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Cytoplasmic carbonic anhydrase isozymes in rainbow trout *Oncorhynchus mykiss*: comparative physiology and molecular evolution

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Summary

It is well established that the gills of teleost fish contain substantial levels of cytoplasmic carbonic anhydrase (CA), but it is unclear which CA isozyme(s) might be responsible for this activity. The objective of the current study was to determine if branchial CA activity in rainbow trout was the result of a general cytoplasmic CA isozyme, with kinetic properties, tissue distribution and physiological functions distinct from those of the red blood cell (rbc)-specific CA isozyme. Isolation and sequencing of a second trout cytoplasmic CA yielded a 780 bp coding region that was 76% identical with the trout rbc CA (TCAb), although the active sites differed by only 1 amino acid. Interestingly, phylogenetic analyses did not group these two isozymes closely together, suggesting that more fish species may have multiple cytoplasmic CA isozymes. In contrast to TCAb, the second cytoplasmic CA isozyme had a wide tissue distribution with high expression in the gills and brain, and lower expression in many tissues, including the red blood cells. Thus, unlike TCAb, the second isozyme lacks tissue specificity and may be expressed in the cytoplasm of all cells. For this reason, it is referred to hereafter as TCAc (trout cytoplasmic CA). The inhibitor properties of both cytoplasmic isozymes were similar (K_i acetazolamide 1.21±0.18 nmol l^{-1} and 1.34±0.10 nmol l^{-1} for TCAc and TCAb, respectively). However, the turnover of TCAb was over three times greater than that of TCAc (30.3±5.83 *vs* 8.90±1.95 e^4 s^{-1}, respectively), indicating that the rbc-specific CA isoform was significantly faster than the general cytoplasmic isoform. Induction of anaemia revealed differential expression of the two isozymes in the red blood cell; whereas TCAc mRNA expression was unaffected, TCAb mRNA expression was significantly increased by 30- to 60-fold in anaemic trout.

Key words: carbonic anhydrase, red blood cell, gill, isozyme, evolution, anaemia.

Introduction

Carbonic anhydrase (CA) is a ubiquitous enzyme that catalyses the reversible hydration/dehydration reactions of carbon dioxide (CO_2). This enzyme has been found in virtually all organisms, but only members of the α-CA family are found in vertebrates (Tashian, 1992; Hewett-Emmett and Tashian, 1996; Hewett-Emmett, 2000). To date, 15 different α-CA isozymes have been characterized in mammals by means of their kinetic properties, subcellular location and/or molecular structure (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 (Henry, 1996; Chegwidden and Carter, 2000; Geers and Gros, 2000). Unlike in mammals, however, very few CA isozymes have been identified in the tissues of non-mammalian groups, such as fish. Although many fish tissues have CA activity (Dimberg et al., 1981; Sanyal, 1984; Conley and Malalatt, 1988; Henry et al., 1988, 1993), few isozymes have been kinetically characterized, and there is little information regarding the molecular structure of these isozymes. Moreover, most of the information that is available pertains to red blood cell (rbc) CA activity, as blood is a particularly abundant source of CA (Henry and Swenson, 2000).

CA activity was originally found in the gills of fish, over 60 years ago (Sobotka and Kann, 1941). Branchial CA activity is thought to be largely cytoplasmic, occurring primarily in pavement cells and chloride cells (Lacy, 1983; Conley and Malalatt, 1988; Rahim et al., 1988; Flügel et al., 1991; Sender et al., 1999; Wilson et al., 2000), with the exception of membrane bound isozymes found in the gills of elasmobranchs (Swenson and Maren, 1987; Gilmour et al., 1997, 2001, 2002; Henry et al., 1997; Wilson et al., 2000), and Antarctic fishes, *Chaenocephalus aceratus* and *Notothenia coriiceps* (Tufts et al., 2002). Evidence is mixed on whether the same CA isozyme

is present in both rbcs and gill tissue. Rahim et al. (1988) provided immunological evidence of a gill CA isozyme in rainbow trout *Oncorhynchus mykiss* and carp *Cyprinus carpio* that was distinct from the rbc CA isozyme. By contrast, Sender et al. (1999) found that the gill and rbc CA enzymes in the flounder *Platichthys flesus* were not immunologically distinct. Recently, however, Esbaugh et al. (2004) provided further evidence of CA activity in the gill cytoplasm of rainbow trout that was not that of the rbc CA isozyme. It is therefore unclear what isozyme is responsible for the gill cytoplasmic CA activity in teleosts.

Blood and branchial CA activities serve different functions. The primary physiological role of rbc CA is to catalyse the hydration of CO_2 to HCO_3^- at the tissue site of production, and dehydration of HCO_3^- to CO_2 at the respiratory surface, to facilitate the transport and excretion of CO_2 from the body (Perry, 1986; Perry and Laurent, 1990; Henry and Heming, 1998; Tufts and Perry, 1998; Henry and Swenson, 2000). Selective pressures preventing the rate of these reactions from limiting CO_2 transport and excretion are believed to be the primary forces driving the increase in rbc CA catalytic rate that is apparent through the fish lineage (Henry et al., 1993; Tufts et al., 2003). In contrast, the primary purpose of cytoplasmic gill CA is to catalyse the hydration/dehydration reactions of CO_2 within the branchial epithelium to provide counter ions for ion exchange processes that regulate acid–base balance and ionic homeostasis (Henry and Heming, 1998; Henry and Swenson, 2000; Marshall, 2002). A notable exception, however, is the membrane-associated CA in the gills of dogfish, which contributes to CO_2 excretion (Gilmour et al., 2001).

