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Characterization of erythrocyte carbonic anhydrase in an ancient fish, the longnose gar (*Lepisosteus osseus*)

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Abstract This study investigates the evolutionary history of vertebrate red blood cell carbonic anhydrase (CA) by characterizing the isozyme properties and nucleotide sequence of an ancient fish, the longnose gar (*Lepisosteus osseus*). The inhibitor sensitivities of gar rbc CA closely resembled those for mammalian CA II, as well as those for CAs from more recently evolved fishes. The kinetic properties of gar rbc CA were not closely aligned with either mammalian CA I and CA II, but fit well into an emerging phylogenetic pattern for early vertebrates. Gar rbc CA cDNA was also amplified from mRNA using 5′ and 3′-RACE and the open reading frame consisted of 786 bp. This sequence shares approximately 65% identity with the nucleotide and amino acid sequences of both mammalian CA I and CA II. When the amino acid sequences within the active site are compared, gar rbc CA differs from mammalian CA I, CA II and CA VII by 9, 4 and 3 of the 36 amino acids, respectively. Phylogenetic analyses suggest that gar rbc CA diverged before the amniotic CAs (CA I, CA II and CA III), but after CA V and CA VII.

Keywords Carbonic anhydrase · Erythrocyte · Gar · *Lepisosteus osseus* · Molecular evolution

Abbreviations *Az* acetazolamide · *CA* carbonic anhydrase · *GTC* guanidium thiocyanate · *MP* maximum parsimony · *NJ* neighbor-joining · *RACE* rapid amplification of cDNA ends · *rbc* red blood cell

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Introduction

Carbonic anhydrase (CA) is a zinc-containing enzyme that catalyzes the reversible hydration and dehydration reactions of CO_2. Since it was first discovered in vertebrate erythrocytes (Brinkman et al. 1932), CA has been found in many different tissues and has been implicated in a wide variety of physiological and biochemical processes (Maren 1967; Sly and Hu 1995; Henry 1996; Chegwidden and Carter 2000). All CAs characterized in the animal kingdom belong to a single gene family referred to as the α-carbonic anhydrases (α-CAs; Hewett-Emmett and Tashian 1996). To date, 14 different α-CA isozymes have been discovered in mammals (Chegwidden and Carter 2000). These CA isozymes have been identified using a number of different approaches including determination of their unique kinetic and inhibitor properties, sub-cellular distribution and/or molecular structure (Maren and Sanyal 1983; Sanyal 1984; Henry et al. 1986, 1993, 1997; Hewett-Emmett and Tashian 1991). In comparison to the situation in mammals, much less is known about the CA isozymes in non-mammalian vertebrates. There is also very little information about the evolution of the different vertebrate CA isozymes.

In most vertebrates, erythrocyte CA plays an important role in blood CO_2 transport and excretion. Since erythrocyte CA is relatively easy to obtain for experimental purposes, it is also probably the most widely studied CA isozyme. The erythrocytes of many mammalian species have been found to possess both low activity (CA I) and high activity (CA II) CA isozymes. In contrast, most early vertebrates are thought to possess only one cytoplasmic CA isozyme within their erythrocytes (Carlsson et al. 1980; Maren et al. 1980; Sanyal et al. 1982; Hall and Schraer 1983; Kim et al. 1983). In agnathans and elasmobranchs, erythrocyte CA resembles mammalian CA I in terms of both activity and sensitivity to inhibitors (Carlsson et al. 1980; Maren et 7al. 1980; Henry et al. 1993). In more recently evolved

teleost fish, however, the kinetic and inhibitor properties of erythrocyte CA are more similar to the mammalian CA II isozyme (Maren et al. 1980; Hall and Shraer 1983; Kim et al. 1983; Henry et al. 1993; Henry and Swenson 2000). It would therefore appear that the slow type I CA isozyme is probably the most ancient erythrocyte isozyme and that it was eventually replaced by a faster type II isozyme after the appearance of the elasmobranchs and prior to the evolution of the teleosts. A recent investigation of the kinetic and inhibitor properties of erythrocyte CA in the bowfin also seems to support this phylogenetic trend (Gervais and Tufts 1999). At present, however, there is very limited information about the properties of erythrocyte CA from other ancient fishes that are phylogenetically intermediate between elasmobranchs and teleosts.

There is also a paucity of information about the molecular structures of erythrocyte CA from early vertebrates. To our knowledge, the only available information in this area includes a partial amino acid sequence for rbc CA from the tiger shark (Bergenhem and Carlsson 1990) and an unpublished mRNA sequence for rbc CA from the European flounder (C. Wright and A.R.C. Cossins; accession number AF093622). A partial cDNA CA sequence from the Atlantic salmon spleen (S.E. Douglas; accession number BG935995) and an mRNA sequence for CA from the zebrafish retina (Peterson et al. 1997) have also been determined, but it is not known whether these are the same CA isozymes that would be found within the rbcs of these species. Thus, the evolutionary relationships between the rbc CAs in early vertebrates and those of mammals have yet to be fully resolved.

On this background, the purpose of the present study was to determine the kinetic and inhibitor properties, as well as the molecular structure, of erythrocyte CA from the longnose gar. The longnose gar is an ancient species of fish, which is phylogenetically intermediate between the elasmobranchs and teleosts. This study will therefore provide important insights about the evolution of erythrocyte CA in vertebrates.

Materials and methods

Animal preparation and blood collection

Longnose gar, *Lepisosteus osseus*, were collected by commercial fishermen on the Bay of Quinte in southeastern Ontario. Gar were killed by a sharp blow to the head, and blood was collected by caudal puncture into a chilled round-bottom flask containing heparinized (40 iu ml^{-1}) saline (in $mmol^{-1}$: 124 NaCl, 10 $NaHCO_3$, 5.5 glucose, 5 KCl, 1.1 $CaCl_2$, 0.5 $MgCl_2$). The erythrocytes were then washed three times in saline, lysed in 50 volumes of distilled water, and frozen for later measurement of CA activity.

