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Function of muscle-type lactate dehydrogenase and citrate synthase of the Galápagos marine iguana, *Amblyrhynchus cristatus*, in relation to temperature

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Abstract

The Galápagos marine iguana, *Amblyrhynchus cristatus*, is unique among lizards in foraging subtidally, leading to activity across a broad range of ambient temperatures (~14–40 °C). To determine whether the marine iguana shows any biochemical changes consistent with maintaining enzyme function at both warm and cold body temperatures, we examined the function of the aerobic enzyme citrate synthase (CS) and the muscle isoform of the anaerobic enzyme lactate dehydrogenase (A_4-LDH) in *A. cristatus* and a confamilial species, *Iguana iguana*, from 14 to 46 °C. We also deduced amino acid sequences from cDNA of each enzyme. In CS, despite two amino acid substitutions, we found no difference in the apparent Michaelis–Menten constant K_m of oxaloacetate at any temperature, indicating that the substrate affinity of CS in *A. cristatus* has not adapted to changes in thermal environment. In A_4-LDH, we used site-directed mutagenesis to show that the substitutions T9A and I283V (*A. cristatus* → *I. iguana*) individually have no effect on kinetics, but together significantly decrease the K_m of pyruvate and catalytic rate constant (k_{cat}) of the *A. cristatus* ortholog. Thus, our data show that *A. cristatus* A_4-LDH has not become cold adapted in response to this species' aquatic foraging behavior, and instead may be consistent with moderate warm adaptation with respect to the *I. iguana* ortholog.

Keywords: A_4-lactate dehydrogenase; *Amblyrhynchus cristatus*; Citrate synthase; *Iguana iguana*; Temperature

1. Introduction

The marine iguana, *Amblyrhynchus cristatus* (Bell), is endemic to the Galápagos archipelago and has a foraging strategy unique among lizards. Although it spends over 95% of its time on land, the marine iguana feeds exclusively on macrophytic marine algae growing along the rocky shores (Wikelski et al., 1997). Smaller iguanas feed intertidally, but larger iguanas will leave the shore to forage subtidally (Trillmich and Trillmich, 1986). In so doing, iguanas have been observed to swim for distances of 800 m, to dive 12 m or more (Hobson, 1965), and to remain submerged for 30 min or more (Bartholomew and Lasiewski, 1965; Wikelski and Trillmich, 1994), although median dive durations are much shorter (Trillmich and Trillmich, 1986). Perhaps the most remarkable aspect of this foraging strategy is that marine iguanas can experience decreases of 20 °C or more in body temperature when transitioning from land to sea.

The Galápagos archipelago is made up of volcanic islands straddling the equator (89–92 w. lat.), and the intense insolation can heat the lava surfaces to 60 °C (White, 1973). Air temperatures during the day often exceed 40 °C, and when on land iguanas carefully control body position to avoid overheating, maintaining a preferred body temperature between 35 and 37 °C (Bartholomew, 1966; White, 1973). In comparison, Bartholomew (1966) showed that the upper lethal temperature for the species is 46 °C (based on data from a single tethered individual), suggesting the need for constant behavioral thermoregulation during the day to avoid overheating.

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Despite the equatorial location of the Galápagos, however, the surface waters surrounding the islands can be relatively cool. The combination of the powerful Humboldt Current, which transports colder water from high latitudes northward along the west coast of South America, and strong upwelling around the archipelago, leads to nutrient-rich waters with temperatures as low as 14 °C (Wikelski and Trillmich, 1994; Houvenaghel, 1978).

A volcanic archipelago has existed over the Galápagos hot spot for at least 17 million years, and there may have been a continuous succession of islands for the past 95 million years (Werner and Hoernle, 2003). This time span appears sufficient to have allowed *Amblyrhynchus* to diverge from its closest relative, the Galápagos land iguana, *Conolophus pallidus* (Rassmann, 1997), as well as to develop behavioral, morphological and physiological adaptations to its aquatic foraging lifestyle. A number of researchers have argued that the marine iguana indeed exhibits such adaptations – morphological differences between *Amblyrhynchus* and other iguanas putatively adapting the marine iguana to swimming and intertidal or subtidal foraging include a dorsoventrally flattened tail, webbed digits, short snout, and long and powerful claws for gripping rocks (e.g., Darwin, 1839; Tracy and Christian, 1985). Potential physiological adaptations include a well-developed nasal salt gland (Schmidt-Nielsen and Fange, 1958), and a pronounced diving bradycardia and peripheral vasoconstriction (Bartholomew and Lasiewski, 1965; Morgareidge and White, 1969). Note, however, that the reported bradycardia was induced in restrained animals, which likely led to artificially high reductions in heart rate compared to freely diving individuals. In contrast to the above studies, Dawson et al. (1977) have argued forcefully that the marine iguana is not especially adapted to a semi-aquatic lifestyle, and that the putative adaptations described above (excepting the nasal salt gland) are within the range of characteristics found in other iguanid lizards.

To the extent that the marine iguana is adapted to aquatic foraging, it seems likely that changes in temperature sensitivity would play an important role. During foraging at low temperatures marine iguanas must maintain the ability to swim and to overcome heavy surf (Bennett et al., 1975), as well as to dive against their own positive buoyancy (their lungs remain filled during dives)(Bartholomew et al., 1976) and grip the rocks to maintain position while feeding (Wikelski and Trillmich, 1994). At the high temperatures experienced on land activity must be maintained to ensure reproductive success (Wikelski and Trillmich, 1994): males must be able to defend territories necessary for maintaining access to a harem (Rauch, 1985), and females must be able to avoid "satellite" males (Wikelski et al., 1996) and dig nests in which to deposit eggs (Bartholomew, 1966; Butler et al., 2002). Thus inactivity at either temperature extreme is not a viable strategy. Furthermore, despite the bradycardia and vasoconstriction described in submerged *Amblyrhynchus*, a number of studies have shown that core body temperatures of subtidally foraging iguanas quickly approach the temperature of the surrounding water (Mackay, 1964; Bartholomew, 1966; Trillmich and Trillmich, 1986). Further, Buttemer and Dawson (1993) concluded that larger, subtidally foraging iguanas time dives to maximize warming rates upon return to land, rather than to minimize cooling *per se* (see also Shepherd and Hawkes, 2005). It appears, therefore, that marine iguanas must maintain appreciable metabolic scope across the wide range of body temperatures, approximately 14–40 °C, that they may experience in their habitat.

In this study, to better describe the degree of adaptation of *A. cristatus* to inter- and subtidal foraging, we have explored differences in temperature sensitivity at the biochemical level between *A. cristatus* and the green iguana, *Iguana iguana* (L.). The green iguana has a preferred temperature range similar to that of *A. cristatus* (~33–38 °C; Van Marken Lichtenbelt et al., 1996; Troyer, 1987), as well as a comparable upper lethal temperature (~46 °C, based on observations of one individual; Brattstrom, 1965). *I. iguana* will bask during the day to maintain its preferred temperature, but rarely experiences the low temperatures *A. cristatus* does when actively diving: nocturnal body temperatures of *I. iguana*, measured on sleeping individuals, appear to correspond with the ambient temperature of their tropical habitats, with reported minima of approximately 24 °C (Van Marken Lichtenbelt et al., 1996; Brattstrom, 1965; Hirth, 1963). Thus it appears that *A. cristatus*, despite having a similar preferred temperature to *I. iguana*, remains active at both lower and higher environmental temperatures.