The main objective of this study was to determine if branchial CA activity in rainbow trout was the result of a general cytoplasmic CA isozyme, with kinetic properties, tissue distribution and physiological functions distinct from those of the rbc-specific CA isozyme. In particular, the trend from agnathans to teleosts towards a faster rbc CA isozyme (Henry et al., 1993; Tufts et al., 2003), leads to the prediction that a trout general cytoplasmic CA isozyme with a wide tissue distribution will exhibit a lower turnover number (slower catalytic rate) than the rbc-specific isozyme. In addition, a second series of experiments tested the differential regulation of the two cytoplasmic CA isozymes in response to a physiological disturbance, anaemia.

Materials and methods

Experimental animals and tissue collection

Rainbow trout *Oncorhynchus mykiss* Walbaum used at Queen's University were obtained from Pure Springs Trout Farm (Belleville, Ontario, Canada); rainbow trout used at the University of Ottawa were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). Prior to experiments, the fish were maintained in large fibreglass tanks supplied with flowing, aerated, dechloraminated (Ottawa) or dechlorinated (Kingston) freshwater at 13°C, and were fed to satiation with commercial trout pellets on alternate days. The photoperiod was 12 h:12 h L:D, and fish were acclimated to the holding facility for at least 2 weeks prior to use.

To induce anaemia, rainbow trout (61.5±1.5 g, N=23; mean ± S.E.M.) were lightly anaesthetised in a solution of ethyl-*p*-aminobenzoate (0.1 g l^{-1}) and 1 ml of blood was removed by caudal puncture. Fish were then placed in a single 0.5 m diameter holding tank. At each of 12 h, 72 h, and 15 days, six fish were removed from the holding tank, and blood and gill tissues were sampled as described below. Haematocrit was measured in duplicate at the time of sampling, using microcapillary tubes centrifuged at 6000 ***g*** for 6 min.

Tissues were collected from individual rainbow trout that were anaesthetized in either CO_2-saturated water (kinetic analyses), or 0.1 g l^{-1} of ethyl-*p*-aminobenzoate (molecular analyses). Blood was collected into a heparinized syringe by caudal puncture and transferred to a microfuge tube. The rbcs and plasma were then separated by centrifugation and immediately frozen in liquid nitrogen. The rbc pellets used in kinetic analyses were washed three times with saline prior to being frozen, to ensure that no other blood components were present. The gills, along with other tissues (heart, brain, gut, liver, spleen, anterior and posterior kidney), 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 peristaltic pump to pump 100 ml of heparinized (50 i.u. ml^{-1} sodium heparin) Cortland's saline (Wolf, 1963) into the body, followed by 1 l of non-heparinized Cortland's saline. Immediately after cannulating the bulbus arteriosus, the ventricle was severed to allow fluid in the circulatory system to drain from the body. Upon sampling, all tissues were carefully examined for blood clots; any observed were removed. Tissue samples were then frozen in liquid nitrogen and stored at –80°C.

Series I: kinetic analysis of rainbow trout gill and rbc cytoplasmic CA

Tissue homogenization and fractionation

To facilitate homogenization, adult trout tissues (1–2 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 using 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 and haemoglobin.

Measurement of carbonic anhydrase activity and haemoglobin concentration

Carbonic anhydrase activity was measured using 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 catalysed 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^{-1} to mol H^+ time^{-1}. The pH was measured using a Radiometer GK2401 C combined pH electrode connected to a Radiometer PHM64 research pH meter. Haemoglobin concentration was measured using Drabkin's method (Sigma, Oakville, CA, USA) with cyanomethaemoglobin (Sigma) as a standard.

Kinetic analysis

To determine the kinetic properties of the rbc and gill cytoplasmic CAs, experiments were conducted 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 enzyme concentration 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, 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 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 et al., 1980, 1993).

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

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

as described by Maren et al. (1980). The inhibition constant for chloride was also calculated, using the method of Dixon (1953). Mean values of k_{cat}, K_m, K_i Az and K_i Cl^- were obtained for the four samples utilised. To examine the sensitivity of each CA isozyme to the rainbow trout plasma inhibitor of CA (pICA), CA activity in the cytoplasm of the rbcs and gills was assayed in the presence of increasing volumes of separated trout plasma.

Series II: molecular analysis of rainbow trout gill cytoplasmic CA

Determination of cDNA sequence

The following procedures were performed independently by groups at Queen's University (using gill tissue), and the University of Ottawa (using whole blood). Total RNA was extracted from rainbow trout gills and whole blood by the acid/phenol method of Chromczynski and Sacchi (1987), as modified for fish blood by Currie et al. (1999), or using Trizol (Invitrogen, Burlington, ON, Canada).

First strand cDNA was synthesized from purified rainbow trout RNA from gill and whole blood using AMV reverse transcriptase (RT) and random primers. A 333 bp internal segment of rainbow trout CA coding region was amplified by PCR at an annealing temperature of 50°C, using a forward primer (CA-F; 5′-CAG TTC CAT TTC CAT TGG GG-3′) and reverse primer (CA-R; 5′-CAG AGG AGG GGT GGT CAG-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* rbc CA (GenBank, AY125007), human CA I (GenBank, X05014), and human CA II (GenBank, J03037). The resulting PCR product was ligated into a pDrive vector (Qiagen, Missaussauga, ON, Canada) and sequenced. This sequence information was used to perform 3′ rapid amplification of cDNA ends (RACE). The cDNA for 3′ RACE was amplified using the 3′ RACE adapter primer (Invitrogen) and Superscript II (Invitrogen). The 3′ sequence was amplified with nested PCR using the Abridged Universal Amplification primer (Invitrogen), and CA forward primers (1st round) (5′-CCT TGC TGT TGT AGG AGT CTT C-3′) and (2nd round) (5′-GGT CCT TGA TGC TTT TGA TG-3′). The 3′ RACE product was ligated into a PCR 2.1 vector (Invitrogen) and sequenced.