Measurement of erythrocyte CA activity

Erythrocyte CA activity was measured using the electrometric Δ pH method (Henry 1991; Henry et al. 1993; Gervais and Tufts 1998). The reaction medium consisted of 10 ml buffer (in mmol l^{-1}: 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.4 with 10% phosphoric acid) held at 4 °C by a circulating water bath attached to the reaction chamber. After the addition of the lysate, the reaction commenced with the addition of 400 µl CO_2 saturated distilled water (~1 °C), delivered from a 1000-µl gas-tight Hamilton syringe. The reaction was then measured over a pH change of approximately 0.15 units using a GK2401C combined electrode (Radiometer) attached to a PHM64 research pH meter (Radiometer). To obtain the true catalyzed reaction rate in the chamber, the uncatalyzed rate (no lysate) was subtracted from the observed rate (lysate present) and the buffer capacity was taken into account to convert from pH units $time^{-1}$ to mol H^+ (CO_2) $time^{-1}$. Buffer capacity of the reaction medium was determined by titrating the reaction buffer with known concentrations of strong acid (0.1 N HCl) in the absence of lysate.

Series I. Inhibition and kinetic properties of CA

In order to determine the inhibition properties of gar erythrocyte CA, samples of rbc lysates were titrated with increasing volumes of the CA inhibitors, acetazolamide (Az; 0.5 µmol l^{-1}), iodide (50 mmol l^{-1}) and copper (20 µmol l^{-1}). The inhibition constant (K_i) for Az was calculated as the slope of the line with the following equation:

$$I_o/i = K_i/(1 - i) + E_o$$

where E_o is the total concentration of free enzyme in the reaction chamber, I_o is the concentration of inhibitor, and i is the fractional inhibition of enzyme activity at a given inhibitor concentration (Easson and Stedman 1937; Maren et al. 1960). The inhibition constants for copper and iodide were determined from a Dixon plot, where inhibitor concentration (i) is plotted against the reciprocal of enzyme activity (v^{-1}; Dixon 1953). The variable K_i is taken as the negative of the i intercept. For comparison purposes, inhibition constants for the mammalian isozymes, human CA I and bovine CA II (Sigma), were also determined under identical experimental conditions. It is important to note, however, that statistical analyses between the results for these mammalian isozymes and gar rbc CA are inappropriate because the mammalian isozymes were obtained from a single source.

In order to determine the substrate affinity (K_m) of erythrocyte CA, its activity was measured against increasing concentrations of CO_2 and then fitted to a Lineweaver-Burk plot (Maren et al. 1980; Henry et al. 1993). The active site turnover rate, K_{cat}, was then determined from the relationship V_{max}/E_o, where V_{max} is the reciprocal of the y-intercept on the Lineweaver-Burk plot (Maren et al. 1980). E_o was also used to estimate the concentration of CA in the erythrocytes. These analyses were also performed on mammalian CA I and CA II (Sigma) in order to provide a basis of comparison under identical experimental conditions.

Series II. Determination of cDNA sequence for gar rbc CA

Total RNA was extracted by the acid/phenol method of Chomczynski and Sacchi (1987), as modified for fish blood by Currie et al. (1999). Packed red blood cells (300 µl) were added to 20 ml guanidinium thiocyanate (GTC), shaken vigorously for 5 min and stored at –80 °C. Upon thawing, the following were added with thorough mixing after each step: 2 ml 2 mol l^{-1} sodium acetate (pH 4.0), 20 ml buffer-saturated phenol (pH 4.0) and 4 ml chloroform/isoamyl alcohol (49:1 by volume). Samples were shaken vigorously for 20 s, left on ice for 15 min, and centrifuged for 30 min at 3,000 *g*. Supernatant was collected and added to an equal volume of isopropanol to precipitate the RNA overnight at –20 °C. The RNA pellet was then obtained by centrifugation for 30 min at 3,000 *g*, dissolved in 5 ml GTC and the phenol extraction/isopropanol precipitation repeated. Following this, the RNA pellet was re-dissolved in 0.3 ml GTC and re-precipitated in 0.3 ml isopropanol, centrifuged (10 min at 3,000 *g*), washed in 75% ethanol [diluted with diethyl pyrocarbonate (DEPC) treated

water], dried and dissolved in 20–60 µl DEPC-treated water. Total RNA was then quantified with a Spectramax Plate Spectrophotometer (Molecular Devices) in triplicate using the absorbance at 260 nm.

First strand cDNA was synthesized from the purified gar rbc total RNA using M-MuLV reverse transcriptase and random primers. A 324 bp internal segment of the gar red blood cell CA coding region was amplified by PCR at an annealing temperature of 50 °C, using a CA forward primer (CA-F), (5′- CAG TTC CAT TTC CAT TGG GG- 3′) and reverse primer (CA-R), (5′- ATA CTC CCG AGA GAC TGG TG- 3′). All PCR reactions involved an initial denaturation (94 °C×30 s) followed by 30 cycles of: 94 °C, 30 s; annealing temperature, 1 min; 72 °C, 1.5 min, followed by a final extension for 10 min at 72 °C. The forward and reverse primers were both designed by determining homologous regions of cDNA between *Drosophila* and zebrafish CA obtained from GenBank and aligned using Clustal W. Where ambiguities were found within homologous regions, nucleotides present in the zebrafish CA sequence were favored. The resulting PCR product was ligated into pCR 2.1 (Invitrogen) and sequenced. This sequence information was used to construct specific primers for use in 5′ and 3′ rapid amplification of cDNA ends (RACE; Boehringer Mannheim).

To determine the 5′ end sequence of gar rbc CA, first strand cDNA synthesis was performed using a gene specific primer (CA-R) and AMV reverse transcriptase (the mRNA was digested by RNase H activity of AMV reverse transcriptase). Purified cDNA was modified by the addition of multiple dATP by the enzyme terminal transferase. This single stranded cDNA was amplified at 52 °C using a gene specific primer SP2 (5′– GCA TCG AGG ACC TTC CGC AGG –3′) and an anchor/oligo dT primer (5′– GAC CAC GCG TCT CGA TCT CGA CTT TTT TTT TTT TTT TTV –3′). This product was then re-amplified at an annealing temperature of 54 °C using a PCR anchor primer, (5′– GAC CAC GCG TAT CGA TGT CGA C–3′) and another gene-specific primer SP3, (5′–CCC CAC AGT GTG CTC TGA TC –3′). A final amplification was performed using the product obtained at 54 °C, SP3, and the PCR anchor primer. This final step was performed at an annealing temperature of 60 °C. A resulting 550 bp PCR product was ligated into pCR 2.1 and sequenced in both directions using primers that were anti-sense to CA-F (5′–CCC CAA TGG AAA TGG AAC TG –3′) and the T7 promoter.