To compare temperature sensitivity at the biochemical level in these two confamilial species, we examined the orthologous enzymes (orthologs) citrate synthase (CS; EC 4.1.3.7), which is associated with aerobic metabolism, and muscle-type lactate dehydrogenase (A_4-LDH; EC 1.1.1.27), which is central to anaerobic metabolism. We also deduced the amino acid sequences of these orthologs in each species, and used site-directed mutagenesis of the *Amblyrhynchus* A_4-LDH ortholog to determine whether structural differences between the A_4-LDHs of the two species are responsible for differences in temperature-related function that we have found.

2. Materials and methods

2.1. Specimen collection

I. iguana samples were collected from specimens of a feral population in Florida, USA, kindly provided by Dr. Martin Wikelski under Princeton University's IACUC protocol. Leg muscle of *A. cristatus* was collected by one of us (M.A.M.) in December 2002 under Louisiana State University surgical IACUC protocol (02-095) and was exported under Dr. Wikelski's CITES permit. Because of the conditions in which samples were collected, tissue could not immediately be frozen. Thus, enzyme activity comparisons between species are not possible. Samples were shipped to Franklin and Marshall College on dry ice and stored at −80 °C.

2.2. Sequencing of ldh-a and cs

Total RNA was purified from approximately 1 g of skeletal muscle tissue of a single individual from each species, using

phenol/chloroform extraction (Trizol reagent; Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. To obtain full-length sequences of the coding region of each transcript, purified total RNA was used as template for a Rapid Amplification of cDNA Ends (RACE) procedure (GeneRacer, Invitrogen), which inserts oligonucleotides of known sequence on the 5′ and 3′ ends of mRNA prior to reverse transcription. The resulting RACE cDNA was then used as template to amplify *ldh-a* or *cs* employing 5′ or 3′ RACE primers provided in the GeneRacer kit. For *ldh-a*, gene-specific primers were designed by homology with *ldh-a* from two iguanids, *Sceloporus undulatus* (GenBank accession no. U28410) and *Dipsosaurus dorsalis* (DQ793218) (forward primer: 5′-TTGTGCAGTTGGCATGGCTTGTG-3′; forward nested primer: 5′-ATGTAGTCAAGTACAGCCCTGAC-3′; reverse primer: 5′-TGTAATTCCTGAGTAGCCCAGG-3′; reverse nested primer: 5′-TTCAGCTTGATAACCTCATAGG-3′). For *cs*, rat, mouse and *C. elegans* sequences (GenBank accession nos. NM_130755, BC029754.1, and NM_066863.1, respectively) were used to design gene-specific primers (forward primer: 5′-GGAACAAGCAAGAATTAAAAG-3′; forward nested primer: 5′-GTTTCCGTGGCTACAGCATTC-3′; reverse primer: 5′-TTTCCAGAGGGAAGCAAGGG-3′; reverse nested primer: 5′-AGCTTGAAGAGAGGATCTTTGG-3′).

Polymerase chain reaction (94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 56–64 °C for 45 s, and 72 °C for 1–2.5 min, with a final 10 min extension at 72 °C) was used to amplify cDNA, and products were examined using 1.2% agarose–ethidium bromide gels. Products showing a single band of the expected size were purified with exonuclease I and shrimp alkaline phosphatase (USB; Cleveland, OH, USA) for 30 min at 37 °C; followed by inactivation at 80 °C for 15 min. Sequencing was performed using the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of the reaction products were determined on an ABI 3700 automated DNA analyzer, and contiguous sequences based on the resultant electropherograms were assembled using Sequencher software (GeneCodes Corp., Ann Arbor, MI, USA). Sequences were aligned using ClustalX (Thompson et al., 1997).

2.3. Cloning and expression of A_4-LDH

Expression of recombinant A_4-LDH was necessary to perform mutagenesis and subsequent kinetic analysis of the *ldh-a* gene. Full-length cDNA clones of *ldh-a* cDNA from *A. cristatus* and *I. iguana* were constructed using standard molecular biology techniques. Primers were designed to insert a BamHI restriction site immediately 5′ to the start codon (for *A. cristatus*: 5′-CCGTAAGGATCCAATGTCTCTCAAGGAGAAGCTGATCACAAATGTTCACAAAGAA-3′; for *I. iguana*: 5′-CCGTAAGGATCCAATGTCTCTCAAGGAGAAGCTGATCGCAAATGTCCACAAAGAA-3′) and an EcoRI site immediately 3′ to the stop codon (for both species: 5′-GGATCCGAATTCCAGGCTTAAAACTGAAGCTCCTTCTGGATTCCCCAGAGTGT-3′). Each gene was inserted into the pTrcHis vector (Invitrogen), creating a construct in which the expressed protein has a poly-histidine sequence attached to the N-terminus of the protein monomer to facilitate purification. After ligation, pTrcHis vector containing the *ldh-a* gene was transformed into TOP10 chemically competent cells (Invitrogen) and plated on LB-agar containing 80 μg mL^{-1} ampicillin. Plasmids were purified from individual colonies grown in 5 mL liquid LB/ampicillin (QiaQuick plasmid miniprep; Qiagen; Valencia, CA, USA), to confirm the presence of the desired gene by automated DNA sequencing. To express protein, 125 mL of LB/ampicillin was inoculated and grown to log phase. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mmol L^{-1}, and cells were allowed to express protein overnight. To purify the recombinant A_4-LDH, cells were centrifuged at 5000 g for 10 min, and the pellet was resuspended in 5 mL CellLytic B (Sigma–Aldrich, St. Louis, MO, USA) per g wet cell paste. Extraction was performed according to the manufacturer's instructions, and the final supernatant was filtered (0.22 μm). The supernatant then was passed over a 1 mL TALON cobalt poly-histidine affinity column (BD Biosciences; San Jose, CA, USA). After the TALON column was washed (25 mL of 50 mmol L^{-1} potassium phosphate, pH 7.0, 300 mmol L^{-1} NaCl), the purified A_4-LDH was eluted with 3.5 mL wash buffer plus 150 mmol L^{-1} imidazole. Fractions with highest enzyme activity were pooled and desalted into storage buffer (50 mmol L^{-1} potassium phosphate, pH 6.8) using PD-10 desalting columns (GE Healthcare; Piscataway, NJ, USA).

2.4. Site-directed mutagenesis of *A. cristatus* A_4-LDH

Site-directed mutagenesis was performed on *A. cristatus* A_4-LDH to examine the effects of the individual amino acid substitutions T9A and I283V on its kinetics. These mutations were chosen because they represent the only differences between *A. cristatus* and *I. iguana* LDH-A. Site-directed mutagenesis was not performed on CS because no differences were found in the kinetics of the CS orthologs of the two species.

Vector containing the *A. cristatus ldh-a* insert was mutated (QuikChange; Stratagene, La Jolla, CA, USA) using oligonucleotides containing the desired codon substitution, in order to create the T9A or the I283V mutant, as well as the double mutant. After mutation, plasmids were transformed into competent *E. coli*, and recombinant protein was expressed and purified as described above. The presence of the desired mutation was confirmed by automated DNA sequencing.

