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Tribute to R. G. Boutilier: Evidence of a high activity carbonic anhydrase isozyme in the red blood cells of an ancient vertebrate, the sea lamprey *Petromyzon marinus*

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Summary

Carbonic anhydrase (CA) is a multi-functional enzyme that catalyzes the hydration/dehydration of carbon dioxide. In the red blood cell (rbc), CA is necessary to facilitate the transport of carbon dioxide out of the body. Results from earlier biochemical studies indicate that ancient vertebrates, such as agnathans, possess a low activity rbc CA isozyme, whereas more recently evolved vertebrates, such as teleost fish, possess a high activity isozyme. At present, however, the changes in the molecular structure that have resulted in this large increase in catalytic efficiency are unknown. The objective of the current study was therefore to determine the molecular structure of rbc CA in lampreys and compare it to that of teleosts in an effort to ascertain how this important enzyme became more efficient over evolutionary time. Isolation and sequencing of cytoplasmic CA from rbc and gill showed only a single isozyme of 789 bp (262 amino acids). This isozyme was also found in brain and kidney, with no evidence of additional cytoplasmic CA isozymes in other tissues. Phylogenetic analysis grouped this isozyme closely to vertebrate CA VII, which is ancestral to the rbc isozymes in other vertebrates. Interestingly, active site analysis revealed a structure similar to high activity isozymes. A comparative kinetic analysis of CA from rbc lysates and CA fusion proteins showed that the traditional method of determining the turnover number may not be appropriate for all vertebrate CAs. In contrast to previous evidence, lamprey CA was found to be a high activity isozyme. These results suggest that the critical functional characteristics of rbc CA have been highly conserved throughout vertebrate evolution.

Key words: carbonic anhydrase, red blood cell, isozyme, sea lamprey, *Petromyzon marinus*, evolution, carp, *Cyprinus carpio*.

Introduction

Carbonic anhydrase (CA) is a ubiquitous metalloenzyme that catalyzes the reversible hydration/dehydration of carbon dioxide. This enzyme has been found in virtually all living organisms, but only members of the α-CA gene family are found in vertebrates (Tashian, 1992; Hewett-Emmett and Tashian, 1996; Earnhardt et al., 1998). To date, 15 different α-CA isozymes have been characterized in mammals by means of their kinetic properties, subcellular location and/or molecular structure (Chegwidden and Carter, 2000; Hewett-Emmett, 2000). These isozymes are found in many different tissues and are involved in a number of homeostatic processes, including carbon dioxide transport, ion exchange and acid–base balance (Chegwidden and Carter, 2000; Geers and Gros, 2000).

In red blood cells (rbcs), CA is the second most abundant protein to haemoglobin and plays a crucial role in CO_2 transport. More specifically, rbc CA catalyzes the hydration of CO_2 to HCO_3^- at the tissue site of production, and the dehydration of HCO_3^- to CO_2 at the respiratory surface, thereby facilitating the transport and excretion of CO_2 from the body (Perry, 1986; Perry and Laurent, 1990; Henry and Heming, 1998; Tufts and Perry, 1998; Geers and Gros, 2000; Henry and Swenson, 2000). In addition, rbc CA also facilitates the linkage of O_2 and CO_2 transport *via* the Bohr effect (Forster and Steen, 1968; Maren and Swenson, 1980). In mammals, there are two cytoplasmic CA isozymes in the rbc, CA I and II. CA II is a high turnover isozyme that is found in cell types of virtually every tissue, while CA I is a low turnover isozyme found mostly in the rbc and intestine (Chegwidden and Carter, 2000). Although both isozymes contribute equally to CO_2 hydration in the rbc, CA I seems to be redundant as CA I-deficiency has no physiological impact (Sly and Hu, 1995). In addition to these two CA isozymes, mammals also possess three other cytoplasmic isozymes (CA III, VII and XIII) that are found in various tissues.

Recent studies on cytoplasmic CA isozymes in fishes have shown evidence for three cytoplasmic CA isozymes. In

rainbow trout, a rbc specific isozyme and a general cytoplasmic isozyme with a wide tissue distribution have been found. In addition, there is genetic evidence of CA VII in zebrafish, although this has yet to be thoroughly investigated. Phylogenetic analyses have shown that these CA isozymes are distinct from mammalian isozymes, with the cytoplasmic fish CAs diverging prior to the gene duplication events that gave rise to the numerous mammalian isozymes (Lund et al., 2002; Esbaugh et al., 2004; Esbaugh et al., 2005). The lone exception is CA VII, which is phylogenetically similar in fishes and mammals. Nonetheless, the rbc CA isozymes in teleosts are all high turnover isozymes that are catalytically comparable to mammalian CA II. In contrast to the situation in mammals, no low turnover CA isozymes have been found in the rbcs of modern teleosts.

Interestingly, earlier biochemical studies on rbc haemolysates indicate that ancient vertebrates, such as agnathans, appear to possess a slow turnover rbc CA isozyme with biochemical properties similar to mammalian CA I (Henry et al., 1993). Agnathan rbcs have also been shown to be deficient in chloride/bicarbonate exchange across the rbc membrane (Nikinmaa and Railo, 1987; Tufts and Boutilier, 1989). The absence of rbc anion exchange in combination with a slow turnover rbc CA isozyme may represent key features of a unique strategy for blood CO_2 transport in early vertebrates (Tufts and Boutilier, 1989; Tufts and Boutilier, 1990; Henry et al., 1993; Tufts and Perry, 1998). At present, however, there is still much to be learned about CA in early vertebrate rbcs. For example, there is currently no molecular sequence information available for agnathan rbc CAs. Thus, very little is known about the structural changes that may have been responsible for this large increase in the catalytic efficiency of rbc CA. The absence of sequence information for any CA isozymes from agnathans also represents an important gap in our understanding of the evolution of this important gene family in vertebrates.