To determine the 3′ end sequence of gar rbc CA, gar cDNA was synthesized using AMV reverse transcriptase and the anchor/oligo dT primer described previously for 5′ RACE. 3′–RACE was performed initially at an annealing temperature of 66 °C, using a second primer designed for gar (SP7), (5′– CTC CTT TCC CAC AAT TCG ACC –3′) and the anchor primer which was used in 5′ RACE. This amplified product (approximately 1 kb in size) was sequenced. Based upon the 5′ and 3′ RACE sequences, one final PCR was performed at an annealing temperature of 54 °C using primers designed from both the 3′ and 5′ non-coding region of the gar rbc CA sequence, CA CDS-F (5′– CTT AAT TCA AAT CTG AAC CAC G –3′) and CA CDS-R (5′ –ATA CAA CTC TGA TGA AGC GTC –3′). This final 786 bp product was ligated into pCR 2.1 and sequenced from the 5′ end using CA CDS-F and from the 3′ end using CA CDS-R. The complete coding sequence of the gar rbc CA was entered in GenBank (accession number AY125007).

Sequence analyses

Gar rbc CA sequence was compared to other vertebrate CAs obtained from GenBank including: zebrafish (accession number U55177), salmon (BG935995), human CA I (X05014) and human CA II (J03037), as well as tiger shark which was from PIR Database (accession number A60519), and consensus CA I and CA II which were obtained from Tashian (1992), Hewett-Emmett and Tashian (1996) and Tashian et al. (2000). Preliminary analysis of the unpublished flounder sequence suggested it was highly, and possibly aberrantly, divergent so we elected to eliminate it from all analyses. Comparisons were made using the homology search program BLASTN. Protein sequences used to construct phylogenetic trees were obtained from Peterson et al. (1997). Accession numbers were as found in Peterson et al. (1997) with the exception of human CA III (Swiss-Prot, accession number P07451), chicken CA II (Swiss-Prot, accession number P07630), rat CA IV (NCBI gi, accession number 544725), human CA IV (NCBI gi, accession number 179790), human CAVB (NCBI gi, accession number 4587222), mouse CA VB (NCBI gi, accession number 9506462), mouse CA VII (NCBI gi, accession number 10304383) and mouse CA VIII (GenBank, accession number X61397).

Alignment of sequences was performed using Clustal W (version 1.8) multiple sequence alignment. A matrix diagram was constructed using the same program whereby reported scores of pairwise alignments are represented as percent identity for both nucleotide and amino acid sequences. Because the salmon and shark sequences are incomplete, they were not included in the matrix diagram. Active site comparisons of gar, zebrafish, salmon, shark, human CA I and CA II were constructed by using consensus CA I, CA II and human CA VII data and CA active site information compiled by Tashian (1992), Hewett-Emmett and Tashian (1996) and Tashian et al. (2000). Alignment used for phylogenetic analysis was also performed using Clustal W, with subsequent deletion of all gaps and ambiguities. We constructed phylogenetic hypotheses using both neighbor-joining (NJ; Saitou and Nei 1987) and maximum parsimony (MP) approaches as implemented in PAUP* (beta test version 4.0b3a; Swofford 2000). MP analysis consisted of a heuristic search with TBR branch-swapping and ACCTRAN character state optimization enforced, and with random stepwise addition and 1,000 random addition replicates. NJ was performed on a matrix of mean character distances. Support for nodes for both analytical approaches was generated using bootstrap analysis with 1,000 pseudoreplicates. All analyses used *Drosophila* CA as the out-group for CA I, CA II, CA III, CA VA, CA VB and CA VII based on earlier analyses by Shah et al. (2000), and were performed with and without the incomplete mouse CA VII and salmon CA sequences. Amino acid sequences used in the analyses had a range in length of 256–265 residues, with the exception of salmon CA and mouse CA VII which consisted of 231 and 232 residues, respectively.

Results

Gar erythrocyte CA activity and inhibitor kinetics

The erythrocytes of the longnose gar were found to have a mean total CA activity of 78 mmol CO_2 min^{-1} ml $rbcs^{-1}$ ($n = 5$). Titration of gar rbc CA with Az and copper produced inhibition patterns that were very similar to those of mammalian CA II (Figs. 1, 2, 3, 4; Table 1). However, the inhibitory effect of iodide on gar rbc CA was intermediate between that of mammalian CA I and CA II (Figs. 3, 4; Table 1). In terms of the kinetic properties of gar rbc CA, the substrate affinity (K_m) resembled that of mammalian CA II, but the turnover number (K_{cat}) was more similar to that of mammalian CA I (Table 2).

Gar rbc CA sequencing and analyses

Isolation and sequencing of the final cDNA product resulted in a complete coding sequence spanning 786 bp from the start codon (underline) to the stop codon (asterisk). This nucleotide and corresponding amino acid

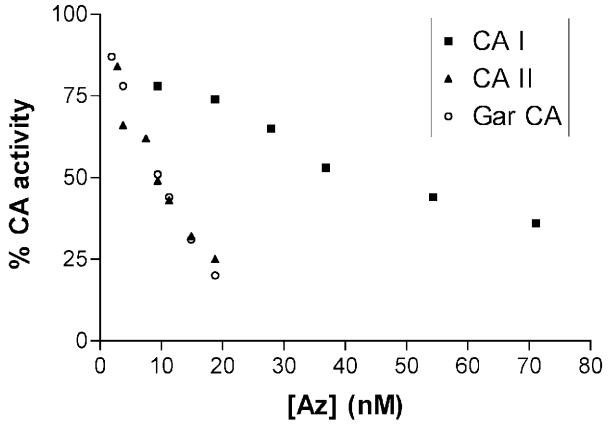

Fig. 1. Representative plot of the inhibition of longnose gar erythrocyte carbonic anhydrase (*CA*) and mammalian CA I and CA II (Sigma) by acetazolamide (*Az*). The data is a representative of four separate experiments using different blood pools for gar erythrocyte CA, and two separate experiments for mammalian CA I and CA