2.5. Measurement of CS K_m^{OAA}

To determine apparent Michaelis–Menten constants of CS for the substrate oxaloacetic acid (K_m^{OAA}), we homogenized approximately 1 g of muscle tissue (Ultra-Turrax T8, IKA Works, Staufen, Germany) in five volumes of 50 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged (20,000 g, 30 min, 4 °C), and after filtration (0.22 μm), the resulting supernatant was used to measure CS activity at 8 °C intervals from 14 to 46 °C. These assay temperatures were

chosen to span the thermal environment experienced by *A. cristatus* both in its terrestrial and marine habitats.

Substrate–saturation curves were generated at each temperature using a reaction cocktail of 50 mM imidazole-Cl (pH 8.2 at 20 °C), 1.5 mM $MgCl_2$, 40 μg mL^{-1} dithio-1,4-nitrobenzoic acid (DTNB), and 100 μg mL^{-1} acetyl coenzyme-A (all chemicals were obtained from Sigma–Aldrich). Imidazole was used as the buffer for the kinetics assays in this study because its pH–temperature relationship is similar to that of intracellular fluids, and maintains the appropriate protonation state of histidyl residues (alpha-stat regulation, Reeves, 1977; Yancey and Somero, 1978), thereby ensuring physiologically realistic enzyme function across a broad range of temperatures. To measure background rates of DTNB reduction, 1.98 mL of cocktail was added to a cuvette, which was temperature-equilibrated for 5 min. Ten μL of homogenate supernatant was added and background change in absorbance was monitored for 1 min at 412 nm in a GE Healthscience Ultrospec 2100pro UV-visible spectrophotometer equipped with a temperature-controlled (±0.1 °C), water-jacketed cuvette holder. The CS reaction was initiated with the addition of 10 μL of oxaloacetate at a final concentration of 0.8, 1.5, 2.5, 3.5, 5.0, or 8.0 μM. Activity was monitored at 412 nm for 2 min and data were corrected for background DTNB reduction rates. Three assays of each concentration of oxaloacetate were performed at each temperature. K_m^{OAA} values were determined from the change in absorbance at different oxaloacetate concentrations using a modified Lineweaver–Burk analysis (Brooks and Suelter, 1986) employing weighted linear regression (Wilkinson, 1961).

2.6. Measurements of A_4-LDH K_m^{PYR} and k_{cat}

Apparent Michaelis–Menten constants for the substrate pyruvate (K_m^{PYR}) were determined for A_4-LDH in tissue homogenate supernatant and for recombinant wild type and mutant *A. cristatus* A_4-LDHs from 14–46 °C. Native polyacrylamide gel electrophoresis followed by staining for LDH activity confirmed that only one isoform of LDH was present in the tissue preparation (data not shown). In addition, catalytic rate constants (k_{cat}) were determined for recombinant enzymes from 14–46 °C. Substrate–saturation curves were created by measuring reaction rate in triplicate in a reaction cocktail consisting of 80 mM imidazole-HCl (pH 7.0 at 20 °C), 150 μM NADH, and one of six pyruvate concentrations, 0.05, 0.1, 0.175, 0.25, 0.4 or 0.6 mM. The LDH reaction was started by adding 10 μL of tissue homogenate or purified recombinant A_4-LDH to 2 mL of temperature-equilibrated reaction cocktail, and reaction rate was determined by monitoring change in absorbance at 340 nm.

A modified Lineweaver–Burk analysis (Brooks and Suelter, 1986; Wilkinson, 1961) provided K_m^{PYR} and V_{max} values. Catalytic rate constants for recombinant A_4-LDHs were calculated using the equation $k_{cat} = V_{max}/[E]$, where $[E]$ is enzyme concentration. Catalytic rate constants could not be determined for enzyme from muscle tissue, because the concentration of active A_4-LDH could not be measured. Enzyme concentrations of the recombinant proteins were determined using a Bradford protein assay (Pierce Biochemicals; Rockford, IL, USA); purity of each recombinant enzyme was confirmed by SDS-PAGE, followed by silver staining (data not shown). The enzyme molecular weights used to calculate molarity included the N-terminal poly-histidine leader that was added to each recombinant protein.

2.7. Statistics

Assays for K_m and k_{cat} were run in triplicate, and results are reported as mean±S.D. For K_m comparisons, a two-way analysis of variance ($\alpha = 0.05$; MATLAB; Mathworks, Natick, MA, USA) was employed, using temperature as one main effect and species and enzyme source (recombinant or muscle tissue) as the other. The ANOVA was followed by a Tukey–Kramer multiple comparisons test. To determine whether k_{cat} values of the recombinant and mutant A_4-LDHs were significantly different, we used a one-way analysis of variance ($\alpha = 0.05$) followed by a Tukey–Kramer test.

2.8. Molecular modeling

We visualized the mutations at residues 9 and 283 within the three-dimensional structure of the *A. cristatus* LDH-A monomer by creating a homology model, using dogfish and pig apo-

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A.c.   MTLLTASSRAAARLLGAKNSSCIIFAARHASTSTNLKDVLANMIPKEQARIKSFRQQYGSTVIGQITVDMLYGGMRGMKGLIYETSVLDPDEGIRFRGYS  100
I.i.   MTLLTASSRAAARLLGAKNSSCIIFAARHASTSTNLKDVLANMIPKEQARIKSFRQQYGSTVIGQITVDMLYGGMRGMKGLIYETSVLDPDEGIRFRGYS

       IPECQKLLPKAPGGAEPLPEGLFWLLVTGEIPSQEQVNWVSREWAKRAALPSHVVTMLDNFPTNLHPMSQLSAAVTALNSESTFARAYSEGISRTKYWEF  200
       IPECQKLLPKAPGGAEPLPEGLFWLLVTGEIPSQEQVNWVSREWAKRAALPSHVVTMLDNFPTNLHPMSQLSAAVTALNSESTFARAYSEGISRTKYWEF

       IYEDSMDLIAKLPCIAAKIYRNLYREGSSIGAIDPALDWSHNFTNMLGYTDTQFIELMRLYLTIHSDHEGGNVSAHTSHLVGSALSDPYLAFAAAMNGLA  300
       IYEDSMDLIAKLPCIAAKIYRNLYREGSSIGAIDPALDWSHNFTNMLGYTDPQFIELMRLYLTIHSDHEGGNVSAHTSHLVGSALSDPYLAFAAAMNGLA

       GPLHGLANQEVLVWLTNLQKELGEDVSDQKLRDFIWNTLNSGRVVPGYGHAVLRKTDPRYTCQREFALKHLPKDPLFKLVAQLYKIVPNVLLEQGKAKNP  400
       GPLHGLANQEVLVWLTNLQKELGEDVSDQKLRDFIWNTLNSGRVVPGYGHAVLRKTDPRYTCQREFALKHLPKDPLFKLVAQLYKIVPNVLLEQGKAKNP