On this background, the main objective of this study was to broaden our understanding of the evolution of vertebrate rbc CA by determining the molecular structure of lamprey rbc CA and comparing it to that of teleosts in an effort to ascertain the structural changes that led to a faster rbc CA isozyme in vertebrates over evolutionary time. The tissue distribution and biochemical properties of rbc CA in this ancient vertebrate species are also determined and compared to those of teleost fish. The molecular sequence information obtained in this study was also used for phylogenetic analyses that examine the evolution of cytoplasmic CA isozymes in vertebrates. These results provide invaluable insights into the evolution of this important gene family in vertebrates.

Materials and methods

Experimental animals and tissue collection

Lamprey *Petromyzon marinus* L. were captured during their spawning migration up the St Mary's River, near Sault St Marie, Ontario, Canada, and were held in freshwater tanks at the Department of Fisheries and Oceans Sea Lamprey Control Center. Individual lamprey were anaesthetized in CO_2 saturated water, and blood was collected in a heparinized syringe after opening the ventral side of the body cavity and exposing the caudal vein. The rbcs and plasma were then separated by centrifugation, and plasma was frozen immediately in liquid N_2. The rbcs were washed three times with saline and frozen in liquid N_2. Lamprey tissues (heart, brain, gut, liver, gill, kidney and muscle), were removed after perfusing the body with saline to clear it of blood. Perfusions were performed by exposing and cannulating the bulbus arteriosus with polyethylene tubing (PE 160; Clay-Adams; Missaussauga, ON, Canada), and using a syringe to push 100 ml of heparinized (50 i.u ml^{-1} sodium heparin) Cortland's saline (Wolf, 1963) through the body, followed by 500 ml of non-heparinized saline. Immediately after cannulating the bulbus arteriosus, the ventricle was severed to allow fluid in the circulatory system to drain from the body. After sampling, all tissues were carefully examined for blood clots; any observed were removed. Tissue samples were then frozen in liquid N_2 and stored at –80°C.

Carp *Cyprinus carpio* L. were obtained from a commercial fisherman on the Bay of Quinte in Southeastern Ontario, Canada. Prior to experiments, the fish were maintained in aerated de-chlorinated freshwater tanks in the animal holding facility at Queen's University. Individual fish were anaesthetized in 0.1 g l^{-1} of ethyl-*p*-aminobenzoate, and were sampled for blood *via* caudal puncture. The red blood cells and plasma were separated by centrifugation, and frozen in liquid nitrogen and stored at –80°C.

Series I: Molecular analyses

Determination of cDNA sequence

Total RNA was extracted from lamprey gills and rbcs and carp rbcs by the acid/phenol method (Chromczynski and Sacchi, 1987), as modified for fish blood (Currie et al., 1999). First strand cDNA was synthesized from purified RNA from lamprey gills and rbcs and carp rbcs using either Omniscript (Qiagen, Missaussauga, ON, Canada), or RevertAid H Minus M-MuLV (MBI Fermentas, Burlington, ON, Canada) reverse transcriptase enzymes and an oligo DT anchor primer. Internal segments (360 bp) of lamprey rbc CA, lamprey gill CA and carp rbc CA coding region were amplified by PCR, using an annealing temperature gradient of 52–60°C, and using degenerate forward primers (5′-CAG TTC CAY TTC CAY TGG G-3′) and degenerate reverse primers (5′-GAG AGY GTC ACM TGG ATC GTY-3′). All PCR reactions involved an initial denaturation at 94°C for 30 s followed by 30 cycles of 94°C for 30 s; annealing temperature for 60 s; 72°C for 90 s, and ending with a final extension for 10 min at 72°C. Both the forward and reverse primers were designed on the basis of high sequence identity among zebrafish CA (GenBank; U55177), gar (*Lepisosteus osseus*) trout CAb (GenBank; AY125007), trout CAc (GenBank AY514870), human CA I (GenBank; X05014), and human CA II (GenBank; J03037). The resulting PCR products were ligated into pDrive vectors (Qiagen) and

sequenced. This sequence information was used to perform 5′ and 3′ rapid amplification of cDNA ends (RACE).

Using the 5′ and 3′ cDNA sequence information, one final PCR was performed using primers designed to the 5′ and 3′ non-coding regions of the lamprey cytoplasmic CA sequence, and carp rbc CA sequence. A final 789 bp lamprey cytoplasmic CA product was amplified, while a final 780 bp carp rbc CA product was amplified. Both were ligated into pDrive vectors, and sequenced. The complete coding region sequences were entered in GenBank (DQ157849 and DQ157850).