The 3′ cDNA sequence and a GenBank 5′ cDNA sequence for a rainbow trout CA homologue (CB94032) were combined to yield a 780 bp coding region (TCAc). The complete coding region sequence was entered in GenBank (AY514870).

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, Missaussauga, ON, Canada) using 20× standard saline citrate (SSC). Membranes were ultraviolet-crosslinked (Fisher UV crosslinker) twice at optimal setting prior to hybridization.

Probes for rbc CA (TCAb) and β-globin (a haemoglobin subunit) were generated from first strand cDNA from rainbow trout rbc mRNA. A 446 base pair probe for β-globin was amplified as described by Lund et al. (2000). Both the TCAb and TCAc probes were 333 base pair fragments that were amplified using the CA-F and CA-R primers, as previously described. Probes were labelled using [α-^{32}P]dCTP (specific activity 10^9 cts min^{-1} 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 cts min^{-1} 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. 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.

Real-time PCR

Total RNA was extracted from 30 mg aliquots of powdered tissue samples using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene). To remove any remaining genomic DNA, the RNA was treated on-column using RNase-free DNase (5 μl) for 15 min at 37°C. The RNA was eluted in 70 μl of nuclease-free H_2O and its quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf, Missaussauga, ON, Canada). cDNA was synthesized from 2 μg of RNA using random hexamer primers and Stratascript reverse transcriptase (Stratagene).

TCAb, TCAc and haemoglobin mRNA levels were assessed by real time PCR on samples of cDNA (0.5 μl) using a Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) and a Stratagene MX-4000 multiplex quantitative PCR system. ROX (Stratagene) was used as a reference dye. The PCR conditions (final reaction volume, 25 μl) were as follows: 0.5 μl cDNA template, 300 nmol l^{-1} forward and reverse primer, 12 μl 2×Master Mix, 1:30000 ROX final dilution. The annealing and extension temperatures over 40 cycles were 58°C (30 s) and 72°C (30 s), respectively. The following primer pairs were designed using Primer3 software: β-actin forward (5′-CCA ACA GAT GTG GAT CAG CAA-3′), β-actin reverse (5′-GGT GGC ACA GAG CTG AAG TGG TA-3′), TCAc forward (5′-CAG TCT CCC ATT GAC ATC GTA-3′), TCAc reverse (5′-CGT TGT CGT CGG TGT AGG T-3′), TCAb forward (5′-TTG GCT TTG TGG ATG ATG TT-3′), TCAb reverse (5′-AGG GGA ACT TGA TTC CAT TG-3′), haemoglobin forward (5′-ATG GTC GAC TGG ACA GAT CC-3′), haemoglobin reverse (5′-CTG AGT CCA TGG AGA CAC GA-3′).

The specificity of the primers was verified by the cloning (TOPO TA cloning kit; Invitrogen) and sequencing of amplified products. To ensure that SYBR green was not being incorporated into primer dimers or non-specific amplicons during the real-time PCR runs, the PCR products were analysed by gel electrophoresis in initial experiments. Single bands of the expected size were obtained in all instances. Furthermore, the construction of SYBR green dissociation curves after completion of 40 PCR cycles revealed the presence of single amplicons for each primer pair. To ensure that residual genomic DNA was not being amplified, control experiments were performed in which reverse transcriptase was omitted during cDNA synthesis. Relative expression of mRNAs was determined (using actin as an endogenous standard) by a modification of the delta-delta Ct method (Pfaffl, 2001). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

Sequence analysis

The TCAc sequence was compared with TCAb (GenBank, AY307082), gar rbc CA, zebrafish retina CA, and dace (*Tribolodon hakonensis*) gill CA (GenBank, AB055617) sequences, as well as human CA I, CA II and CA VII (GenBank, AY075019). Alignment of the amino acid sequences was performed using ClustalW (version 1.8) multiple sequence alignment. In addition, a comparative analysis of the active sites was performed between TCAc, TCAb, gar rbc CA and dace gill CA, as well as human CA VII and consensus CA I and CA II sequences, as reported by Tashian et al. (2000).

A phylogenetic analysis of amino acid sequences was also carried out, which included rainbow trout TCAb and TCAc, gar rbc CA, dace gill CA and zebrafish retina CA. This analysis also included: mouse CA I (GenBank, NM_009799), CA II (GenBank, BC055291), CA III (GenBank, NM_007606), CA Vb (GenBank, NM_019513) 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; rat CA I (GenBank, XM_226922), CA II (GenBank, NM_019291), CA III (GenBank, NM_019292), CA V (GenBank, NM_019293) and CA VII (GenBank, XM_226204), and chicken CA II (GenBank, X12639), *Xenopus* CA II (GenBank, BC041213) and zebrafish CA VII (BC049309). Alignment used for the phylogenetic analysis was 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 mouse and human CA VII as outgroups, as previously described by Hewitt-Emmett and Tashian (1996).

Gaps in sequence alignment were accounted for in three distinct series of analyses. In the first analysis, all possibly 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, with no major differences arising.

Statistical analysis

Values are expressed as means ± S.E.M. Statistical

differences in the kinetic properties and inhibitor sensitivities of TCAb and TCAc were analysed using unpaired Student's *t*-tests. One-way analysis of variance (ANOVA) followed by *post hoc* multiple comparisons using the Bonferroni test, as appropriate, were used to statistically analyse the effect of anaemia or acid infusion on relative rbc or gill mRNA expression of TCAb and TCAc. In all analyses, the fiducial level of significance was 5%.