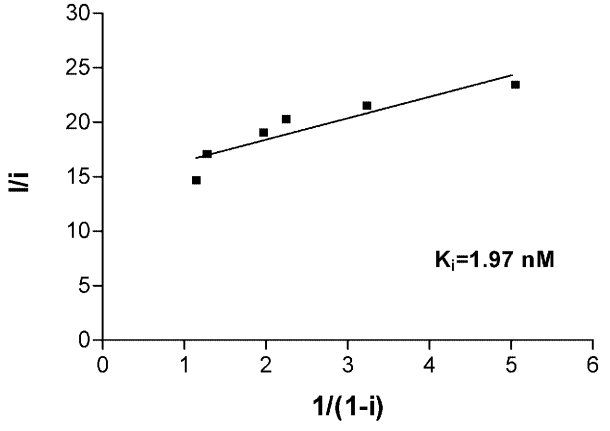

Fig. 2. Representative Easson-Stedman plot of Az inhibition of longnose gar erythrocyte CA II ($r^2 = 0.83$). The data is a representative of four separate experiments using different blood pools for gar erythrocyte CA

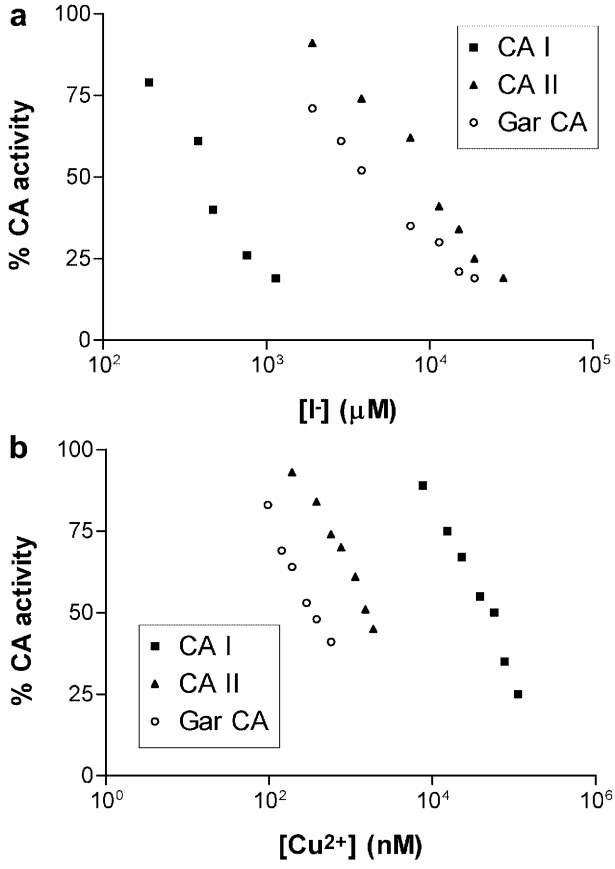

Fig. 3. Representative plots of the inhibition of longnose gar erythrocyte CA and mammalian CA I and CA II (Sigma) by (**A**) iodide and (**B**) copper. The data are representative of four separate experiments using different blood pools for gar erythrocyte CA, and two separate experiments for mammalian CA I and CA II

sequence is illustrated in Fig. 5. A matrix diagram (Table 3) was then constructed comparing the nucleotide and amino acid identity of gar rbc CA with that of zebrafish, human CA I, human CA II and human CA VII. The complete coding region of gar rbc CA most closely resembled zebrafish CA in respect to both nucleotide (72%) and amino acid identity (77%). The gar rbc CA amino acid sequence exhibited 62%, 65% and 58% identity with the amino acid sequences for CA I, CA II and CA VII, respectively.

Active site comparisons (Table 4) showed that gar rbc CA differs from a consensus CA I (Tashian 1992) in 9 out of the 36 amino acid residues, and by only 4 out of 36 amino acids in comparison to a consensus CA II (Tashian 1992; Hewett-Emmett and Tashian 1996; Tashian et al. 2000). Gar rbc CA was most similar, however, to human CA VII, differing in only 3 out of the 36 active site amino acids. When gar rbc CA was compared to consensus CA I, consensus CA II, and human CA VII with respect to only those amino acids that are conserved in active CAs (Tashian 1992; Hewett-Emmett and Tashian 1996; Tashian et al. 2000), no differences were observed.

NJ analysis produced a generally well supported phylogenetic tree, the branches of which are drawn to scale with their length representing mean character differences (Fig. 6). Because the inclusion of the incomplete mouse CA VII and salmon CA sequences did not affect either the topology, or the degree of support, both of these sequences remain in the final NJ tree. The tree generated by MP analysis showed an almost identical topology (tree not shown). Our phylogenetic analyses suggest that CAs I, II and III comprise a single monophyletic clade, albeit one that has only 65% bootstrap support. Further, our results indicate that the fish CA clade is basal to the clade comprised of CA I, CA II and CA III. In the fish clade, gar CA diverged first, before the two teleost CAs. CAs V and VII and CAs IV, VI and VIII, respectively, each comprise single monophyletic clades although only the latter clade was robustly supported through bootstrap analysis (98%).