Expression of carbonic anhydrase fusion proteins

The calmodulin affinity protein expression and purification system (Stratagene, Missaussauga, ON, Canada) was used to create lamprey and rainbow trout rbc CA fusion proteins. This allowed the proteins to be easily expressed, purified and quantified. In short, primers containing *Nco*I and *Bam*HI restriction sites were used to amplify both the trout CAb and lamprey CA coding regions. The resulting insert was ligated into a pDrive vector (Qiagen), and subsequently double digested with *Nco*I and *Bam*HI. The resulting insert was then directionally ligated into the expression vector pCAL-c (Stratagene), which encodes for a C-terminal calmodulin affinity tag. The plasmids were then transfected into BL21-RIPL expression cells (Stratagene). Expression of fusion proteins for both trout CAb and lamprey CA was induced in culture by exposure to 1 mmol l^{-1} IPTG (isopropyl β-D-thiogalactopyranoside). The induced bacterial cultures were pelleted by centrifugation and resuspended in $CaCl_2$ binding buffer (50 mmol l^{-1} Tris-HCl, 150 mmol l^{-1} NaCl, 10 mmol l^{-1} β-mercaptoethanol, 1 mmol l^{-1} magnesium acetate, 1 mmol l^{-1} imidazole, 2 mmol l^{-1} $CaCl_2$). The concentrated culture was then lysed using a french press (2 passes at 15 000 p.s.i.), and cell debris was removed by centrifugation. The remaining fraction, which contained soluble proteins, was run over a column packed with calmodulin affinity resin. The column was then washed with 100 column volumes of binding buffer to ensure all non-fusion proteins were removed. The fusion protein was then eluted by exposure to a buffer containing 2 mmol l^{-1} EDTA and 150 mmol l^{-1} NaCl. Protein purity was assessed by SDS-PAGE using Coomasie Blue staining, with the CA fusion protein forming a 32 kDa band. If any other bands were seen, the protein sample was diluted in $CaCl_2$ binding buffer and re-run over the calmodulin affinity resin column. The protein was concentrated using the Amicon Ultra centrifugal filter device (Millipore, Cambridge, ON, Canada). Protein concentration was determined *via* the Coomasie Blue protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard, and diluted to a working stock of 250 nmol l^{-1} prior to biochemical assays.

Northern blot analysis

For northern blots, 10 μg of total RNA was fractionated by glyoxal/dimethyl sulphoxide (DMSO) denaturing electrophoresis on a 1% agarose gel and transferred to a Duralon nylon membrane (Stratagene) using 20× standard saline citrate (SSC). Membranes were ultraviolet-crosslinked (Fisher UV crosslinker, Ottawa, ON, Canada) twice at optimal setting prior to hybridization.

Probes for lamprey CA and lamprey hemoglobin were generated from first strand cDNA from lamprey rbc mRNA. The lamprey hemoglobin probe was a 427 bp fragment that was amplified using the forward primer (5′-GGA AGT GTT GCG CCT CTG ATG-3′) and reverse primer (5′-GGC GGA CCT GAG CAG GAT G-3′), at an annealing temperature of 57°C. The lamprey CA probe was a 360 bp fragment that was amplified as described above. Probes were labelled using [α-^{32}P]dCTP (specific activity 10^9 c.p.m. μg^{-1} DNA) and the Ready-To-Go labelling system (Pharmacia; Piscataway, NJ, USA). Membranes were prehybridized at 60°C for 3 h in Church's buffer. Blots were then hybridized overnight in the same solution at 60°C, with approximately 10^9 c.p.m. of denatured probe. The blots were then washed twice using 1× SSC/0.1% SDS solution (20 min, 60°C) and once using 0.25× SSC/0.1% SDS (20 min, 60°C). Finally, blots were exposed to a phosphor screen (Kodak; Rochester, NY, USA) and visualized and quantified using a phosphoimager (Molecular Devices; Sunnyvale, CA, USA) driven by ImageQuant software (Molecular Devices). All membranes were also probed with a human 18S rRNA probe (Battersby and Moyes, 1998) to correct blots for loading differences, and were expressed relative to the band with the greatest density.

Sequence analysis

A phylogenetic analysis of amino acid sequences was also carried out, which included lamprey CA, rainbow trout TCAb and TCAc, gar rbc CA, dace gill CA, zebrafish retina CA, zebrafish cytoplasmic CA (GenBank; NM_199215) and zebrafish CA VII (GenBank; BC049309). This analysis also included: mouse CA I (GenBank; NM_009799), CA II (GenBank; BC055291), CA III (GenBank; NM_007606) and CA VII (GenBank; NM_053070); human CA I, CA II, CA III (GenBank; NM_005181), CA Va (GenBank; NM_001739), CA Vb (GenBank; NM_007220) and CA VII. Alignments used for the phylogenetic analysis were performed by ClustalX (version 1.81). Phylogenetic hypotheses were constructed using both neighbour joining (NJ) (Saitou and Nei, 1987) and maximum parsimony (MP) as performed by PAUP* (beta test version 4.0b10) (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 1000 random addition replicates. NJ was performed on a matrix of mean character distances. Support for nodes for both analytical procedures was performed using the bootstrap analysis with 1000 pseudoreplicates. All analyses were performed using human CA V as an outgroup, as previously described (Hewett-Emmett and Tashian, 1996).

Gaps in sequence alignment were accounted for in three distinct series of analyses. In the first analysis, all possible informative gaps were included and treated as missing data. In the second analysis, all gaps were removed, and in the third analysis, all gaps were treated as a distinct character state.

The final analysis could only be performed using MP analysis. All subsequent trees were compared qualitatively for differences.

Series II. Biochemical analyses

Tissue homogenization and fractionation

To facilitate homogenization, lamprey tissues (0.4–1.5 g; N=4) were cut into fine pieces using scissors and a scalpel. The tissue was then added to 8 volumes of refrigerated Tris buffer (in mmol l^{-1}: 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.4 with 10% phosphoric acid) per gram tissue and homogenized using a motor driven Teflon-glass homogenizer until no pieces of tissue remained (approximately 5 passes). Next, the crude homogenate was centrifuged (100 000 ***g*** for 90 min, Beckman L8-55M ultracentrifuge) (Henry et al., 1993) at 4°C to remove cellular debris, mitochondria and membrane fractions from the tissue cytoplasmic fraction. The cytoplasmic fractions were then examined to determine the relative levels of CA.

Measurement of carbonic anhydrase activity

Carbonic anhydrase activity was measured *via* the electrometric ΔpH method (Henry, 1991; Henry et al., 1993). The reaction medium consisted of 10 ml of Tris buffer kept at 4°C. After the enzyme source was added, the reaction was started by the addition of 400 μl of CO_2 saturated distilled water from a 1000 μl gas tight Hamilton syringe. The reaction velocity was measured over a pH change of 0.15 units. To obtain the true catalyzed reaction rate, the uncatalyzed rate was subtracted from the observed rate, and the buffer capacity was taken into account to convert the rate from pH units/time to mol H^+/time. The pH was measured using a Radiometer GK2401 C combined pH electrode connected to a Radiometer PHM64 research pH meter (Lyon, France).