Results

Sequence analysis

Isolation and sequencing of the final cDNA product for the rainbow trout gill CA (TCAc) yielded a complete coding sequence of 780 base pairs, or 259 amino acids (Fig. 1). The coding region of TCAc was aligned with and compared to gar *Lepisosteus osseus* rbc, zebrafish *Danio rerio* retina, dace *Tribolodon hakonensis* gill, and rainbow trout rbc (TCAb) CA sequences, as well as to human CA I, II and VII. The TCAc sequence closely resembled all of the fish CA sequences, with amino acid percentage identities ranging from 74 to 76%. Comparisons with human CA sequences revealed that TCAc most closely resembled CA II, with 62% amino acid identity.

NJ and MP analyses of vertebrate cytoplasmic CAs produced generally well supported phylogenetic trees of similar topology (Fig. 2). These analyses suggested that CAs I, II and III constitute a single monophyletic clade, while the fish cytoplasmic CAs (with the exception of zebrafish CA VII) constitute a separate clade. The fish cytoplasmic CA clade is basal to that of other vertebrate CAs (CA I, II, and III), but appears after the divergence of CA V and VII. Within the fish CA group, slight differences in topology were obtained with the two different approaches. NJ analysis revealed that TCAb and zebrafish retina CA were closely grouped, and TCAc and dace gill CA grouped together. The gar rbc CA was the most ancestral sequence. The tree formed by MP analysis showed a similar topology (tree not shown), with the exception that dace gill CA and TCAc did not group together, but diverged after gar rbc CA and prior to the TCAb/zebrafish retina CA group. It should also be noted that zebrafish CA VII grouped most closely with mammalian CA VII rather than with the fish cytoplasmic CA clade.

```
1   atgtctcatgcatggggatacgcaccggacaatggacccgacaaatggtgtgaaggcttc
     M  S  H  A  W  G  Y  A  P  D  N  G  P  D  K  W  C  E  G  F
61  ccaattgccaacggaccccgccagtctcccattgacatcgtacctggggaggctgccttc
     P  I  A  N  G  P  R  Q  S  P  I  D  I  V  P  G  E  A  A  F
121 gacgcagccttgaaggcgctcactttgaagtacgacccttccacctccattgacattctc
     D  A  A  L  K  A  L  T  L  K  Y  D  P  S  T  S  I  D  I  L
181 aacaacggacattcctttcaagtgacctacaccgacgacaacgacaactcaactctgaca
     N  N  G  H  S  F  Q  V  T  Y  T  D  D  N  D  N  S  T  L  T
241 gggggccccatttcagggacgtacaggctaaagcagttccacttccactggggcgccagc
     G  G  P  I  S  G  T  Y  R  L  K  Q  F  H  F  H  W  G  A  S
301 gacgacaggggttctgagcataccgtggccgggaccaagtatgctgccgagctccacctg
     D  D  R  G  S  E  H  T  V  A  G  T  K  Y  A  A  E  L  H  L
361 gtacactggaacaccaagtaccccagctttggtgatgctgctagcaagtctgatggcctt
     V  H  W  N  T  K  Y  P  S  F  G  D  A  A  S  K  S  D  G  L
421 gctgttgtaggagtcttcctccaggttggaaatgaaaatgccaatcttcagaaggtcctt
     A  V  V  G  V  F  L  Q  V  G  N  E  N  A  N  L  Q  K  V  L
481 gatgcttttgatgccattaaagccaagggcaagcagacctctttcgagaattttgacccc
     D  A  F  D  A  I  K  A  K  G  K  Q  T  S  F  E  N  F  D  P
541 accatcctgctccccaagtccctagactactggacttacgacggctccctgaccacacct
     T  I  L  L  P  K  S  L  D  Y  W  T  Y  D  G  S  L  T  T  P
601 cctctgctggagagtgtcacctggatcgtctgcaaggagtcaatcagcgtcagccctgcc
     P  L  L  E  S  V  T  W  I  V  C  K  E  S  I  S  V  S  P  A
661 cagatgggcaaattccggagcctgcttttctctggagagggcgaggccgcctgctgcatg
     Q  M  G  K  F  R  S  L  L  F  S  G  E  G  E  A  A  C  C  M
721 gtggacaactaccgccccctcagccctcaagggccgcgctgtgcgtgcatccttcaaa
     V  D  N  Y  R  P  P  Q  P  L  K  G  R  A  V  R  A  S  F  K
781 taa
     *
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Fig. 1. Nucleotide and deduced amino acid sequence of carbonic anhydrase (CA) from the rainbow trout gill. Sequence shown is coding region only, from start codon (underlined) to stop codon (asterisk) as determined through RACE (rapid amplification of cDNA ends).

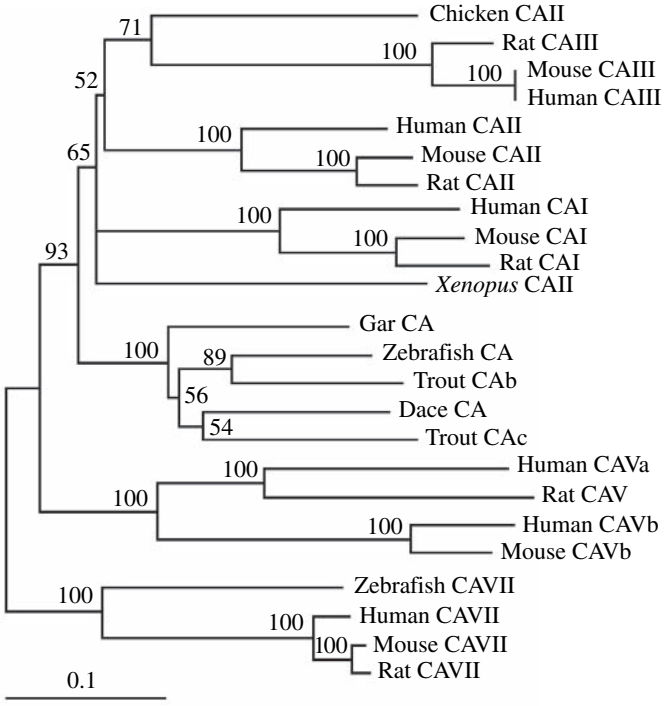

Fig. 2. Phylogenetic analysis of rainbow trout cytoplasmic carbonic anhydrase (TCAc) and other α-CA isozymes. The phylogenetic tree was constructed using neighbour joining analysis with support for nodes assessed using bootstrap analysis. The tree was ordered using mouse, rat and human CA VII as a monophyletic outgroup. Branches are drawn to scale with the length of 0.1 approximating replacement of 10% of the amino acids in the protein alignment (no Poisson correction for multiple hits).