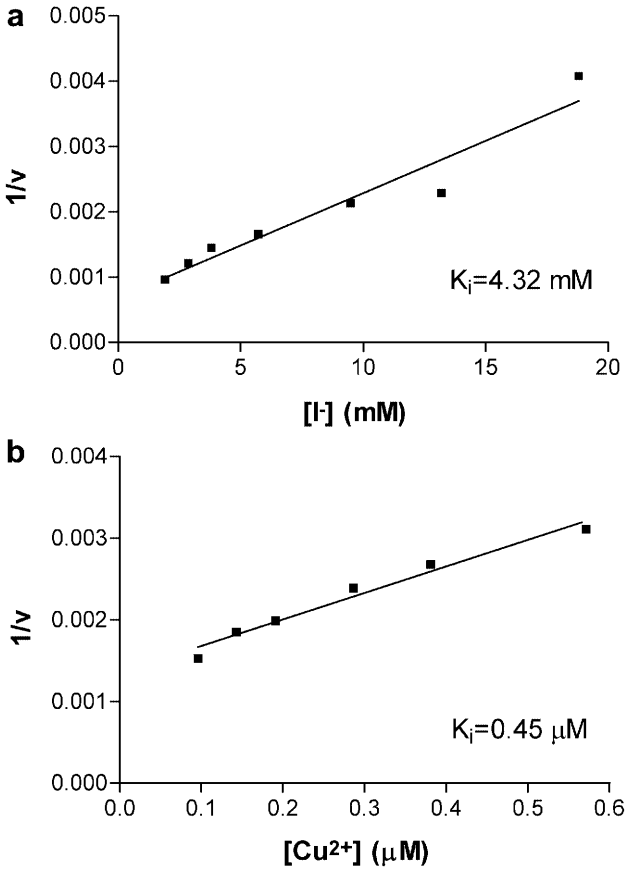

Fig. 4. Representative plots of (**A**) iodide and (**B**) copper inhibition of longnose gar erythrocyte CA ($r^2 = 0.93$ and 0.97, respectively). The K_is for both inhibitors were calculated as the –x intercept of the line generated by measuring the reciprocal of CA activity (v) against increasing concentrations of the inhibitors

Discussion

To date, information about the evolution of CA in animals has been largely based on sequence data obtained from mammals. To our knowledge, this is the first study to determine both the biochemical properties and complete nucleotide sequence of an erythrocyte CA isozyme from an early vertebrate.

In terms of its sensitivity towards the CA inhibitors used in this study, gar rbc CA most closely resembles the rapid-turnover mammalian rbc isozyme, CA II (Figs. 1, 3; Table 1). It is noteworthy, however, that the sensitivity of gar rbc CA towards one of the inhibitors, iodide, was intermediate between the values obtained for the two mammalian rbc isozymes. Unlike the inhibitor results, the kinetic properties of gar rbc CA did not exhibit striking similarities towards either of the mammalian rbc isozymes (Table 2). These findings suggest that some characteristics of gar rbc CA are also different from both CA I and CA II of mammals.

Inter-species comparisons of the biochemical properties of fish rbc CAs (Table 5) indicate that gar possess a rbc CA isozyme that is very similar, in many respects, to that recently characterized in another closely related species of fish, the bowfin (Gervais and Tufts 1999). In terms of the turnover rates of their active sites, the rbc CAs of both gar and bowfin are intermediate between the high activity CA isozymes of teleost rbcs and the low

Table 1. Acetazolamide (*Az*), iodide (I^-) and copper (Cu^{2+}) inhibition constants of erythrocyte carbonic anhydrase (*CA*) from mammals (CA I and CA II) and gar. Values are mean ± SEM for gar ($n = 4$ for gar and $n = 2$ for CA I and CA II)

	K_i Cu^{2+} (μM)	K_i Az (nM)	K_i I^- (mM)
Gar	0.5 ± 0.1	1.7 ± 0.2	4.5 ± 0.4
CA I	36.2	33.2	0.6
CAII	1.2	2.1	8.8

Table 2. Substrate affinities and turnover numbers of mammalian CA I, CA II and gar erythrocyte CA. Values are mean ± SEM for gar ($n = 4$ for gar and $n = 2$ for CA I and CA II)

CA isozyme	Substrate affinity (mM CO_2) (K_m)	Turnover number (e^4 s^{-1}) (K_{cat})
Gar	16.8 ± 1.5	7.7 ± 2.2
CA I	2.2	1.0
CA II	10.2	30.9

```
1    ATGTCTCACTCATGGGGATATGCAGCTAACAACGGACCAGACAAATGGCATGAGAAATTT
       M  S  H  S  W  G  Y  A  A  N  N  G  P  D  K  W  H  E  K  F
61   CCAATTGCTCAAGGACCCAGGCAGTCTCCCATTGACATTGTGCCCTCCCAGGCACAGCAC
       P  I  A  Q  G  P  R  Q  S  P  I  D  I  V  P  S  Q  A  Q  H
121  GACCCAGATTTGAAGCCACTCCGTATCGTTTATGATCCCAGCACATCCAAAGGGATTTTG
       D  P  D  L  K  P  L  R  I  V  Y  D  P  S  T  S  K  G  I  L
181  AACAATGGACATTCCTTCCAAGTTGACTTTGCGGATGAAAACGACAGCTCGACACTGCAA
       N  N  G  H  S  F  Q  V  D  F  A  D  E  N  D  S  S  T  L  Q
241  GGAGGACCAATTTCCGGCGTTTACAGGCTGCGTCAGTTCCACTTCCATTGGGGTGCTAGC
       G  G  P  I  S  G  V  Y  R  L  R  Q  F  H  F  H  W  G  A  S
301  GATGAAAGAGGATCAGAGCACACTGTGGGGGGAGTGAAGTATGCAGCTGAGCTTCATCTG
       D  E  R  G  S  E  H  T  V  G  G  V  K  Y  A  A  E  L  H  L
361  GTTCACTGGAATGCTGGGAAATACGCGAGCTTTGGAGATGCTGCAAAGGCACCAGATGGG
       V  H  W  N  A  G  K  Y  A  S  F  G  D  A  A  K  A  P  D  G
421  TTGGCTGTTGTCGGCGTTTTCCTCAAGATTGGCGCCGCCAGTCCTAACCTGCAGAAGGTC
       L  A  V  V  G  V  F  L  K  I  G  A  A  S  P  N  L  Q  K  V
481  CTCGATGCCCTTGATGCGATTAAGACAAAGGGAAAGCAAACTCCTTTCCCACAATTCGAC
       L  D  A  L  D  A  I  K  T  K  G  K  Q  T  P  F  P  Q  F  D
541  CCCAAGATTCTACTTCCTTCCTCTCTGGACTTCTGGACATATGAGGGGTCTCTGACAACC
       P  K  I  L  L  P  S  S  L  D  F  W  T  Y  E  G  S  L  T  T
601  CCACCCCTGCTGGAGAGTGTCACCTGGATCGTCCTCAAAGAGCCCATCACTGTCAGCTCA
       P  P  L  L  E  S  V  T  W  I  V  L  K  E  P  I  T  V  S  S
661  GAACAGATGGCGAAATTCCGCAGTCTCCTGTTCACCGCAGAGGGCGAGACCGCTTGCTGC
       E  Q  M  A  K  F  R  S  L  L  F  T  A  E  G  E  T  A  C  C
721  ATGGTGGACAACTACCGCCCACCCCAGCCCCTCAAGGGAAGGAAGGTCCGAGCCTCCTTC
       M  V  D  N  Y  R  P  P  Q  P  L  K  G  R  K  V  R  A  S  F
781  AAATGA - 786
       K  *
```