Kinetic analysis

Lamprey rbc lysates were prepared by diluting 1 volume of rbcs in 100 volumes of ice cold double distilled water. The concentration of CA in the lysates was obtained by measuring CA activity in the presence of different concentrations of acetazolamide (Az), a potent CA inhibitor. These data were then plotted on an Easson–Stedman plot (Easson-Stedman, 1937), using the equation:

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

where I_o is the inhibitor concentration, i is the fractional inhibition at a given inhibitor concentration, K_i is the inhibition constant, and E_o is the concentration of enzyme (Maren et al., 1960; Maren and Swenson, 1980; Henry et al., 1993). For each inhibitor concentration, assays were performed in duplicate and the mean activity was plotted. E_o and K_i Az values were calculated for each sample. For each trial, the eu (enzyme units) value was determined and a ratio of E_o/eu was then calculated; the E_o of further samples could then easily be determined based on the calculated eu (Maren and Swenson, 1980; Maren et al., 1993).

Experiments were then conducted on both rbc lysates and fusion proteins to examine the velocity of CO_2 hydration at increasing concentrations of CO_2. The reciprocals of these values were plotted on a Lineweaver–Burke plot (Maren et al., 1980; Henry et al., 1993), from which the V_{max} and K_m values were obtained. The enzyme units (eu) were kept between 1 and 2 (Maren et al., 1960), and these values were recorded for each trial.

The catalytic rate constant (k_{cat}) was then calculated using the formula:

$$k_{cat} = V_{max} / E_o ,$$

as previously described (Maren et al., 1980). The inhibition constant for copper and iodide was also calculated, using the method of Dixon (Dixon, 1953). Mean values of k_{cat}, K_m, K_i Az, K_i Cu^{2+} and K_i I^- were obtained for the four samples. For all analyses, all points were assayed in duplicate. The turnover number for fusion proteins was calculated in duplicate to ensure repeatability.

Statistical analysis

All values are expressed as means ± s.e.m. (N=4), with the exception of the fusion protein k_{cat} numbers that are means of duplicate tests. The cytoplasmic CA activity from various tissues was evaluated for significant differences using an ANOVA. If the ANOVA indicated significant differences between tissues, a PLSD *post hoc* test was used to determine which values were significantly different. The fiducial level of significance was 5%.

Results

Isolation and sequencing of the final cDNA product for lamprey rbc CA and cytoplasmic gill CA resulted in identical products, which consisted of a complete coding region of 789 bp, or 262 amino acids (Fig. 1). For comparative purposes, a high activity rbc CA isozyme from carp (*Cyprinus carpio*) was also isolated and sequenced, which resulted in a 780 bp, or 260 amino acid, coding region.