       WPNVDAHSGVLLQYYGMKEMNYYTVLFGVSRALGVLSQLIWSRALGFPLERPKSMSTDGLMVLVGAKSVL                                469
       WPNVDAHSGVLLQYYGMKEMNYYTVLFGVSRALGVLSQLIWSRALGFPLERPKSMSTDGLMVLVGAKSGL
```

Fig. 1. Amino acid sequences of CS orthologs from *Amblyrhynchus cristatus* (A.c.) and *Iguana iguana* (I.i.), deduced from *cs* cDNA sequences (data not shown; NCBI accession nos. DQ829807 and DQ829808, respectively). The two substitutions between the orthologs, T252P and V469G, are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

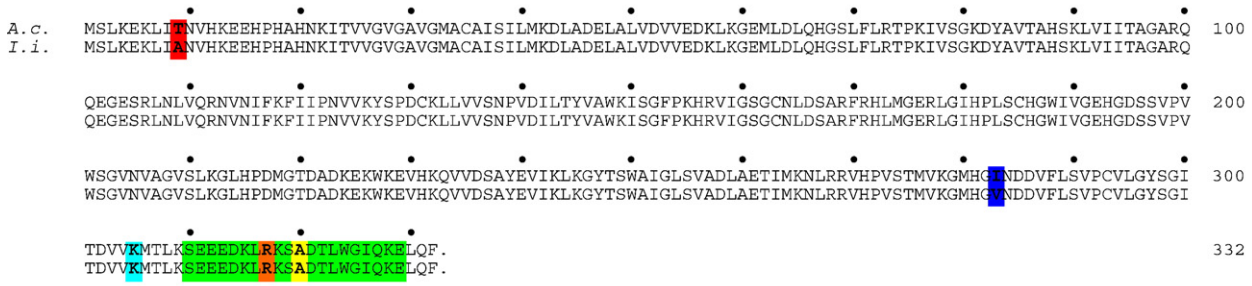

Fig. 2. Amino acid sequences of LDH-A orthologs from *Amblyrhynchus cristatus* (A.c.) and *Iguana iguana* (I.i.), deduced from *ldh-a* cDNA sequences (data not shown; NCBI accession nos. DQ829809 and DQ829810). The two substitutions between the orthologs, T9A and I283V, are highlighted in red and blue, respectively. Helix αH, whose mobility can affect substrate affinity and catalytic rate, is highlighted in green. Residues interacting with T9A (305 K, cyan) and I283V (317R, orange; and 320A, yellow) are also highlighted (see also Fig. 7). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

LDH-A (PDB accession numbers 6LDH and 9LDT, respectively) (Abad-Zapatero et al., 1987; Dunn et al., 1991) as templates onto which the primary structure of *A. cristatus* LDH-A was threaded. SwissModel software (Schwede et al., 2003; Guex and Pietsch, 1997) was used to create the model, and mutations were modeled using SwissPDBViewer (Guex and Pietsch, 1997). The resulting structures were visualized with SwissPDBViewer and VMD software (Humphrey et al., 1996).

3. Results

3.1. Deduced amino acid alignments of CS and LDH-A

The coding region of the *cs* gene comprises 1467 bp in both *A. cristatus* (GenBank accession no. DQ829807) and *I. iguana* (DQ829808), and the two genes differ at 32 nucleotide positions (97.8% identity). Thirty of these differences are synonymous, however, and the deduced amino acid sequences of the two 469-amino acid orthologs differ at only two residues, T252P and V469G (*A. cristatus* → *I. iguana*) (Fig. 1). The high degree of structural similarity between the proteins is unsurprising, considering the confamilial status of the two species.

The substitution of a glycine for valine occurs at the C-terminus of the molecule, and may be expected to have minimal impact on the structure and kinetics of CS. The mutation of the polar threonine to non-polar and relatively rigid proline at position 252 has a greater likelihood of modifying the structure and flexibility of the surrounding peptide chain. However, examination of this residue within the three-dimensional structure of the homologous chicken (*Gallus gallus*) CS monomer (PDB 5CTS; Karpusas et al., 1990; approximately 87% amino acid sequence identity with *A. cristatus* CS) indicates that position 252 is located on a solvent-exposed loop far from the active site. Given the minimal steric constraint at this site and its lack of interaction with structures involved in catalysis, we expected that a substitution here would have little impact on substrate binding.

The 999 bp *A. cristatus* and *I. iguana ldh-a* cDNA sequences (accession nos. DQ829809 and DQ829810, respectively) differ at 21 nucleotide positions (97.9% identity). Again, only two of these differences are nonsynonymous, leading to substitutions T9A and I283V between the *A. cristatus* and *I. iguana* LDH-A monomers (Fig. 2). As is discussed below, each of these mutations has the potential to impact the function of A_4-LDH through interactions with a mobile region near the active site, helix αH (Abad-Zapatero et al., 1987), which is highlighted in Fig. 2. The *I. iguana ldh-a* sequence reported here differs by two amino acids from a sequence that had been determined by other researchers earlier (Hsu and Li, unpublished; GenBank accession no. AY130429). A second mRNA isolation from the same *I. iguana* individual, followed by cDNA synthesis and sequencing, confirmed the accuracy of our *ldh-a* nucleotide sequence.

3.2. Temperature effects on kinetics of CS and A_4-LDH

We measured apparent Michaelis–Menten constants in CS and A_4-LDH of both *A. cristatus* and *I. iguana*, to determine how temperature affects function in these enzymes, and to determine whether the amino acid changes described above lead to functional differences in either pair of orthologs. Fig. 3 shows K_m^{OAA} values of both *A. cristatus* and *I. iguana* CS at five temperatures between 14 and 46 °C. A two-factor analysis of variance indicates that temperature has a significant effect on

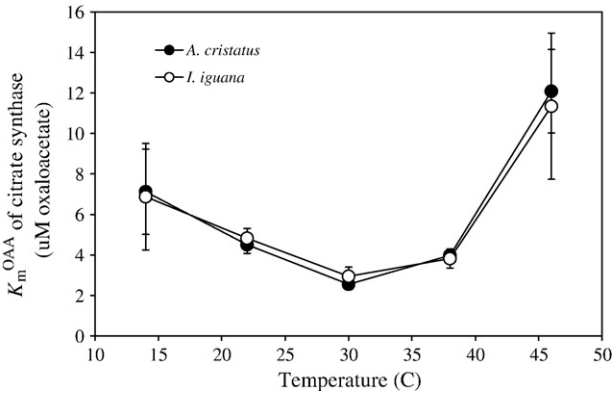

Fig. 3. Apparent Michaelis–Menten constants for the substrate oxaloacetate (K_m^{OAA}) of citrate synthase in leg muscle tissue homogenate supernatant of *A. cristatus* and *I. iguana*, measured from 14 to 46 °C. Error bars represent S.D. of three replicate measurements.

K_m^{OAA} in both orthologs ($P<<0.001$), and that K_m^{OAA} at 30 °C is significantly lower than values measured at 14 or 46 °C. However, there is no difference in K_m^{OAA} values between the CS of the two species at any of the experimental temperatures ($P=0.925$). From these results we conclude that, although CS is sensitive to temperature, changes in affinity for OAA have not occurred in *A. cristatus* CS during the time the marine iguana has been foraging inter- or subtidally at relatively low temperatures. Because we found no functional difference between the CS orthologs, we did not pursue site-directed mutagenesis to test the effects of the amino acid substitutions we had found in this enzyme.

