these barracudas affect the energy changes associated with loop movement.

A suggestion as to how certain of the differences in amino acid sequence among the LDH-As of the barracudas might affect thermal stability can be obtained from the three-dimensional structure of the dogfish enzyme (Holbrook et al., 1975; Abad-Zapatero et al., 1987). On the basis of the structural analysis of the dogfish homolog, residue 8 in the barracuda LDH-As is proposed to lie immediately C-terminal to the short N-terminal helix, α-A, which interacts with as many as five residues along the *R*-axis of the tetramer and stabilizes intersubunit interactions (Holbrook et al., 1975). Among the residues with which a site-8 residue would interact in the three barracuda homologs is Asp-301. For LDH-A of *S. lucasana*, the Asp present at position 8 would likely result in charge repulsion, thus weakening intersubunit interactions. This effect might account for the reduced thermal stability of the LDH-A of *S. lucasana* relative to homologs of *S. argentea* and *S. idiastes*, in which Gly and Asn residues, respectively, are present at position 8. This conjecture is supported by our observation that the thermal stability of LDH-A of *S. lucasana* is increased when the Asp at position 8 is replaced with a Gly (clone C-8).

The differences in K_m among the barracudas' LDH-A homologs are less easily explained in structural terms. Because the only difference in the deduced sequences of the homologs of *S. lucasana* and *S. idiastes* is at position 8, where Asn and Asp residues are found, respectively, we conclude that changes in strengths of intersubunit interactions may translate into changes in the K_m of pyruvate, the K_m of NADH, k_{cat}, and thermal stability. However, our site-directed mutagenesis studies show that substitutions at positions 61 and/or 68 may also influence the K_m of pyruvate (Table 2). These positions lie within α-helix C (α-C), which is involved in intersubunit interactions along the Q axis of the enzyme (Abad-Zapatero et al., 1987). α-C is located in the static core of the enzyme which undergoes very little displacement (approximately 0.33 Å) during formation of the ternary complex of dogfish LDH-A (Gerstein & Chotia, 1991). This displacement is over an order of magnitude less than the displacements associated with movements of the loop. Although this helix lies in a region of the molecule that exhibits minimal movement during binding and catalysis,

our finding that substitutions at positions 61 and/or 68 can affect the K_m of pyruvate suggests that even relatively static regions of LDH-A can either influence the energy changes associated with catalytically important conformational changes or influence the stereochemical and/or charge characteristics of the ternary complex (Nobbs et al., 1994).

It is difficult to predict the exact effects of the Ala to Val and Ser to Gly shifts found at positions 61 and 68, respectively. Matthews et al. (1987) have shown that replacement of a Gly by an Ala within a helix can enhance thermal stability. Shifts to Val or Ser could stabilize the helix due to hydrophobic effects, but the addition of these larger side chains relative to Ala and Gly could disrupt packing of the folded protein. Because we observed no significant differences between the thermal stabilities of the unmodified LDH-A of *S. lucasana* and the C-61−68 chimeric enzyme (Figure 4), the influences of the changes at positions 61 and 68 found on the K_m of pyruvate are not accompanied by any measurable change in enzyme stability. Whatever the mechanism(s) whereby changes in sequence of relatively static regions of an enzyme influence its kinetic properties or stability, the key point to emphasize is that changes in these regions can be of importance in evolutionary adaptation to temperature.

The near-identity of the interactions between LDH active site residues, substrate, and cofactor in prokaryotic and eukaryotic LDHs (Deng et al., 1994) indicates that the geometry of the active site in the ternary complex differs little among LDH homologs. The conservation of active site sequence and geometry suggests that evolutionary alterations in the flexibility of the molecule, notably changes that affect movement of the loop region, may play a key role in establishing interspecific differences in the kinetic and stability properties of LDHs. Although it remains conjectural as to how substitutions at regions remote from the active site transmit their effects to binding, catalytic, and stability properties of the enzymes, we believe that our data support the hypothesis of Deng et al. (1994) that the so-called "active site" of LDH-A must be viewed as an "extended unit" that involves much, and possibly most, of the enzyme's structure.

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