Tissue distribution

The tissue distribution of the cytoplasmic CA sequenced from lamprey rbc and gills was examined *via* northern blot analysis of RNA from perfused lamprey tissues (Fig. 2). This analysis showed that lamprey cytoplasmic CA was highly expressed in both the rbc and gills, and also in the brain and kidney. Expression levels of CA in the gills and rbcs were approximately equal, while levels in the brain and kidney were considerably lower. Lamprey haemoglobin expression levels were also examined to verify that blood, and thus rbc CA, was successfully removed from the tissues during perfusion. To ensure that the distribution of cytoplasmic CA activity in lamprey tissues corresponded to the levels of cytoplasmic CA expression, an additional biochemical analysis of the cytoplasmic CA activity in tissues was performed (Fig. 3). The levels of CA activity were highest in the rbc, followed by the

```
1      atgtccggacatcactgggggtatggcgaggagaacggccccgccgagtggcacaaggac
        M  S  G  H  H  W  G  Y  G  E  E  N  G  P  A  E  W  H  K  D
61     ttccagatcgccaaaggggagcggcagtcgcccatcgacatccagccgggcgaggcgacg
        F  Q  I  A  K  G  E  R  Q  S  P  I  D  I  Q  P  G  E  A  T
121    tacgatgccaccctgaagcccctctcggtgatctacgaccccgcgtctgccctcagcatg
        Y  D  A  T  L  K  P  L  S  V  I  Y  D  P  A  S  A  L  S  M
181    ggcaacaacggccactccttctccgtggagtacgacgactccggcgagaagtgcgtgttg
        G  N  N  G  H  S  F  S  V  E  Y  D  D  S  G  E  K  C  V  L
241    agtgggggggcccctgcccaacccgtacaagctgaagcagttccacttccactgggggct
        S  G  G  P  L  P  N  P  Y  K  L  K  Q  F  H  F  H  W  G  A
301    gcggacggcagtggctctgagcacaccgtggccggcaagacgtactccgctgagctgcac
        A  D  G  S  G  S  E  H  T  V  A  G  K  T  Y  S  A  E  L  H
361    ctcgtccactggaactcggccaagtacaagagcttcgcggaggcggccaacaagagcgat
        L  V  H  W  N  S  A  K  Y  K  S  F  A  E  A  A  N  K  S  D
421    ggcctcgccgttctgggcgtcttcctcgaggctggtgcggagaaccctggtctgaaaaag
        G  L  A  V  L  G  V  F  L  E  A  G  A  E  N  P  G  L  K  K
481    gtcacggacactctgaacatcatcaggagcaagggagccaaggtggacttcctcgactat
        V  T  D  T  L  N  I  I  R  S  K  G  A  K  V  D  F  L  D  Y
541    gacccctcggttctcctgcccaagtcactggacttctggacctacctgggctcgctcacg
        D  P  S  V  L  L  P  K  S  L  D  F  W  T  Y  L  G  S  L  T
601    accccgccgctcttcgagagcgtcacctggatcgtgttcaaggagccgatccccgccagc
        T  P  P  L  F  E  S  V  T  W  I  V  F  K  E  P  I  P  A  S
661    aaggagcagttggcgcgcttccgcgagctgctcttcacgtgcgagggggacagcgagaac
        K  E  Q  L  A  R  F  R  E  L  L  F  T  C  E  G  D  S  E  N
721    tgcatggtggacaactaccgtccgccacagcccctgggcggacgcacggtgcgcgcctcc
        C  M  V  D  N  Y  R  P  P  Q  P  L  G  G  R  T  V  R  A  S
781    ttccagtga
        F  Q  *
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Fig. 1. Nucleotide and deduced amino acid sequence of lamprey rbc carbonic anhydrase. The sequence shown is the coding region only, from start codon (underlined) to stop codon (asterisk), as determined through RACE (rapid amplification of cDNA ends).

gill and kidney. The heart, liver and muscle had negligible levels of cytoplasmic CA activity.

Sequence analysis

The coding region sequences for both lamprey and carp CA were aligned with, and compared to, CA sequences for rainbow trout CAb and CAc, as well as gar rbc CA (Table 1). With the exception of lamprey CA, all the fish CA sequences had a high degree of similarity that ranged from 70–76% nucleotide, or 73–78% amino acid. In contrast, lamprey cytoplasmic CA was less similar, with alignment scores ranging from 61–65% nucleotide, or 58–61% amino acid. When the fish CAs were compared to human CA II and CA VII, however, all sequences showed approximately equal similarity, with nucleotide alignment scores ranging from 58–65% for CA VII and 61–65% for CA II (not shown).

NJ analysis of vertebrate cytoplasmic CA isozymes produced a generally well-supported phylogenetic tree (Fig. 4). This analysis grouped the carp rbc CA sequence within the previously described fish cytoplasmic CA clade, with it being most closely grouped with a zebrafish CA sequence from the retina. Lamprey CA, however, did not group within the fish CA clade, instead grouping most closely with vertebrate CA VII. MP parsimony analyses produced similar phylogenetic trees with the exception that lamprey CA formed a polytomy with vertebrate CA VII, instead of being included within the clade.

The final aspect of sequence analysis consisted of an examination of the amino acid residues of the active site pocket (Table 2). Only three amino acid differences were found when the active site pocket of lamprey CA was compared to the high activity cytoplasmic CA isozymes of a representative teleost, the rainbow trout. Similarly carp rbc CA had only two amino acid differences when compared to trout isozymes, while lamprey and carp differed from each other by only four amino acids. In addition, lamprey only differed from the high activity human CA II and VII isozymes by four amino acids. In contrast, lamprey CA differed from the low activity human CA I isozyme by nine amino acids. The high activity carp CA isozyme also only differed from human CA II by four amino acids and only differed from human CA VII by two amino acids.

Kinetic analyses

The kinetic properties and inhibitor characteristics of lamprey cytoplasmic CA from rbc lysates were also examined (Table 3). The K_i values for acetazolamide and copper were

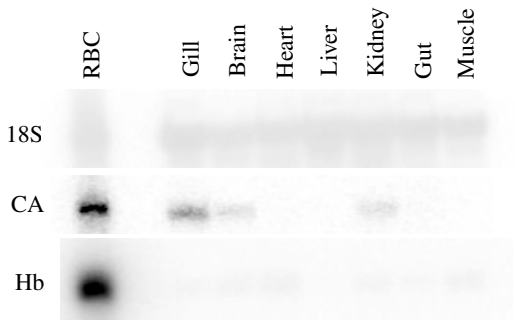

Fig. 2. Representative northern blots for lamprey cytoplasmic carbonic anhydrase (CA) and haemoglobin (Hb) mRNA, and 18S rRNA from perfused adult lamprey tissues (N=4). RBC, red blood cell.