In contrast to CS, K_m^{PYR} of A_4-LDH from *A. cristatus* is significantly different from the *I. iguana* isoform, whether recombinant A_4-LDH or A_4-LDH from muscle tissue homogenate is compared (Fig. 4). For recombinant A_4-LDH, at each temperature measured with the exception of the highest, 46 °C, the K_m^{PYR} of *A. cristatus* A_4-LDH is significantly lower (two-factor analysis of variance, $P<<0.01$). For K_m^{PYR} values measured directly from muscle tissue homogenate supernatant, the same pattern appears – *A. cristatus* A_4-LDH K_m^{PYR} is significantly lower than that of the *I. iguana* ortholog at all temperatures tested ($P<<0.01$). Both rWT and muscle tissue A_4-LDHs have comparable K_m^{PYR} profiles with respect to temperature, and the relationship between K_m^{PYR} values of the two species is similar regardless of the source of the enzyme; however, in each species rWT A_4-LDH has higher K_m^{PYR} values compared to the enzyme prepared from muscle tissue ($P<<0.01$). The relative increase in K_m^{PYR} values in rWT A_4-LDH may be due to the addition of the N-terminal poly-histidine leader in this enzyme preparation, or to stabilizing effects of covalent or non-covalent posttranslational modifications to the wild type enzyme. Regardless of the source of the difference in substrate affinity between rWT and muscle tissue enzymes, however, the results confirm that A_4-LDH from *I. iguana* has higher K_m^{PYR} values than that of *A. cristatus* across the temperature range experienced by *A. cristatus*. As discussed below, this relationship does not support the conclusion that *A. cristatus* A_4-LDH has become adapted to function at colder temperatures than the *I. iguana* ortholog.

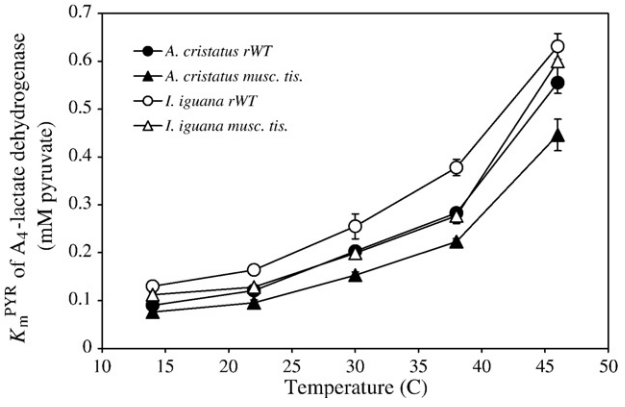

Fig. 4. Apparent Michaelis–Menten constants for the substrate pyruvate (K_m^{PYR}) of recombinant wild type (rWT) *A. cristatus* and *I. iguana* A_4-LDHs (circles), as well as A_4-LDHs in leg muscle tissue homogenate supernatant (musc. tis.) (triangles), measured from 14 to 46 °C. Error bars represent S.D. of three replicate measurements.

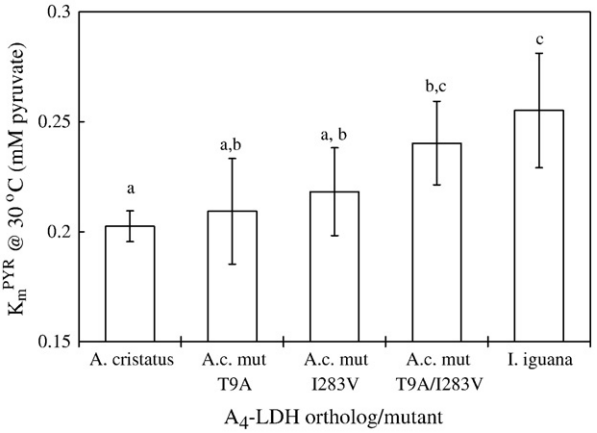

Fig. 5. Apparent Michaelis–Menten constants for pyruvate of recombinant A_4-LDHs from *A. cristatus* and *I. iguana*, as well as the mutants T9A, I283V, and the double mutant T9A/I283V of *A. cristatus* A_4-LDH, measured at 30 °C (mean ± S.D.; $n=3$). Columns that share a letter designation are not significantly different (single factor ANOVA, $\alpha=0.05$); all other comparisons are significantly different.

3.3. Effects of T9A and I283V mutations on A_4-LDH

To determine the effects of the T9A and I283V amino acid substitutions between the two A_4-LDH orthologs (each in the *A. cristatus* → *I. iguana* direction), we used site-directed mutagenesis to insert each mutation individually into recombinant *A. cristatus* A_4-LDH, and we also constructed the double mutant. We measured K_m^{PYR} of each mutant at 30 °C, a temperature within the thermal range of each species, and already shown to differ between the A_4-LDH orthologs (Fig. 4), to determine the effects of the two substitutions on substrate affinity. We confirmed that recombinant *I. iguana* A_4-LDH has a K_m^{PYR} significantly higher that that of *A. cristatus* (0.255 ± 0.026 vs. 0.203 ± 0.007 mM pyruvate; $n=3$ for all means) (Fig. 5).

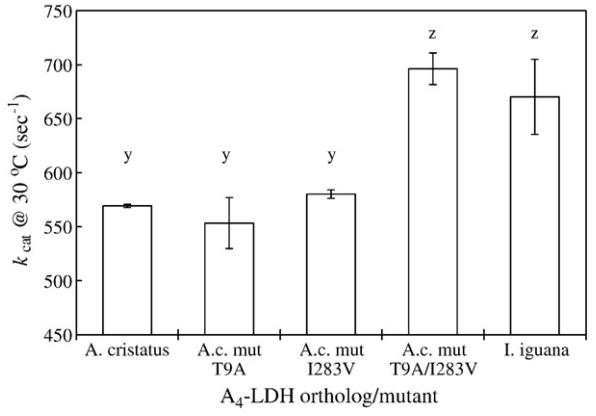

Fig. 6. Catalytic rate constants (k_{cat}) of recombinant A_4-LDHs from *A. cristatus* and *I. iguana*, as well as the mutants T9A, I283V, and the double mutant T9A/I283V of *A. cristatus* A_4-LDH, measured at 30 °C (mean ± S.D.; $n=3$). Columns that share a letter designation are not significantly different (single factor ANOVA, $\alpha=0.05$); all other comparisons are significantly different.

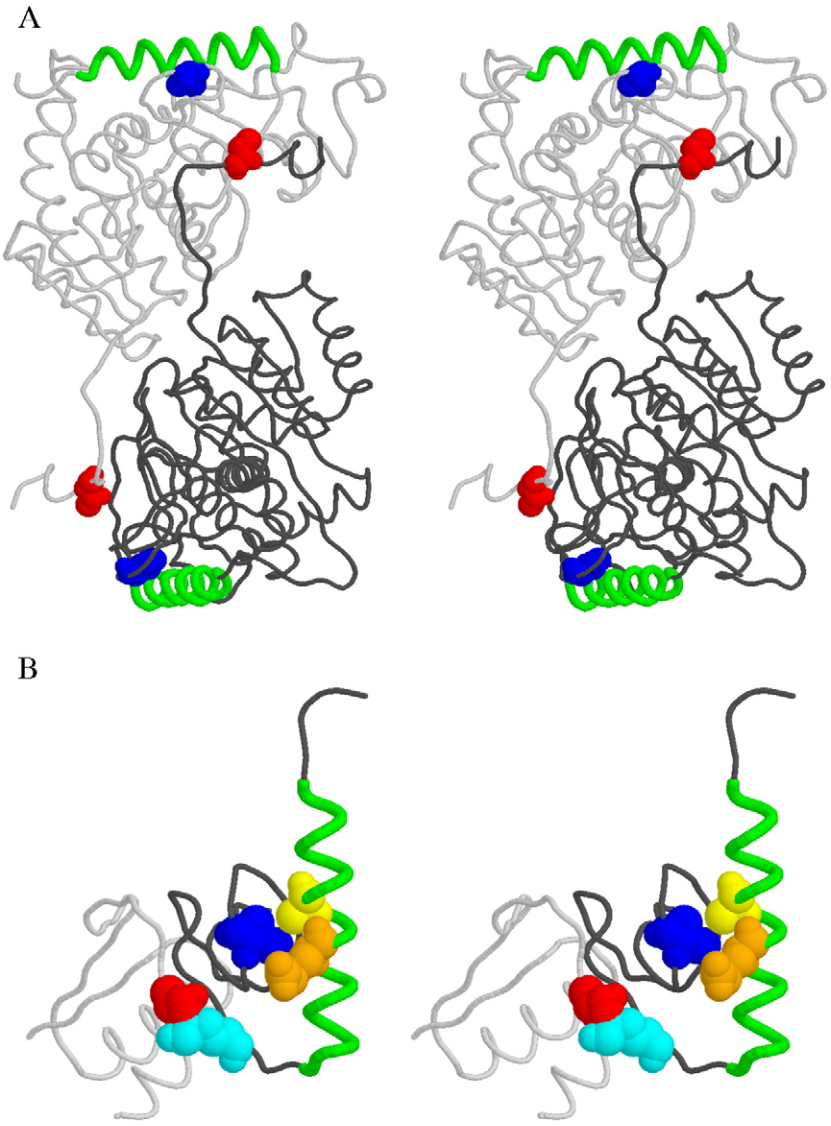

Fig. 7. (A) Stereo image of a three-dimensional model of two monomers of the homotetrameric *A. cristatus* A_4-LDH, based on homology modeling using pig and dogfish LDH-As as templates. The monomers are shown in light and dark gray, to illustrate the relationship of the subunits within the active homotetramer. The residues found to vary between *A. cristatus* and *I. iguana* are shown in red (T9A) or blue (I283V) spacefill. The mobile helix αH is highlighted in green. (B) A magnified view of the region in which residue 9 (red spacefill; threonine in *A. cristatus* and alanine in *I. iguana*) interacts with a lysine at position 305 (cyan) in the neighboring subunit, and residue 283 (blue; isoleucine in *A. cristatus* and valine in *I. iguana*) interacts with arginine 317 (orange) and alanine 320 (yellow) of the mobile helix αH (green) within the same subunit (see Fig. 2 and Discussion). Images were visualized with Rasmol software (Sayle and Milner-White, 1995).

We found that the K_m^{PYR} of the *A. cristatus* A_4-LDH mutant T9A (0.209±0.024 mM) is not significantly different than that of the wild type. The mutant I283V has a K_m^{PYR} (0.218± 0.020 mM) that is somewhat higher and intermediate between the *A. cristatus* and the *I. iguana* orthologs, but also is not significantly different than the *A. cristatus* enzyme. The T9A/I283V double mutant, in contrast, has a K_m^{PYR} at 30 °C (240.3± 0.019 mM) that is significantly higher than the *A. cristatus* ortholog, but is not significantly different from *I. iguana* A_4-LDH. These results suggest that there is a synergistic interaction between the two mutations, such that neither alone affects substrate affinity significantly, but in combination they reduce substrate affinity (i.e., increase K_m^{PYR}).

To further explore differences in function between the A_4-LDHs of *A. cristatus* and *I. iguana*, we measured k_{cat} values for each recombinant enzyme, as well as for the two single mutants and the double mutant, at 30 °C (Fig. 6). We found that the A_4-LDH of *I. iguana* has a significantly higher k_{cat} at this temperature (670.2±34.7 s^{-1}) than does the *A. cristatus* ortholog (569.3±1.3 s^{-1}). Both the T9A and the I283V mutants have k_{cat} values (553.3±23.6 and 580.1±3.9 s^{-1}, respectively) that are not significantly different from the recombinant *A. cristatus* enzyme. The double mutant T9A/I283V, however, has a significantly higher k_{cat} (696.2±14.6 s^{-1}), one that is statistically indistinguishable from the k_{cat} of the recombinant *I. iguana* ortholog. These results are analogous to our findings

for K_m^{PYR} (Fig. 5), where the *A. cristatus* wild type A_4-LDH and both single mutants show similar values, but the two mutations together act synergistically to produce a substrate affinity not significantly different from the *I. iguana* A_4-LDH.

3.4. Location of mutations within the three-dimensional structure of A_4-LDH