The last aspect of the sequence analyses involved a comparison of the active site of TCAc with those of other fish CAs, as well as those from consensus CA I and II (Tashian et al., 2000) and human CA VII sequences. The active site of the TCAc sequence was most similar to the TCAb and dace gill CA sequences, differing at only one amino acid residue, while differing at two amino acid residues from the gar rbc CA sequence (Fig. 3). When compared to the mammalian CA active sites, the TCAc sequence was most similar to CA VII, differing by three amino acid residues.

Tissue distribution

The tissue distributions of both TCAb and TCAc were examined using northern blot analysis (Fig. 4) and real-time RT-PCR (Fig. 5) of perfused trout tissues. These analyses

TCAc	Y	S	N	N	H	S	F	Q	T	K	Q	H	H	E	H	E	H	V	F	L	V	G	W	Y	L	T	T	P	P	L	S	V	W	V	N	R		
	*	*						*			*	*	*	*	*	*	*							*		*	*							*		*	*	aa
				+	+	+		+				z	z				z	~		~	~				~							~	~					diff
TCAb	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		1
Dace	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.		1
Gar	.	.	.	.	.	.	.	.	D	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.		2
CA I	.	.	.	V	.	.	.	H	N	F	.	.	.	.	.	.	.	.	L	.	I	.	.	.	.	H	.	.	H	.	.	.	I	.	.		9
CA II	.	.	.	.	.	.	.	N	E	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.		4
CA VII	.	.	.	.	.	.	V	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.		3

Fig. 3. Comparison of the active sites of trout cytoplasmic catalytic anhydrase (CA) with those of trout red blood cell CA (TCAb), dace CA, gar CA, concensus trout CA I, CA II and human CA VII. Identical amino acids are indicated by a dot. aa diff, number of amino acid differences. *, putative active site; z, zinc binding ligand; +, proton shuttling associated ligand; ~, substrate associated pocket.

indicated that the TCAb isozyme was expressed almost exclusively in the rbc. Low levels of expression in the spleen and anterior kidney (Fig. 4), or heart and brain (Fig. 5) could be accounted for by blood that was not removed during saline perfusion. Blood contamination is, in fact, indicated by corresponding expression of haemoglobin in the spleen, anterior kidney (Fig. 4), brain and heart (Fig. 5). By contrast, the TCAc isozyme exhibited a wider tissue distribution. Although gill was the predominant site of expression of TCAc, brain tissue also displayed substantial expression, with low levels found in the kidney, gut, liver and muscle (Figs 4 and 5). Unlike northern blot analysis, real-time RT-PCR also revealed low TCAc expression in the rbcs. A comparison of the abundance of each isozyme in the rbcs, using real-time RT-PCR, indicated that TCAb was 666±183 times (N=6) more abundant than TCAc.

Kinetic analysis

The kinetic properties and inhibitor sensitivities of cytoplasmic CA isozymes from gill and rbc lysates were examined (Table 1). The K_i values for Az and chloride were similar for both the rbc and gill CA isozymes. However, when the cytoplasmic fractions of gill and rbc lysates were assayed in the presence of increasing volumes of trout plasma, which contains an endogenous CA inhibitor (Dimberg, 1994; Haswell and Randall, 1976; Henry et al., 1997), the rbc CA isozyme was found to be significantly more sensitive than the gill CA isozyme (Fig. 6). Moreover, both the turnover value (k_{cat}) and substrate affinity value (K_m) of the rbc CA isozyme were significantly higher than corresponding values for the gill CA

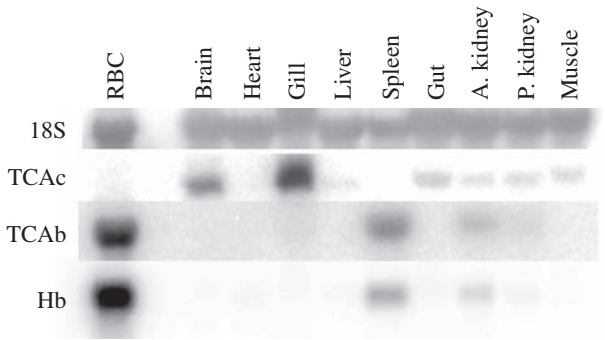

Fig. 4. Representative northern blots for rainbow trout red blood cell carbonic anhydrase (TCAb), cytoplasmic carbonic anhydrase (TCAc) and β-globin (Hb) mRNA and 18S rRNA from adult rainbow trout tissues (N=4). RBC, red blood cells; A, anterior; P, posterior.

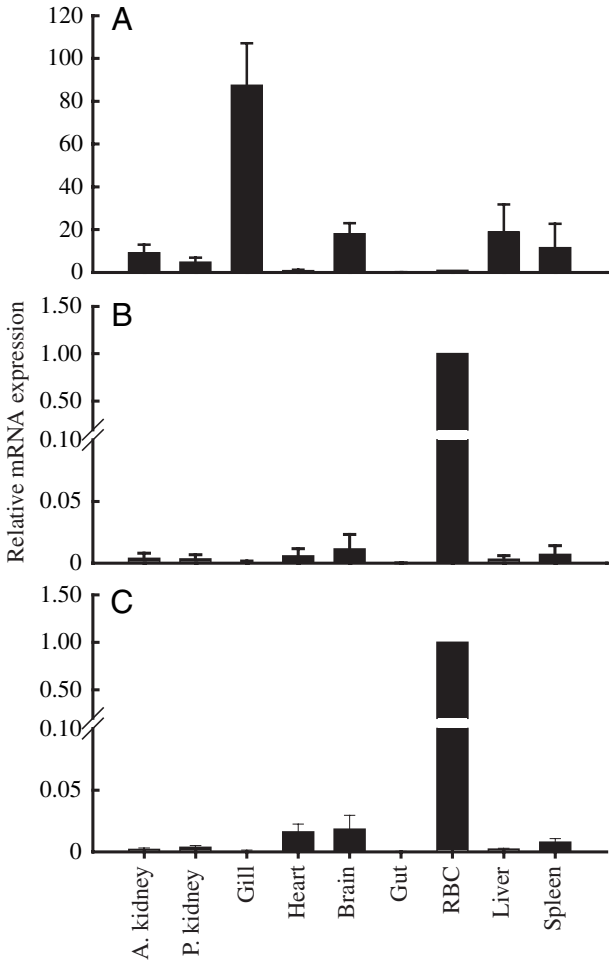

Fig. 5. Relative mRNA expression (mean ± S.E.M.) of cytoplasmic carbonic anhydrase (TCAc; A) and red blood cell carbonic anhydrase (TCAb; A) and haemoglobin (C) in rainbow trout tissues, as determined by real time RT-PCR (N=6). The red blood cell (RBC) mRNA expression was set to 1 in all cases.

Table 1. *Comparison of the catalytic and kinetic properties of cytoplasmic carbonic anhydrase isozymes from rainbow trout, using gill tissue and red blood cells as enzyme sources*

	K_m (mmol l^{-1})	k_{cat} (e^4 s^{-1})	K_i Az (nmol l^{-1})	K_i Cl^- (mmol l^{-1})
Gill	8.72±1.03	8.90±1.95	1.21±0.18	62.03±6.75
RBC	23.26±5.22*	30.28±5.83*	1.34±0.10	61.34±2.41

Values are mean ± S.E.M. (N=4), with statistically different groups indicated by an asterisk (unpaired t-test; P<0.05).
RBC, red blood cells.

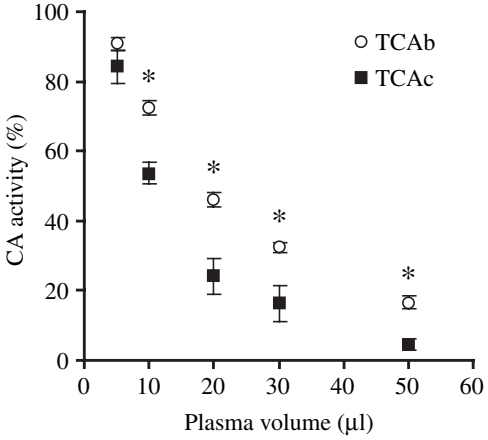

Fig. 6. The relative effect of plasma [which contains an endogenous carbonic anhydrase (CA) inhibitor] on the red blood cell (circles) and gill (squares) CA activity (mean ± S.E.M.) of rainbow trout (N=4). Significant differences are indicated by an asterisk (unpaired t-test; P<0.05).