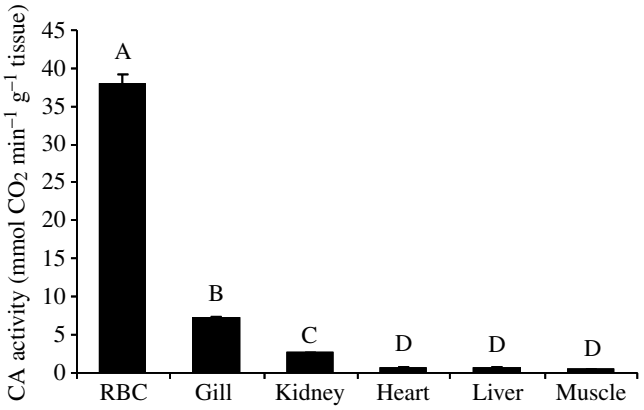

Fig. 3. Carbonic anhydrase (CA) activity in the cytosolic fraction of perfused adult lamprey tissues. Values are means ± s.e.m. (N=4), with statistically different groups indicated by different letters (P<0.05). RBC, red blood cell.

both higher than those previously described for rainbow trout, and an intermediate fish, the longnose gar. In contrast, the K_i value for iodide was approximately equal to values previously described for other fish. The turnover number (k_{cat}) of lamprey CA was approximately 70 times lower than that of the high activity trout CAb, and about 17 times lower than longnose gar rbc CA. The substrate affinity number (K_m) of lamprey rbc CA was also lower than numbers previously reported for trout and gar. In addition, the total amount of CA activity in lamprey rbcs was about 10 times lower than that in trout rbcs (Table 4). In contrast, fusion proteins of both trout CAb and lamprey CA were found to have almost identical turnover numbers, and both were consistent with high activity isozymes (Table 5).

Table 1. *Matrix diagram comparing nucleotide/amino acid sequence identity of lamprey red blood cell carbonic anhydrase and other fish cytoplasmic carbonic anhydrase isozymes*

	Nucleotide/amino acid sequence identity (%)			
	Trout CAc	Trout CAb	Gar CAb	Carp CAb
Lamprey	65/61	61/58	62/60	61/59
Trout CAc		73/78	70/77	70/73
Trout CAb			72/74	76/78
Gar CAb				71/75

Isozymes isolated from red blood cells are represented as CAb, and general cytoplasmic isozymes CAc.

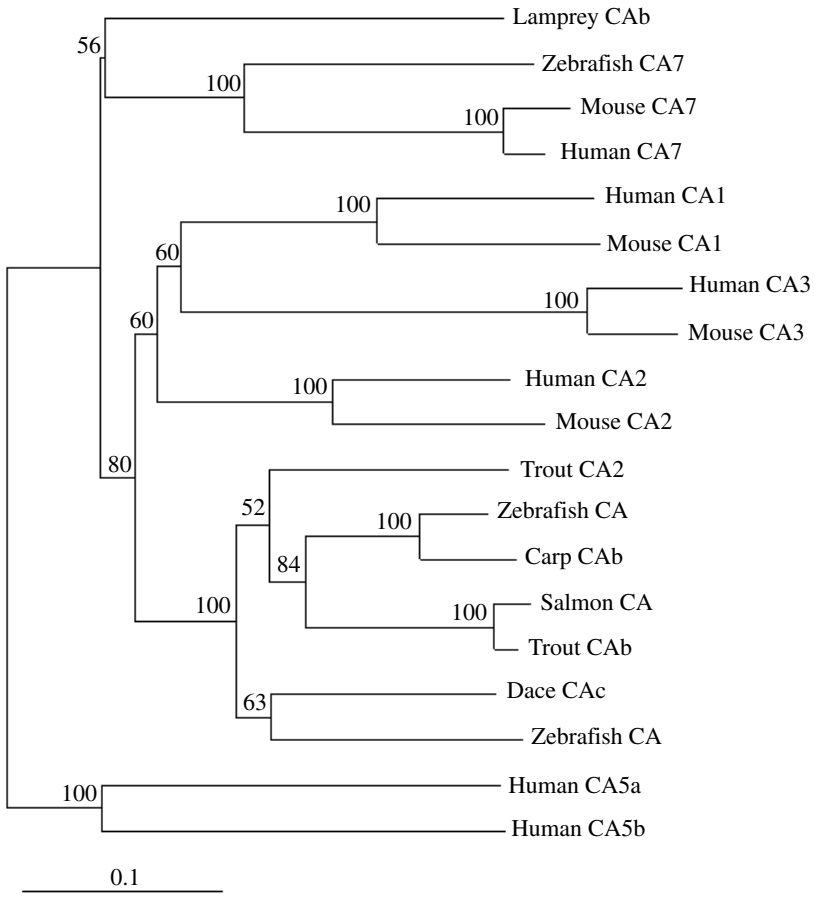

Fig. 4. Phylogenetic analysis of cytoplasmic lamprey carbonic anhydrase and other cytoplasmic α-carbonic anhydrase isozymes. The phylogenetic tree was constructed using neighbour joining analysis with support for nodes assessed using bootstrap analysis. The tree was ordered using human carbonic anhydrase Va and Vb as a monophyletic outgroup. Branches are drawn to scale (bar) with the length of 0.1 approximating replacement of 10% of the amino acids in the protein alignment (no Poisson correction for multiple hits).

Discussion

The results of this study suggest that lamprey have only a single cytoplasmic CA isozyme that is expressed in numerous tissues throughout the body. Despite previous evidence, this isozyme is, in fact, a high turnover isozyme similar to that found in modern teleosts, such as rainbow trout.

The lamprey cytoplasmic CA isozymes from rbcs and gills both had the same 789 bp (262 amino acids; Fig. 1) coding region. This is in contrast to what has been shown in a representative teleost, the rainbow trout, which has a distinct high activity isozyme in the rbcs and a second high activity isozyme that is widely expressed throughout the body, including in the gills (Esbaugh et al., 2005). Further examination of the lamprey cytoplasmic CA revealed that it was also highly expressed in the brain, and to a lesser extent in the kidney (Fig. 2). The cytoplasmic CA found in the rbc is primarily involved in facilitating CO_2 transport (Geers and Gros, 2000), while cytoplasmic CA activity in the gill and kidney are primarily involved in providing counter ions for transport processes (Chegwidden and Carter, 2000; Marshall, 2002). Cytoplasmic CA in the brain has numerous functions (Chegwidden et al., 2000). Unlike rainbow trout and other vertebrates, however, there is no measurable expression of cytoplasmic CA in the lamprey muscle, liver or gut (Esbaugh et al., 2005). To verify this finding and ensure that a second CA isozyme is not present in these tissues, cytoplasmic fractions of various tissue homogenates were tested for CA activity (Fig. 3). Similar to the northern blot analysis, cytoplasmic CA activity was negligible in heart, liver and muscle. The lack of cytoplasmic CA activity in the gut and liver, where cytoplasmic CA usually provides protons and bicarbonate for numerous processes (Chegwidden et al., 2000), may be due to the life cycle stage of the lamprey that were studied. It is well known that during the spawning stage of the lamprey life cycle, the digestive system degrades (Sidon and Youson, 1983a; Sidon and Youson, 1983b). Thus, higher levels of CA may be expressed in

Table 2. *Comparison of the active site for lamprey red blood cell carbonic anhydrase (CA) to isozymes from trout and carp, as well as to consensus CA I and II[1] and human CA VII*

Lamprey	Y	S	N	N	H	S	F	S	E	K	Q	H	H	E	H	E	H	V	F	L	V	G	W	Y	L	T	T	P	P	F	S	V	W	V	N	R	
	*	*						*			*	*	*	*	*	*	*							*		*	*						*		*	*	aa
					+							z	z				z	~		~	~				~							~	~			difference
Trout B	.	.	.	.	.	.	.	Q	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	3
Trout C	.	.	.	.	.	.	.	Q	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	3
Carp B	.	.	.	.	.	.	.	Q	D	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.	.	.	.	4
CA I	.	.	.	V	.	.	.	H	N	F	.	.	.	.	.	.	.	.	L	.	I	.	.	.	.	H	.	.	H	.	.	.	I	.	.	9
CA II	.	.	.	.	.	.	.	N	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	C	.	.	.	.	.	4
CA VII	.	.	.	.	.	.	V	Q	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	4

[1]Tashian et al., 2000.
*Putative active site; z, zinc binding ligand; +, proton shuttling ligand; ~, substrate associated pocket.

lamprey during the feeding stage of their life cycle. It remains unclear, however, whether other CA isozymes may be present at very low levels in various lamprey tissues, or if very low turnover isozymes, analogous to mammalian CA III, may be present.