Fig. 5. Gar CA nucleotide and deduced amino acid sequence. Sequence shown is the coding region only, from the start (*underline*) to stop codon (*asterisk*) as determined through 5′ and 3′ RACE (rapid amplification of cDNA ends)

Table 3. Matrix diagram comparing nucleotide/amino acid sequence identity of gar rbc CA, zebrafish CA and human CA I, CA II and CA VII

	Zebrafish	CA I	CA II	CA VII
Gar	72/77	64/62	65/65	60/58
Zebrafish		63/60	65/63	60/60
CA I			63/60	59/50
CAII				62/56

Table 4. Active site comparisons of gar rbc CA, zebrafish CA, salmon CA, shark CA and human CA I and CA II with a consensus CA I and CA II sequence (Tashian 1992; Hewett-Emmett and Tashian 1996; Tashian et al. 2000) and with human CA VII

Consensus																																				Differences	
CA I	Y	S	N	V	H	S	F	H	N	F	Q	H	H	E	H	E	H	V	L	L	I	G	W	Y	L	T	H	P	P	H	S	V	W	I	N	R	from CA I	
	+	+			+			+			+	Z	Z	+	+	+	Z							+		+	+							+		+	+	All	Cons.
		*	*								*	*	*	*	*	*	*	*						*		*		*	*					*		*	*	Sites	(*)
Gar	.	.	.	N	.	.	.	Q	D	R	.	.	.	.	.	.	.	.	F	.	V	.	.	.	.	.	T	.	.	L	.	.	.	V	.	.	9/36	0/17
Zebrafish	.	.	.	N	.	.	.	Q	D	R	.	.	.	.	.	.	.	.	F	.	V	.	.	.	.	.	T	.	.	L	.	.	.	V	.	.	9/36	0/17
Salmon	.	.	.	N	.	.	.	Q	G	K	.	.	.	.	.	.	.	.	F	.	V	.	.	.	.	.	T	.	.	L	.	.	.	V	--	--	9/34	0/15
Shark	--	.	--	--	--	--	--	--	--	R	.	.	.	.	.	.	.	.	F	.	V	.	.	.	.	.	T	.	.	L	.	.	.	V	.	.	6/28	0/16
CA I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	V	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	3/36	0/17
CA II	.	.	.	N	.	A	.	N	E	I	.	.	.	.	.	.	.	.	F	.	V	.	.	.	.	.	T	.	.	L	C	.	.	V	.	.	11/36	0/17

Consensus																																						Differences	
CA II	Y	S	N	N	H	S	F	N	E	I	Q	H	H	E	H	E	H	V	F	L	V	G	W	Y	L	T	T	P	P	L	C	V	W	V	N	R	from CA II	
	+	+			+			+			+	Z	Z	+	+	+	Z							+		+	+							+		+	+	All	Cons.
		*	*								*	*	*	*	*	*	*	*						*		*	*	*	*					*		*	*	Sites	(*)
Gar	.	.	.	.	.	.	.	Q	D	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	4/36	0/18
Zebrafish	.	.	.	.	.	.	.	Q	D	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	4/36	0/18
Salmon	.	.	.	.	.	.	.	Q	G	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	--	--		4/34	0/16
Shark	--	.	--	--	--	--	--	--	--	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	2/28	0/17
CA I	.	.	.	V	.	.	.	H	N	F	.	.	.	.	.	.	.	A	L	.	.	.	.	.	.	.	H	.	.	S	.	.	.	.	.	.	8/36	1/18
CA II	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1/36	0/18

Human																																						Differences	
CA VII	Y	S	N	N	H	S	V	Q	D	K	Q	H	H	E	H	E	H	V	F	L	V	G	W	Y	L	T	T	P	P	S	S	V	W	V	N	R	from CA VII	
	+	+			+			+			+	Z	Z	+	+	+	Z							+		+	+							+		+	+	All	Cons.
		*	*								*	*	*	*	*	*	*	*						*		*	*	*	*					*		*	*	Sites	(*)
Gar	.	.	.	.	.	.	F	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	3/36	0/18
Zebrafish	.	.	.	.	.	.	F	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	3/36	0/18
Salmon	.	.	.	.	.	.	F	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	--	--	3/34	0/16
Shark	--	.	--	--	--	--	--	--	--	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	2/28	0/17
CA I	.	.	.	V	.	.	F	H	N	F	.	.	.	.	.	.	.	A	L	.	.	.	.	.	.	.	H	.	.	L	.	.	.	.	.	.	9/36	2/18
CA II	.	.	.	.	.	A	F	N	E	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	C	.	.	.	.	.	7/36	0/18

+ = active site H bond network
* = conserved in active CA's
Z = zinc liganded histidine
-- = data not available

activity CAs found in the rbcs of elasmobranchs and agnathans. In contrast, the inhibition characteristics of the rbc CAs in gar and bowfin are clearly more similar to those of the rbc CAs found in the more recently evolved teleost fish.

Although these initial results provide important information about the biochemical characteristics of gar rbc CA and their relationship to those of other vertebrate CAs, it must be acknowledged that methodological factors may also influence some of these comparisons. For example, comparisons involving biochemical values obtained in different studies, such as those presented in Table 5, may be affected by methodological differences between studies. Within the present study, it should also be noted that experiments on gar rbc CA were conducted on very dilute lysates whereas experiments on mammalian CA I and CA II were performed on purified (Sigma) enzymes. In our experience, it is unlikely that these methodological factors would have substantial impact on the obvious phylogenetic trends in these biochemical values. Nonetheless, it is important that molecular sequence information can now also be considered in conjunction with biochemical data in this type of study. In the present study, the associated molecular analyses provide further support for some of our initial biochemical findings, as well as several additional insights about the relationships between gar rbc CA and other vertebrate CAs.