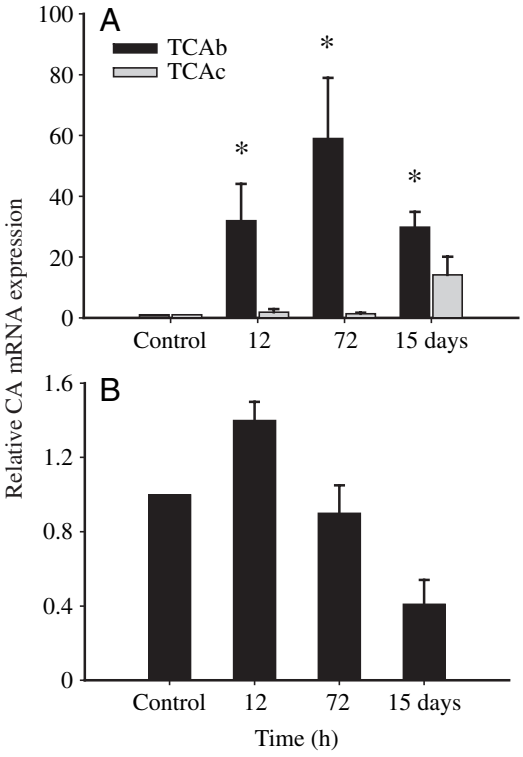

Fig. 7. The effects of anaemia on the relative mRNA expression (mean ± S.E.M.) of cytoplasmic carbonic anhydrase (TCAc) and red blood cell carbonic anhydrase (TCAb) in the red blood cells (A), and cytoplasmic carbonic anhydrase (TCAc) in gills (B) of rainbow trout, as determined by real time RT-PCR (N=6). Significant differences are indicated by an asterisk (P<0.05). Control values were set to 1 in all cases.

isozyme (Table. 1); thus, the rbc-specific isozyme appears to be a faster enzyme with a lower substrate affinity than the cytoplasmic isozyme.

Functional analysis

Changes in mRNA expression in whole blood and gill tissue for both TCAb and TCAc in response to the induction of anaemia were examined using real-time RT-PCR. Withdrawal of 1 ml of blood was sufficient to decrease haematocrit significantly (one-way ANOVA, P<0.05) by approximately 45–55%, from the control value of 47.6±3.7% (N=6) to 28.0±2.8% (N=6) at 12 h, 23.5±1.8% (N=6) at 72 h and 21.5±4.1% (N=5) at 15 days. The response to this induction of anaemia was a significant increase in rbc TCAb mRNA expression at all sample times (Fig. 7A), in the absence of any significant change in rbc or gill TCAc mRNA expression (Fig. 7A and B).

Discussion

The results of the present study show that a general cytoplasmic CA isozyme, that is distinct from the rbc-specific isozyme in structure, catalytic properties and distribution, is found in the tissues of rainbow trout. The general cytoplasmic CA isozyme (TCAc) has a lower turnover (lower catalytic activity) than the rbc-specific CA isozyme (TCAb), is found in high abundance in the gills and brain and is widely distributed in other tissues, including rbcs, in lower amounts.

The general cytoplasmic CA isozyme has a coding region of 780 base pairs (259 amino acids; Fig. 1), and high sequence identity to other known fish CA sequences (74–76%). The high sequence identity between the TCAb and TCAc isozymes (76%) may explain the results of Sender et al. (1999), who found no evidence for the presence of two distinct CA isozymes in the gill cytoplasm and rbc of flounder (*Platichthys flesus*). The gill CA activity in flounder was believed to be caused by the same isozyme found in the rbc, because antibodies produced to CA from both sources were cross-

reactive. Assuming that two isozymes do, in fact, occur in flounder, cross-reactivity between antibodies could be the result of high protein similarities.

To date, molecular analyses of the phylogeny of the α-CA gene family have been limited by the small amount of CA sequence information from non-mammalian vertebrate species (Lund et al., 2002; Tufts et al., 2003). When TCAc was included in a phylogenetic analysis of cytoplasmic α-CA isozymes, it joined a previously described fish CA monophyletic clade (Peterson et al., 1997; Lund et al., 2002; Tufts et al., 2003; Esbaugh et al., 2004). These findings suggest that the current trend of using mammalian nomenclature (slow type CA I and fast type CA II) to identify fish CA isozymes may be inappropriate (Tufts et al., 2003). In agreement with previous studies, the fish cytoplasmic CA group emerged prior to the gene duplication event that gave rise to the mammalian CA I, II and III genes, as well as the divergence of other tetrapod vertebrate isozymes, such as *Xenopus* and chicken CA II (Fig. 2). The analyses also agree with previous studies that show that the fish cytoplasmic CA group diverged after the emergence of CA V and VII in vertebrates. Unlike previous studies, however, this analysis contains two cytoplasmic CA isozymes from a single species. Interestingly, the two cytoplasmic CA isozymes from rainbow trout do not group most closely together; rather, TCAc groups closely with a gill isozyme from dace, whereas TCAb is most closely associated with a zebrafish retina sequence. There is evidence that the common ancestor of salmonid fish underwent a genome duplication event (Hinegardner and Rosen, 1972; Allendorf and Thorgaard, 1984). However, the topology of the trees formed by both NJ and MP analysis suggest that the duplication event that gave rise to the two cytoplasmic CA isozymes in trout occurred in the ancestor common to both zebrafish and trout, and possibly also dace. Either a gene duplication event that occurred at some point in the evolution of teleost fish, or a genome duplication event that occurred at the origin of modern fishes, could account for the presence of two cytoplasmic CA isozymes in trout (Amores et al., 1998; Meyer and Schartl, 1999; Robinson-Rechavi et al., 2001; Taylor et al., 2001). It is therefore necessary to examine whether multiple cytoplasmic CAs are present in more teleost families, as well as in other fish lineages, such as agnathans, dipnoans and non-teleost actinopterygians.