The lamprey cytoplasmic CA sequence was moderately similar to both trout CAb and CAc, and gar rbc CA, with nucleotide and amino acid alignment scores ranging from 58–65% (Table 1). This is considerably less similar than other fish isozymes are to each other, with alignment scores ranging from 70–77% for nucleotide and amino acid alignments. For comparative purposes, a carp rbc CA was also sequenced, which yielded a 780 bp coding region. This sequence also had very high sequence similarity to the other fish CA isozymes for both nucleotide (70–76%) and amino acid (73–78%) alignments. In contrast, the carp and lamprey sequences were less similar. Interestingly, lamprey CA and the other fish CAs showed equal similarity when compared to the high turnover human isozymes, CA II and CA VII. In addition, phylogenetic analyses of vertebrate cytoplasmic CAs did not group lamprey CA closely to other cytoplasmic fish CA sequences (Fig. 4). Unlike carp CA, which grouped within the previously described fish cytoplasmic CA clade (Lund et al., 2002; Tufts et al., 2003; Esbaugh et al., 2004; Esbaugh et al., 2005), lamprey CA grouped closely with vertebrate CA VII. Interestingly, vertebrate CA VII is thought to be ancestral to both mammalian CA I, II and III, as well as the fish cytoplasmic CAs (Lund et al., 2002; Esbaugh et al., 2004; Esbaugh et al., 2005), and is not known to have any physiological function (Lakkis et al., 1996; Lakkis et al., 1997; Earnhardt et al., 1998). It is unclear whether lamprey CA is actually a CA VII-like isozyme due to the discrepancies between the NJ and MP methods; however, it is certain that the lamprey isozyme is ancestral to cytoplasmic isozymes found in more derived vertebrates. This is consistent with the fact that lamprey are modern day representatives of an ancient vertebrate lineage.

The final analysis of the molecular structure of lamprey CA examined the active site pocket, which is the site of enzymatic activity, and compared it to that of other cytoplasmic CA isozymes (Table 2). Interestingly, the low activity lamprey CA isozyme had only three amino acid differences from the active site pocket of the two rainbow trout isozymes, which are characterized as high turnover isozymes (Maren et al., 1980; Henry et al., 1993; Tufts et al., 2003; Esbaugh et al., 2004; Esbaugh et al., 2005). In addition, lamprey CA had only four amino acid differences from the high turnover carp rbc CA (Esbaugh et al., 2004). Even more intriguing, none of the

Table 3. *Biochemical characteristics of carbonic anhydrase from red blood cell lysates of lamprey, trout and gar*

	K_m (mmol l^{-1})	k_{cat} (e^4 s^{-1})	K_i Az (nmol l^{-1})	K_i Cu^{2+} (μmol l^{-1})	K_i I^- (mmol l^{-1})
Lamprey	13.3±4.5	0.44±0.12	4.4±0.2	0.87±0.02	3.51±0.35
Trout	23.3[1]	30.28[1]	1.3[1]	0.1[2]	3.3[2]
Gar[2]	16.8	7.7	1.7	0.5	4.5

[1]Esbaugh et al., 2005; [2]Lund et al., 2002.

Table 4. *The overall carbonic anhydrase activity in red blood cells from rainbow trout and lamprey*

	CA activity (mmol ml^{-1} rbc)
Lamprey	16.73±0.49
Trout	155.43±9.67

Values are means ± s.e.m., *N*=4.

Table 5. *Catalytic characteristics of carbonic anhydrase from lamprey and trout red blood cell lysates, and fusion proteins of lamprey and trout red blood cell carbonic anhydrase*

	Lysates		Fusion proteins	
	K_m (mmol l^{-1})	k_{cat} (e^4 s^{-1})	K_m (mmol l^{-1})	k_{cat} (e^4 s^{-1})
Lamprey	13.3±4.5	0.44±0.12	23.9	9.5
Trout	23.3[1]	30.28[1]	18	7.7

Lamprey lysate values: *N*=4; fusion protein values represent means of duplicate protein purifications.
[1]Esbaugh et al., 2005.

mentioned amino acid differences appear in residues that have been implicated in enzyme function (Stams and Christianson, 2000). The same trend arises when lamprey CA is compared to mammalian CA isozymes I, II and VII. Only four amino acid differences occur when compared to the high activity CA II and VII isozymes, again with no differences occurring in residues thought to be critical for enzyme function. There are nine amino acid differences, however, when lamprey CA is compared to low activity human CA I. These differences include Val-62, His-67 and His-200, which cause CA I to have a reduced active site cavity, interrupting the high levels of proton transfer found in CA II and CA VII (Lindskog and Silverman, 2000; Stams and Christianson, 2000). In contrast to the previous biochemical data, these molecular analyses suggest that lamprey CA is not a low activity isozyme, but is in fact a high turnover isozyme.

To further examine this issue, a biochemical analysis of lamprey CA was performed to examine its catalytic efficiency and kinetic properties (Table 3). The inhibition constants of lamprey CA to iodide and copper are both similar to values reported for high turnover isozymes from rainbow trout and gar (Lund et al., 2002). It is also noteworthy that these values are closer to mammalian CA II than to CA I (Lund et al., 2002). Similar to what has been previously reported for agnathans (Henry et al., 1993; Maren et al., 1980), lamprey CA is more resistant to inhibition by acetazolamide than other fish isozymes (Gervais and Tufts, 1999; Lund et al., 2002; Esbaugh et al., 2004). It should be noted, however, that the inhibition constant reported here is substantially lower than that reported previously for lampreys (Henry et al., 1993), although both are much higher than those of teleosts. Interestingly, reduced sensitivity towards acetazolamide and other sulfonamide inhibitors is characteristic of low turnover CA I. In addition, the turnover number of lamprey CA is approximately two orders of magnitude lower than reported for rainbow trout (Maren et al., 1980; Esbaugh et al., 2004; Esbaugh et al., 2005), and the total amount of CA activity in lamprey rbcs is approximately 10 times less than that in trout rbcs (Table 4). These biochemical data therefore concur with previous reports that agnathans have a low turnover isozyme in their rbcs.