The results of our site-directed mutagenesis experiments indicate that the two substitutions between the *A. cristatus* and *I. iguana* A_4-LDHs, at positions 9 and 283, together cause the differences in K_m^{PYR} and k_{cat} we have found. To better understand the possible mechanisms by which these substitutions may affect substrate affinity and catalytic rate, we created a homology model of *A. cristatus* A_4-LDH to visualize the positions of these substitutions within the three-dimensional structure of the enzyme (Fig. 7).

Within the monomer, the conversion of polar threonine to non-polar alanine at position 9 is far from the active site on the N-terminal arm; however, this structure wraps around a neighboring subunit within the homotetramer, allowing position 9 to interact with helix αH on that subunit (Fig. 7A). Helix αH encloses one side of the active site (Abad-Zapatero et al., 1987), and, as discussed below, is integral to the movements associated with catalysis in A_4-LDH (Abad-Zapatero et al., 1987; Gerstein and Chothia, 1991). Residue 9 interacts with a residue, 305 K, located on a loop structure N-terminal to helix αH (Figs. 2 and 7B)(Gerstein and Chothia, 1991) that may help control the mobility of the helix.

The substitution of valine for isoleucine at position 283 is conservative – both side chains are non-polar – and so might be expected to impact enzyme function minimally. However, our three-dimensional model indicates that residue 283 interacts directly with two residues on helix αH as well, 317A and 320R (see Figs. 2 and 7B). Again, this interaction may provide a mechanism by which even minor changes at position 283 can affect substrate affinity and catalytic rate.

4. Discussion

Beginning with Darwin's observations in the Galápagos Islands (1839), investigators have remarked on the unusual behavior of the marine iguana, and speculated on possible adaptations of the species to its unique aquatic foraging strategy. While some have suggested that *A. cristatus* is morphologically and physiologically distinct from other iguanids, allowing more efficient swimming, diving, and temperature regulation (e.g., Darwin, 1839; Bartholomew and Lasiewski, 1965; Tracy and Christian, 1985), others have argued that attributes of the marine iguana usually supposed to enhance swimming and diving ability actually fall within the range found for other members of the family (Moberly, 1968; Bennett et al., 1975; Dawson et al., 1977). To explore this question further at the biochemical level, we have examined the function of two metabolic enzymes of *A. cristatus*, relating substrate affinity and catalytic rate to the cold temperatures in which this species forages. The data we present address two questions: Are orthologs of CS and A_4-LDH in *A. cristatus* and *I. iguana* poised to function optimally at different temperatures? And to what extent can the structural differences we have found between the A_4-LDH orthologs explain changes in K_m^{PYR} and k_{cat}?

4.1. Are A. cristatus enzymes poised for optimal function at low temperature?

If *A. cristatus* has adapted to remain highly active at the low temperatures of the marine environment, we might expect that the kinetics of central anaerobic or aerobic enzymes would show evidence of cold adaptation, specifically measured as increases in catalytic rate and decreases in substrate affinity at any measurement temperature (Hochachka and Somero, 2002). Instead, the data we present here suggest that neither CS nor A_4-LDH of *A. cristatus* function better at cold temperatures compared to the corresponding orthologs of the terrestrial *I. iguana*.

The K_m^{OAA} values of CS in the two iguanid species we compared are nearly identical from 14 to 46 °C (Fig. 3), indicating that a relative increase in substrate affinity has not occurred in the *A. cristatus* enzyme. In both orthologs substrate affinity does respond to temperature, with K_m^{OAA} at 30 °C less than half that found at 14 or 46 °C. This is noteworthy, because the aerobic scope of *A. cristatus* is maximal at 35 °C, near the species' preferred body temperature (Bennett et al., 1975), but aerobic scope decreases at both lower and higher temperatures, in a pattern analogous to what we have found for CS substrate affinity.

While 46 °C approximates the upper lethal temperature for both species (Bartholomew, 1966; Brattstrom, 1965), *A. cristatus* can forage actively in water at 14 °C (Wikelski and Trillmich, 1994). Furthermore, *A. cristatus* swims aerobically when traveling to and from subtidal foraging sites (Bennett et al., 1975), which indicates adequate CS activity even at low temperatures. Thus, although CS function is at least somewhat sensitive to temperature (Fig. 3), the lack of difference in K_m^{OAA} between the *A. cristatus* and *I. iguana* CS orthologs suggests that any loss of function at low temperature is not sufficient to induce selection to alter the substrate affinity of the enzyme.

A number of previous studies have shown that CS activity levels in ectotherms are sensitive to environmental temperature (e.g., Crockett and Sidell, 1990; Kawall et al., 2002), with increased activity found in those species occupying colder habitats. The results of these studies indicate that one possible mode of temperature adaptation in CS is through a modification in enzyme concentration, rather than in kinetic parameters. Unfortunately, due to the quality of *A. cristatus* tissue available for this study (our tissue came from an individual that was not immediately frozen after death), it was not possible for us to accurately determine fresh tissue CS activity levels. A further factor that may affect CS kinetics is allosteric regulation. Eukaryotic CS may be regulated by a number of metabolites, including ATP, ADP and NADH, and while it is unlikely that the concentration of these effectors would differ substantially in the tissues of the two iguanid species, it is possible that the CS homologs would be differentially susceptible to them. Because

the enzyme assays used here involved a 2500-fold dilution of tissue extract, we expect that the K_m^{OAA} values we measured were unaffected by allostery. Nevertheless, a more complete examination of changes in CS activity in *A. cristatus* with respect to terrestrial iguanids must await future studies.