When the nucleotide and deduced amino acid sequences of gar rbc CA are compared to those of zebrafish CA and human CA I, CA II and CA VII (Fig. 5; Table 3), it is apparent that gar rbc CA is most similar to the CA obtained from zebrafish retina. Although information on the distribution of different CA isozymes in fish is currently unavailable, these comparisons suggest that the CA isozyme in the zebrafish retina is probably the same isozyme that is present in the zebrafish rbc, but this has yet to be confirmed. As

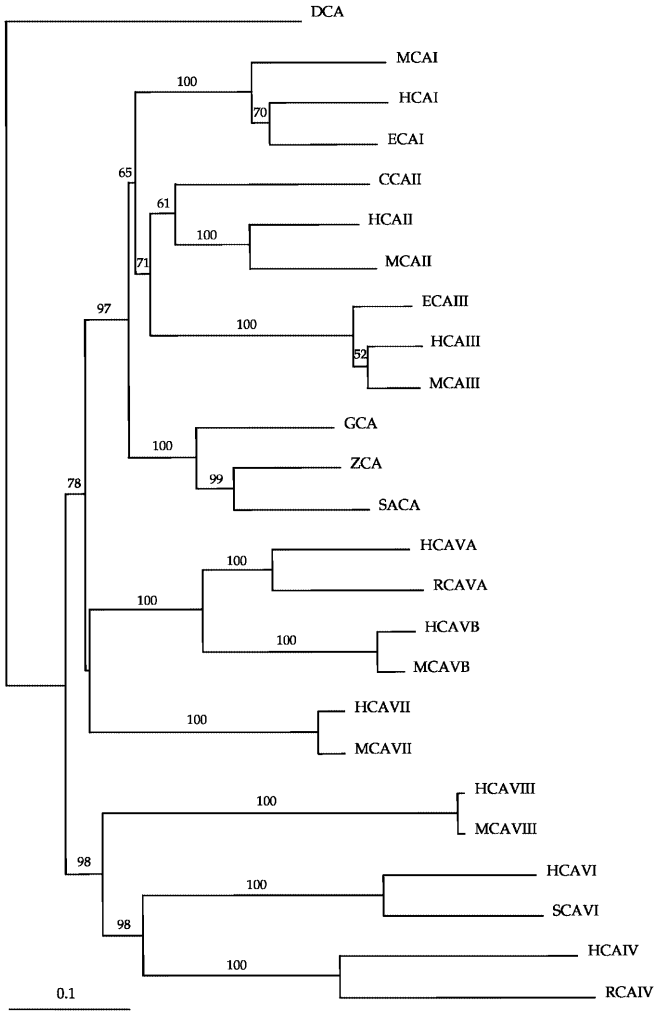

Fig. 6. Phylogenetic analysis of gar CA (*GCA*) and other α-CA isozymes. The phylogenetic tree was constructed using neighbor-joining analysis with support for nodes assessed using bootstrap analyses. The tree was ordered using *Drosophila* CA as the out-group for CA I, CA II, CA III, CA VA, CA VB and CA VII, based on earlier analyses by Shah et al. (2000). Branches are drawn to scale with the length of 0.1 approximating replacement of 10% of the amino acids in the protein alignments (no Poisson correction for multiple hits). Amino acid sequences ranged in length from 256 to 265 residues, with the exception of salmon CA and mouse CA VII which consisted of 231 and 232 residues, respectively. (*C* chicken, *D* Drosophila, *E* horse, *H* human, *G* gar, *M* mouse, *R* rat, *S* sheep, *SA* salmon, *Z* zebrafish)

might be anticipated from the biochemical data within this study, the sequence information obtained does not provide an obvious indication that gar rbc CA is more similar to either CA I or CA II (Table 2). Gar rbc CA shares 64% nucleic acid and 62% amino acid identity with human CA I, 65% identity for both nucleotides and amino acids when compared to human CA II, and 60% nucleotide and 58% amino acid identity with human CA VII. When the active sites of different CAs were compared, however, it was apparent that gar rbc CA was more similar to human CA VII and consensus CA II than to CA I.

The active site of gar rbc CA differed from consensus CA I and CA II by nine and four amino acids, respectively, and from human CA VII by only three amino acids (Table 4). These results are entirely consistent with the view that the structural organization of the CA II and CA VII active sites are highly conserved (Hewett-Emmett and Tashian 1996). Moreover, our findings also provide evidence to support the case made by these authors that the structural organization of the active site in the ancestral vertebrate CA was probably closer to CA VII than CA II. The fact that gar rbc CA does show several amino acid differences from consensus CA I and CA II probably explains why the kinetic properties of the gar rbc isozyme were somewhat different than both CA I and CA II (Table 2). In this regard, it is also noteworthy that the turnover number for the active site in gar rbc CA and mammalian CA VII are both intermediate between mammalian CA I and CA II (Table 2; Earnhardt et al. 1998). Within the non-mammalian vertebrate CAs included in the active site comparison (Table 4), the CA from zebrafish retina was again the most similar to gar rbc CA. The active site amino acids from the sequence translated from the incomplete CA cDNA from salmon spleen were also very similar to gar CA, differing at only two sites. For these active site comparisons, it should also be considered that no CA proteins from fish have yet been analyzed by x-ray crystallography, so the possibility exists that active sites for the fish CA isozymes may be different than those for mammalian CA I and CA II. This possibility seems unlikely, however, because of the high degree of similarity in active site amino acids.