Interestingly, the biochemical and molecular data for lamprey cytoplasmic CA appear to be in conflict. The biochemical results indicate that lamprey rbcs possess a low turnover isozyme similar to that of human CA I. In contrast, molecular results indicate that the molecular structure of lamprey CA very closely resembles that of a high activity isozyme. Two explanations can be proposed to clarify the disparity in the biochemical and molecular data. The first explanation is that changes in the molecular structure outside the active site substantially altered the formation of the active site, giving lamprey CA an entirely novel catalytic mechanism. This, however, seems unlikely as the residues of the active site are almost entirely conserved, suggesting a conserved function. The second explanation is that the traditional method (Maren et al., 1960; Maren and Rittmaster, 1977; Maren et al., 1980; Sanyal et al., 1982; Henry et al., 1993; Maren et al., 1993; Gervais and Tufts, 1999; Lund et al., 2002) of estimating enzyme concentration during biochemical assays is affected by the low sensitivity of lamprey CA to acetazolamide, thus causing an overestimate of enzyme concentration in assay preparations. In brief, the traditional method for testing the turnover number of CA from tissue lysates involves titrating CA with acetazolamide, a potent CA inhibitor, to estimate enzyme concentration *via* an Easson–Stedman plot. This method, however, has been widely used since 1960, and is the basis for almost all comparative CA work with regard to catalytic efficiency.

To examine the possibility that the traditional method of evaluating the catalytic properties of CA may cause an underestimate of the turnover number in lamprey, an experiment was performed using trout and lamprey rbc CA fusion proteins. By creating and purifying fusion proteins for both isozymes, a known concentration of each enzyme could be added directly to CA assay preparations. This removed any possible effect of differing acetazolamide sensitivities on turnover number calculations. When this experiment was performed, the turnover numbers of trout and lamprey rbc CA fusion proteins were found to be almost identical (Table 5). Despite previous reports to the contrary, the results of this experiment unequivocally show that lamprey CA is, in fact, a high turnover isozyme. The observed differences in overall CA activity in trout and lamprey rbcs (Table 4) are, therefore, likely due to variation in the amount of enzyme within the rbcs of each species.

The results of this study have a profound impact on the current theories of evolution of rbc CA isozymes in vertebrates. This study has shown that a high activity CA isozyme was likely present in rbcs very early in vertebrate evolution. The combined results of this study, including biochemical analyses, tissue expression results and phylogenetic hypotheses, also suggest that early vertebrates may have had only a single high activity cytoplasmic CA isozyme. This idea is based on the unique placement of lamprey CA in the phylogenetic analysis of the α-CA gene family, and the broad tissue expression of this isozyme in lamprey, accounting for the CA activity in most major tissues. After the divergence of gnathostomes, this single isozyme may have proceeded to evolve into the plethora of cytoplasmic CA isozymes that are found in modern vertebrates. To investigate this idea, additional work should be performed on both lamprey and hagfish, to examine tissues for cytoplasmic CA isozymes that may have very low expression, or very low catalytic efficiency. In addition to these important implications, these findings also contradict the idea that the evolution of high activity CA in the rbc coincided with the adoption of high activity anion exchange in the rbc membrane (Henry et al., 1993). Instead, it seems that CA is a remarkably conserved protein throughout the vertebrate lineage, and that variation in rbc CA activity is simply the result of variation in enzyme abundance. This idea is also supported by the recent finding that differences in rbc CA activity between species of teleost fish can be entirely attributed to differences in CA concentration, rather than differences in CA catalytic properties (Esbaugh et al.,

2004). This is in stark contrast to other important rbc proteins, such as haemoglobin, band 3, and the Na^+/H^+ exchanger, which show major variation among vertebrates.

In conclusion, our combined molecular and biochemical analysis of the rbc CA isozyme from the sea lamprey, an extant member of a very ancient vertebrate lineage, has filled an important gap in our knowledge of the early evolution of rbc CA. Lamprey rbc CA is a widespread, high activity CA isozyme, that is closely related to vertebrate CA VII. Thus, a high activity rbc CA probably evolved early in vertebrate evolution, and the critical elements of its structure have been highly conserved. Variation in rbc CA activity appears to be simply due to variation in abundance, and not to major structural changes in the enzyme itself.

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