In contrast to CS, we do find differences in K_m^{PYR} and k_{cat} between the A_4-LDH orthologs of *A. cristatus* and *I. iguana* (Figs. 4, 5, 6). However, the differences we have found are not consistent with cold adaptation in the *A. cristatus* ortholog. We base this statement on the results of earlier studies that have examined A_4-LDHs from ectothermic vertebrates across a broad range of environmental temperatures, and have established a strong pattern of increasing K_m^{PYR} at any measurement temperature as A_4-LDH orthologs from colder environments are examined (Hochachka and Somero, 2002). For example, A_4-LDHs from species experiencing widely different temperatures, such as Antarctic and South American notothenioid fishes (Fields and Somero, 1998), temperate barracudas (Graves and Somero, 1982; Holland et al., 1997), tropical and temperate damselfish (Johns and Somero, 2004), subtropical gobies (Fields and Somero, 1997) and desert iguanas (Fields, 2001) have been examined, and in each case, orthologs from species adapted to warmer environments have lower K_m^{PYR} values when measured at a common temperature (Fig. 8). However, when measurements are made within the physiological temperature range of each species, K_m^{PYR} values are similar in each of the A_4-LDH orthologs, indicating temperature compensation in substrate affinity. This pattern of temperature compensation has also been shown to extend to enzymes other than A_4-LDH, including cytosolic malate dehydrogenase (Dahlhoff and Somero, 1993; Fields et al., 2006) and α-amylase (D'Amico et al., 2001).

Combining our K_m^{PYR} results with a representative selection of these earlier findings (Fig. 8), it is clear that A_4-LDH of *A. cristatus*, like CS, shows no shift in substrate affinity that would indicate adaptation to the colder temperatures this species experiences when foraging in the ocean. Instead, the reduction in K_m^{PYR} of *A. cristatus* A_4-LDH relative to that of *I. iguana* – i.e., the right-shift of the K_m^{PYR} temperature profile of *A. cristatus* A_4-LDH (Fig. 8) – is consistent with adaptation to temperatures ~5–8 °C higher in the A_4-LDH of the marine iguana.

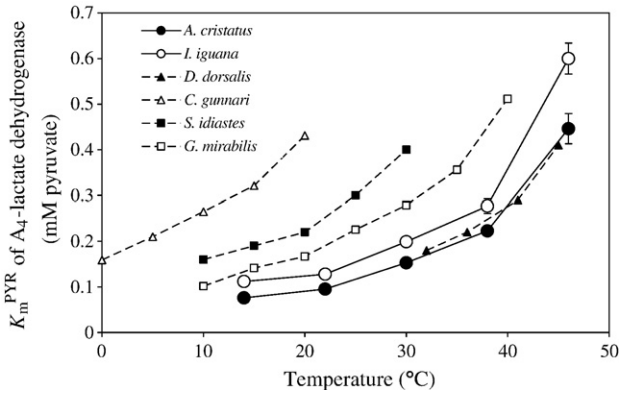

Fig. 8. Comparison of K_m^{PYR} of A_4-LDHs from ectothermic vertebrates, illustrating the relationship between substrate affinity and habitat temperature. *Amblyrhynchus cristatus* and *I. iguana* muscle tissue A_4-LDH data are from Fig. 4; other species include *Champsocephalus gunnari* (an Antarctic notothenioid teleost, maximum environmental temperature ~2 °C; Fields and Somero, 1998), the temperate barracuda *Sphyraena idiastes* (~22 °C; Holland et al., 1997), the warm temperate goby *Gillichthys mirabilis* (~30 °C; Fields and Somero, 1997) and the desert iguana *Dipsosaurus dorsalis* (~42 °C; Fields, 2001).

The k_{cat} data we have collected provide further evidence that A_4-LDH of *A. cristatus* is not cold adapted relative to the *I. iguana* form. As with K_m^{PYR}, the pattern we have found instead is consistent with function optimized to relatively warm temperatures. Generally, as habitat temperature increases, k_{cat} values of enzymes in ectotherms decrease (Fields and Somero, 1998; D'Amico et al., 2001; Hochachka and Somero, 2002; Fields et al., 2006), and here we have shown that the catalytic rate of the rWT *A. cristatus* ortholog is significantly lower than that of the *I. iguana* form at 30 °C (Fig. 6). The correlated decreases in catalytic rate and K_m found in warm-adapted orthologs have been argued to be a result of increased stability in these enzymes (Somero, 1995; Fields, 2001; D'Amico et al., 2002; Fields and Houseman, 2004). This is because function at higher temperatures is optimized by selection for greater stability in order to maintain an active site with appropriate geometry for binding (increasing substrate affinity), but the increased rigidity necessarily reduces the rate at which the catalytic cycle can progress (decreasing k_{cat}).

Although our A_4-LDH kinetics data are consistent with warm adaptation in the *A. cristatus* ortholog, we recognize that there are alternative hypotheses that may be equally consistent with these data. For example, the differences we have found may simply represent selectively neutral mutations that have accrued in the time since the two species diverged. In other words, it is possible that the K_m^{PYR} and k_{cat} differences in *A. cristatus* A_4-LDH relative to the *I. iguana* ortholog provide no functional advantage in either the aquatic or terrestrial environment. Although we were unable to obtain tissue from the Galápagos land iguana, *C. pallidus*, its A_4-LDH would provide a useful test of this question, since the two species share the same terrestrial habitat, but *C. pallidus* never experiences the cold temperatures of the sea. Thus, it would be valuable in a future study to compare the K_m^{PYR} and k_{cat} of *C. pallidus* A_4-LDH with data presented here for the *A. cristatus* and *I. iguana* orthologs, thereby helping to determine whether the shifts in kinetics of the marine iguana ortholog are selectively neutral or instead are indicative of adaptation to the warm terrestrial environment of the Galápagos. Of course, enzymes other than A_4-LDH and CS are equally likely to be under selective pressure to alter kinetics in response to changes in environmental temperature. Thus the data presented here do not preclude the possibility of thermal adaptation in other enzymes or biochemical pathways in *A. cristatus*.

Ultimately, temperature adaptation in the marine iguana is complicated by the trade-offs that individuals face both during different activities, and at different stages of their life histories. For example, younger, smaller individuals forage intertidally and are less likely to experience low body temperatures due to immersion in seawater, while larger males cool significantly upon each dive. Similarly, although high levels of activity at cold temperatures may

be advantageous during foraging, high activity levels at high temperatures likely are necessary for reproductive success. Thus selective pressures for thermal adaptation may be conflicting, with maintenance of activity in the warm terrestrial environment (where the iguanas spend most of their time) outweighing optimization of activity during subtidal foraging.