Examination of the amino acid residues that are specific to the active site pocket, as described by Tashian et al. (2000), can also yield insight into the functional evolution of CA isozymes. A comparison of the active site amino acid residues (Fig. 3) indicates not only that the fish cytoplasmic CA isozymes are highly similar at the amino acid level, but that the critical elements of enzyme function are almost entirely conserved, with only one amino acid difference between the teleost CA isozymes. In addition, the active site of TCAc and gar rbc CA differ by only two amino acids. It is also noteworthy that none of these amino acid differences fall within sections of the sequence that have been directly implicated in the catalytic mechanism (reviewed by Stams and Christianson, 2000).

Despite the high sequence similarity between the two trout cytoplasmic CA isozymes, significant differences were detected in their kinetic properties and inhibitor sensitivities, supporting the contention that the sequences represent discrete isozymes, with potentially physiologically distinct roles. It should be noted that because the mRNA, and presumably protein, level of TCAb in the rbc greatly exceeded that of TCAc, the kinetic properties of rbc lysates were taken to be representative of TCAb. TCAc was significantly more sensitive to the endogenous inhibitor in trout plasma than was TCAb (Fig. 6), while both isozymes exhibited similar inhibition constants when exposed to Az and chloride ions (Table 1). The inhibition constant for Az for both isozymes was within the range previously described for rainbow trout rbc CA (Henry et al., 1993; Esbaugh et al., 2004), while the value for chloride was slightly lower than that reported by Henry et al. (1993). Calculation of the turnover (k_{cat}) and substrate affinity constant (K_m) revealed that, as predicted, both were lower for the general cytoplasmic CA than for TCAb (Table 1). Indeed, the rbc-specific TCAb reaction rate was over three times faster than that of the more widely distributed TCAc. This difference in catalytic efficiency is most probably related to the different roles of the two isozymes. The primary role of the faster TCAb isozyme is to catalyse the dehydration/hydration reactions of CO_2 for the purpose of CO_2 transport and excretion (Perry, 1986; Tufts and Perry, 1998; Henry and Swenson, 2000). A single pass of blood through the gill vasculature (approximate transit time of 0.5–2.5 s; Cameron and Polhemus, 1974) can remove up to 35% of the total blood CO_2 load (Perry, 1986). Thus, an emphasis on the rapid translation of HCO_3^- from the plasma to the rbc and its conversion to molecular CO_2 is critical. Current theory contends that CO_2 excretion is rate-limited by the speed of the Cl^-/HCO_3^- exchanger (Perry, 1986; Perry and Gilmour, 1993; Desforges et al., 2001), and Henry et al. (1993) proposed that the evolution of faster rbc CA isozymes was related to the incorporation of a rapid Cl^-/HCO_3^- exchanger into the rbc membrane (see also Tufts et al., 2003). The data from the present study are certainly consistent with this hypothesis – a faster rbc isozyme related to the presence of a rapid rbc membrane anion exchanger (Cameron, 1978; Romano and Passow, 1984) and involved in CO_2 transport and excretion, *versus* a slower cytoplasmic isozyme that may play a role in numerous different functions (Henry, 1996). For example, within the gill, the general cytoplasmic isozyme probably plays a major role in acid–base balance and ionic regulation, where high activity may not be as crucial as it is to CO_2 excretion. It should also be noted that TCAb and TCAc do not display the dramatic differences in catalytic and kinetic properties associated with mammalian CA I and II, suggesting that TCAc may play a more catalytically demanding role in fish than the low activity CA I isozyme plays in mammals.

Differences between trout and mammals are also apparent in the tissue distribution of the CA isozymes. In mammals, CA I