In combination with previous experiments that have characterized the altered function of mammalian CA I and CA II in the presence of sulfonamides and inorganic anions, the sequence information obtained in this study can also be used to explain the sensitivity of gar rbc CA towards the different CA inhibitors. Sulfonamide inhibitors of CA, such as Az, complex with both mammalian CA I and CA II by replacement of the zinc-bound water molecule in the active site with their ionized NH group and through the formation of a hydrogen bond with the OH group of Thr-199 (Lindskog 1997). In the case of CA II, the thiadiazole ring of Az is also in van der Waals contact with Val-121, Leu-198 and Thr-200, while the carbonyl oxygen of its amido group forms a hydrogen bond with the side chain amide of Gln-92 and its methyl group interacts with Phe-131 (Vidgren et al. 1990; Lindskog 1997). All of these residues are present in the same positions in gar rbc CA and therefore explains why the Az sensitivity of the rbc isozyme in this ancient species is virtually identical to that for mammalian CA II (Table 1). In contrast, the reduced sensitivity of CA I towards Az can be, at least partially, explained by the absence of a hydrogen bond to Gln-92 (Chakravarty and Kannan 1994), as well as the substitution of Val-121 by Asp-121, Phe-131 by Leu-131, and Thr-200 by His-200. Site-directed mutagenesis studies have also shown that replacement of Thr-200 in human

Table 5. Characteristics of erythrocyte CA from different fish taxa (*NA* not available)

Fish Taxa	K_m ($mMCO_2$)	K_{cat} (e^4s^{-1})	K_i Az (nM)	K_iCu^{2+} (μM)	K_iI^- (mM)
Agnathan					
(Hagfish)	NA	1.3[2]	9 [3]	9.6[8]	1.4[8]
(Lamprey)[4]	5	1.5	22	NA	NA
Elasmobranch					
(Dogfish)	5[2]	2.5[2]	200[2]	NA	1 [5]–9[2]
(Bull Shark)		0.5–1.1[6]			
(Tiger Shark)		0.9–1.4[6]			
Holostean					
(Bowfin)[7]	8.4	5.1	1.6	0.4	5.6
(Gar)[1]	16.8	7.7	1.7	0.5	4.5
Teleost					
(Trout)	10[2,4]	70[2]	2[4]	0.1[8]	3.3[8]

[1]Present study; [2]Maren et al. 1980; [3]Carlsson et al. 1980; [4]Henry et al. 1993; [5]Maren and Rittmaster 1977; [6]Maynard and Coleman 1971; [7]Gervais and Tufts 1999; [8]M.R. Gervais and B.L. Tufts, unpublished data

CA II with His results in an enzyme that more closely resembles CA I (Behravan et al. 1990; Behravan et al. 1991).

The metal ions mercury and copper, are believed to inhibit human CA II by binding to the imidazole side chain of His-64, blocking its role in proton transfer from the zinc-bound water molecule to buffer molecules located outside of the active site region (Magid 1967; Tu et al. 1981). Crystallographic and mutation analyses have confirmed this mechanism of inhibition for CA II (Eriksson et al. 1988; Tu et al. 1989), but similar analyses have not been performed on CA I. The presence of His-64 in gar rbc CA may therefore explain why the sensitivity of this CA isozyme towards copper is virtually identical to that of mammalian CA II (Table 1).

Similar to sulfonamide inhibitors, iodide displaces the zinc-bound water molecule from the CA active site, but it does not hydrogen bond to Thr-199 (Lindskog 1997). As demonstrated in the current study (Table 1), mammalian CA I binds anionic inhibitors approximately one order of magnitude more strongly than CA II. The higher affinity of CA I for anion inhibitors may be due, in part, to the presence of His at position 200 instead of Thr, which is found in CA II (Behravan et al. 1990, 1991; Engstrand et al. 1995). Similar to CA II, gar rbc CA possesses Thr-200. As observed in the present study (Table 1), it would therefore be expected that gar rbc CA would have a lower affinity for iodide than mammalian CA I. Interestingly, however, the sensitivity of gar rbc CA towards iodide does not appear to be as low as that for mammalian CA II. These results may indicate that other amino acids also play an important role in determining the anion sensitivity of CAs.

To date, molecular analyses of the phylogeny of CA isozymes have been primarily based on information from mammals. These studies have shown that the CA I, CA II and CA III isozymes were the result of recent gene duplications, while the CA V, CA VII and CA - VIII isozymes diverged much earlier (Tashian 1989, 1992; Hewett-Emmett and Tashian 1996). Sequence information for CAs from the rbcs of the tiger shark (Bergenhem and Carlsson 1990) and the retina of zebrafish (Peterson et al. 1997) have also provided some insights about the gene duplication events leading to the evolution of the CA I, II and III genes. A recent phylogenetic analysis by Peterson et al. (1997) showed that zebrafish retina CA diverged after CA V and CA VII, and before CA I, CA II and CA III, as did tiger shark CA (Tashian 1989). The information obtained for gar rbc CA in the present study now makes it possible to conduct a phylogenetic analysis of CAs that includes the full sequence for a fish rbc CA. This analysis indicates that CA from the gar rbc diverged slightly before the teleost CAs from the zebrafish retina and the salmon spleen (Figure 6). Assuming that the CAs from the zebrafish retina and from the salmon spleen are the same isozymes that are found within the rbcs of these species, it is also likely that this node in the tree now begins to describe the point of emergence of the rbc CA isozymes in the more recently evolved fishes, such as teleosts. However, further sequence information for the rbc CAs from other fish species is clearly required before this can be verified. The present analysis also implies that the CA I, CA II and CA III genes diverged after the appearance of the holosteans and the teleosts (Radinsky 1987; Peterson et al. 1997) and therefore supports previous studies that place the time of CA I and CA II divergence at approximately 340–320 million-years ago (Fraser and Curtis 1986; Peterson et al. 1997). The present topology and grouping of gar CA in the same node as zebrafish and salmon CA also supports the view that the CA V and CA VII genes appeared after the insects (represented here by *Drosophila*), but before the elasmobranchs, holosteans and teleosts (Peterson et al. 1997).

In conclusion, the results of this investigation show that gar rbc CA is a unique CA isozyme with biochemical properties that fit well into an emerging phylogenetic pattern for early vertebrates. The close similarity at the molecular level between the CAs in the gar rbc and the zebrafish retina and salmon spleen suggest that the CAs from these two teleosts may also be rbc CA isozymes. Phylogenetic analyses suggest that the CA isozymes found in these fish species diverged before CA I, CA II and CA III, but after CA V and CA VII. Although these analyses begin to identify the point of

emergence of the fish rbc CAs, it is obvious that there is still much to be learned about the evolution of the different vertebrate CA isozymes.

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