4.2. Structural correlates of altered K_m and k_{cat} in *A. cristatus* A_4-LDH

We have shown that there are statistically significant differences in K_m^{PYR} and k_{cat} between *A. cristatus* and *I. iguana* A_4-LDHs, and our site-directed mutagenesis experiments indicate that synergistic interactions between residues at positions 9 and 283 are responsible for these functional differences. A number of previous studies have related minor changes in A_4-LDH structure to changes in kinetics, and have found a small number of areas within the monomer where temperature-adaptive changes are most likely to occur. These areas include helices controlling access to the active site, including helix αH (see Fig. 7) (Fields and Somero, 1998; Fields and Houseman, 2004; Fields et al., 2006), and loops contacting these helices (Holland et al., 1997; Johns and Somero, 2004). The differences we have found in the structure of A_4-LDH from *A. cristatus* and *I. iguana*, T9A and I283V, fit into this pattern of temperature adaptation in A_4-LDH.

Residue 283 occurs within a loop that contacts helix αH (Fig. 7), a helix that must move outward to allow entry of pyruvate and NADH to the active site, and then inward to help form the catalytic vacuole (Dunn et al., 1991; Gerstein and Chothia, 1991). Tight interactions between residues on αH and neighboring structures help control the movement of the helix (Gerstein and Chothia, 1991), and therefore impact substrate binding and catalytic rate. While the substitution of isoleucine for valine at position 283 in *A. cristatus* A_4-LDH is conservative – the two differ only by an extra methylene group in isoleucine – the increased efficiency of packing and hydrophobic interactions between 283I and residues 317R and 320A on helix αH may increase the stability and decrease the mobility of the latter, and thus reduce catalytic rate while increasing substrate affinity. A number of studies have shown that increased residue size, hydrophobicity and packing efficiency can enhance protein stability (Haney et al., 1999; Vieille and Zeikus, 2001), and it is the *A. cristatus* ortholog, which has K_m^{PYR} and k_{cat} values indicative of increased stability, that has the bulkier and more hydrophobic isoleucine residue. Further evidence in support of the importance of this region for the modification of A_4-LDH kinetics comes from previous studies of A_4-LDH orthologs from Antarctic notothenioid fishes. In these species, the substitution V317Q on helix αH, which closely interacts with position 283, significantly increased K_m^{PYR} and k_{cat} (Fields and Somero, 1998; Fields and Houseman, 2004). To determine whether the efficiency of packing and residue size indeed play a role in stabilizing helix αH in the iguana A_4-LDH, it may be possible in a future study to create an additional mutant, I283A. In this case, the relatively small hydrophobic alanyl residue would replace bulky isoleucine, and in response we would predict that helix αH would be destabilized to a greater extent than found in the 283V isoform. The expected result would be higher k_{cat} and K_m^{PYR} values than we have found in the *I. iguana* A_4-LDH (283V).

Residue 9 is much further from the active site in the LDH-A monomer, but in the active homotetramer this residue is part of a helix, αA, that abuts helix αH of a neighboring monomer (Abad-Zapatero et al., 1987) (see Fig. 7). In this case, the residue at position 9 interacts with a lysine residue at position 305 on the loop immediately N-terminal to helix αH, and this loop plays a role in controlling the position and movement of the helix. The terminal hydroxyl group of threonine found at position 9 in *A. cristatus* A_4-LDH potentially can hydrogen bond with the side chain amino group of 305 K, thereby stabilizing the loop region leading to helix αH. This potential hydrogen bonding is lost in *I. iguana* A_4-LDH, where an alanine occupies position 9. Interestingly, a previous study on A_4-LDHs of temperate barracudas (Holland et al., 1997) showed that temperature-adaptive differences in K_m^{PYR} between orthologs from a subtropical and a temperate species were caused by a single mutation at position 8, directly neighboring the residue 9 mutation we have found to affect kinetics in the two iguanid species. Thus we suggest that the differences in kinetics of A_4-LDH of *A. cristatus* and *I. iguana* with respect to temperature are due to changes in the stability and mobility of helix αH, mediated by substitutions at positions 9 and 283.

5. Conclusions

We have compared the function of CS and A_4-LDH between the Galápagos marine iguana, *A. cristatus*, and the green iguana, *I. iguana*. We have found that the K_m^{OAA} of CS, while temperature-sensitive, is identical between the two species from 14–46 °C. However, both K_m^{PYR} and k_{cat} of A_4-LDH are lower across this temperature range in the *A. cristatus* ortholog. These differences in A_4-LDH kinetics can be ascribed to two amino acid mutations between the orthologs, T9A and I283V, which appear to affect temperature sensitivity synergistically through interactions with a helix, αH, that must move during catalysis. The differences in kinetics between the two A_4-LDH orthologs are not consistent with adaptation to high activity levels at the cold temperatures *A. cristatus* experiences during swimming and diving, supporting the arguments of Dawson et al. (1977) that *A. cristatus* does not show specific adaptations to its unique marine foraging lifestyle. Determining whether the decrease in K_m^{PYR} and k_{cat} of *A. cristatus* A_4-LDH with respect to the *I. iguana* ortholog represents adaptation to warmer terrestrial temperatures or selectively neutral drift will require kinetics data from CS and A_4-LDH of more iguanid species, especially *C. pallidus.*

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samples was supported by NSF grant DEB 0545744 "Collaborative Research: Monitoring Stress and Survival in Galapagos Marine Iguanas" to M. Wikelski.

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