and CA II are found in the cytoplasm of the rbcs as well as many other tissues throughout the body (Chegwidden and Carter, 2000; Parkkila, 2000; Swenson, 2000). In contrast, the major trout rbc CA isozyme, TCAb, appears to be found only in the rbc (Figs 4 and 5). Unlike TCAb, however, TCAc exhibits a widespread distribution among different tissues, with greatest expression occurring in the brain and gills, and lower expression in the liver, gut, white muscle and the anterior and posterior kidney. Presumably, TCAc has numerous physiological roles (Henry, 1996), similar to mammalian CA I and II (Chegwidden and Carter, 2000; Parkkila, 2000; Swenson, 2000). The finding that TCAc is present with TCAb in the rbcs of trout is in contrast to the prevailing belief that teleost rbcs express only one CA isozyme (Maren et al., 1980; Sanyal et al., 1982; Kim et al., 1983; reviewed by Henry and Heming, 1998), but is in accordance with a few studies that presented evidence for two isozymes (Girard and Istin, 1975; Carter et al., 1976). Whereas mammalian rbcs express two cytoplasmic CA isozymes that differ by an order of magnitude in catalytic activity, both trout rbc cytoplasmic isozymes appear to be higher activity enzymes. It is unclear why two isozymes with the potential for overlapping function would be expressed in the same tissue, although it is possible that their roles are differentiated by protein interactions. Such protein interactions – for example, with the rbc membrane Cl^-/HCO_3^- exchanger – might also explain why the slower TCAc isozyme is the more widespread isozyme in trout, while the higher activity TCAb is specific to the rbc. In mammalian rbcs, cytoplasmic CA II interacts with numerous transport proteins, while CA I is unable to do so (Vince et al., 2000; Reithmeier, 2001; Sterling et al., 2001, 2002a,b; Li et al., 2002). It remains to be determined whether an analogous system also evolved in modern fish.

Previous studies indicated that CA expression in fish is regulated in response to a variety of internal and external conditions, such as salinity (Girard and Istin, 1975; Dimberg et al., 1981; Kultz et al., 1992), temperature (Houston and McCarty, 1978; Houston and Mearow, 1979), and acid–base status (Dimberg and Hoglund, 1987). With this in mind, a preliminary study was performed to examine whether the two cytoplasmic CA isozymes in the rbc were differentially regulated in response to anaemia.

In the rbc, a dramatic increase in TCAb mRNA expression occurred in response to the induction of anaemia (Fig. 7A), whereas no significant change in TCAc mRNA expression was observed in the rbc (Fig. 7A) or gill (Fig. 7B). It should, however, be noted that young rbcs were probably released into the bloodstream over the duration of the experiment, and these young rbcs are known to have 10-fold higher levels of mRNA transcripts (Lund et al., 2000). The extent to which young rbcs may affect the results of these experiments is unclear as the haematocrit of all animals remained depressed throughout the 15-day experimental period, suggesting that few young rbcs were released into the bloodstream. In addition, if the increased expression levels of TCAb mRNA observed throughout anaemia were, in fact, due to the increased mRNA transcript levels inherent in young rbcs, then the levels of TCAc mRNA in the rbcs would also be expected to increase. The expression levels of TCAc mRNA, however, did not significantly change throughout the course of the experiment. Although these data do not unequivocally demonstrate differential regulation of the two CA isozymes, it remains plausible. The differential regulation of the two rbc CA isozymes would support the idea that they serve different functions within the rbc, which is similar to mammalian rbcs where CA II is responsible for the majority of CO_2 hydration/dehydration (Maren et al., 1976). Further experiments should be performed to more carefully quantify the effect of young rbcs, as well as the roles the two cytoplasmic isozymes may play.

In conclusion, the results of the present study demonstrate that, in rainbow trout, the CA isozyme in the rbcs is distinct in structure, tissue distribution, kinetic properties, and probably physiological role, from a second, more widely distributed or general cytoplasmic isozyme. Although both isozymes exhibited similar inhibitor sensitivities, the catalytic activity of the rbc CA isozyme was threefold higher than that of the general cytoplasmic form, likely owing to selective pressures specific to the demands of CO_2 excretion. Selective up-regulation of the rbc-specific CA isozyme within rbcs in response to anaemia supports the hypothesis of two discrete isozymes with different functions.

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