Type IV carbonic anhydrase is present in the gills of spiny dogfish (*Squalus acanthias*)

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Running title: Carbonic anhydrase in dogfish gills
Abstract

Physiological and biochemical studies have provided indirect evidence for a membrane-associated carbonic anhydrase (CA) isoform, similar to mammalian type IV CA, in the gills of dogfish (*Squalus acanthias*). This CA isoform is linked to the plasma membrane of gill epithelial cells by a glycosylphosphatidylinositol anchor and oriented towards the plasma, such that it can catalyze the dehydration of plasma HCO$_3^-$ ions. The present study directly tested the hypothesis that CA IV is present in dogfish gills in a location amenable to catalyzing plasma HCO$_3^-$ dehydration. Homology cloning techniques were used to assemble an 1127 base pair cDNA that coded for a deduced protein of 306 amino acids. Phylogenetic analysis suggested that this protein was a type IV CA. For purposes of comparison, a second cDNA (1107 base pairs) was cloned from dogfish blood; it encoded a deduced protein of 260 amino acids that was identified as a cytosolic CA through phylogenetic analysis. Using real-time PCR and *in situ* hybridization, mRNA expression for the dogfish type IV CA was detected in gill tissue, and specifically localized to pillar cells and branchial epithelial cells that flanked the pillar cells. Immunohistochemistry using a polyclonal antibody raised against rainbow trout type IV CA revealed a similar pattern of CA IV immunoreactivity, and demonstrated a limited degree of co-localization with Na$^+$.K$^+$-ATPase immunoreactivity. The presence and localization of a type IV CA isoform in the gills of dogfish is consistent with the hypothesis that branchial membrane-bound CA with an extracellular orientation contributes to CO$_2$ excretion in dogfish by catalyzing the dehydration of plasma HCO$_3^-$ ions.

**Key words:** carbonic anhydrase, type IV carbonic anhydrase, dogfish, CO$_2$ excretion, gill
Introduction

Several lines of evidence suggest that a membrane-associated carbonic anhydrase (CA) isoform similar to the mammalian type IV CA is present in the gills of spiny dogfish (*Squalus acanthias*), and probably other chondrichthyans (17). Mammalian type IV CA is a high activity enzyme that is linked to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor such that the enzyme itself projects into the extracellular environment where it can catalyze the reversible dehydration/hydration reactions of CO₂ (1, 32, 33, 74; reviewed by 47). Mammalian CA IV can be released from its membrane association by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), and this feature, together with its unusual resistance to the detergent sodium dodecylsulphate (SDS), a property conferred by the presence of one or more stabilizing intramolecular disulphide bonds, is often used as diagnostic in characterizing an unknown CA activity as CA IV-like (33, 66, 74; reviewed by 47).

In dogfish, the presence of plasma-accessible CA activity in the gills has been inferred from several studies that have examined the effects of selectively inhibiting extracellular CA activity (14-16, 53, 57, 71; but see also 55 for an opposing point of view). For example, Swenson et al. (53, 57) found that the branchial clearance of an infused HCO₃⁻ load was significantly reduced following treatment of dogfish with a CA inhibitor, polyoxyethylene-aminobenzolamide (F3500), that is restricted to the extracellular environment. Similar use of F3500 or the sulphonamide inhibitor benzolamide, which only slowly permeates cell membranes, revealed a role for extracellular CA activity in CO₂ excretion (15, 16), as well as in determining the pH equilibrium status of post-branchial blood in dogfish (14). The plasma of elasmobranch fish, unlike most vertebrates, contains CA (14, 17, 19, 73) and therefore a
difficulty with these *in vivo* approaches was that they were unable to distinguish between the contributions of plasma and branchial membrane-associated CA activities. The use of an *in situ* saline-perfused dogfish gill preparation provided clear evidence that branchial CA activity was available to catalyze perfusate CO₂ reactions (71). In particular, Wilson et al. (71) reported that passage through an *in situ* dogfish gill preparation of a saline solution exhibiting a pH disequilibrium was sufficient to establish an equilibrium condition, but that inclusion of the CA inhibitor acetazolamide in the perfusate eliminated this effect.

In addition to the physiological evidence supporting the presence in dogfish gills of membrane-associated CA activity with an extracellular orientation, biochemical evidence suggests that this CA activity can be released from its membrane linkage by PI-PLC (16). Differential centrifugation of gill homogenates from rainbow trout and dogfish revealed that the CA activity of the microsomal or membrane fraction was significantly higher in dogfish than in trout (16, 19). Subsequent incubation of microsomal fractions with PI-PLC resulted in the significant transfer of CA activity to the supernatant in dogfish but not in trout, suggesting that the membrane-associated CA activity of dogfish gills is a type IV-like enzyme (16).

A strong case can therefore be made from the available physiological and biochemical evidence that a type IV-like CA isoform is present in the gills of dogfish. However, the available evidence is indirect, and recent work on fish red blood cell cytosolic CAs has underlined the fact that CAs of similar biochemical properties may have distinct phylogenetic origins (7, 8, 64). Thus, the objective of the present study was to directly test the hypothesis that a type IV-like CA is present in the gills of dogfish by cloning dogfish CA IV and examining its mRNA and protein distribution in gill tissue.
Materials and methods

Experimental animals

Pacific spiny dogfish (*Squalus acanthias*) were collected by net during trawls by local fishermen and transported to a holding facility at Bamfield Marine Station where they were held in a 75,000 L circular tank supplied with full-strength seawater at 13°C. To obtain tissue samples, dogfish were euthanized by immersion in an anaesthetic solution (ethyl-\(p\)-aminobenzoate; 1 g L\(^{-1}\)). Blood samples were withdrawn by caudal puncture, frozen in liquid N\(_2\) and stored at -80°C until use. Gill, kidney, intestine, rectal gland, liver, heart, brain and white muscle tissues were then harvested, frozen in liquid N\(_2\) and stored at -80°C until use. In addition, gill tissue was immersion fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH = 7.4) overnight, then transferred to 15% sucrose followed by 30% sucrose for 2 h in each case. These tissues were kept at 4°C throughout the fixation procedure, and were then stored in 30% sucrose at 4°C until use. Cryoprotected gill tissue pieces were frozen in Shandon Cryomatrix embedding medium (Fisher). Thin sections (10 µm) from the trailing edge of the gill, obtained using a cryostat (CM 1850; Leica) at -15°C, were collected onto electrostatically charged slides (SuperFrost Plus; VWR International), air-dried for 30 min and stored at -20°C until use.

Molecular cloning of dogfish CA isoforms

Total RNA was extracted from gill tissue or whole blood using Trizol (Invitrogen) according to the instructions of the manufacturer. Spectrophotometry (Eppendorf BioPhotometer; VWR International) was used to verify RNA concentrations, and cDNA was
then synthesized from 5 µg of total RNA using Superscript II reverse transcriptase (Invitrogen) and random hexamer primers. PCR was performed using 1 µL of cDNA template in 25 µL reaction mixtures containing 3.5 mmol L⁻¹ MgCl₂, 200 µmol L⁻¹ of each dNTP, 250 nmol L⁻¹ of each primer and 1 unit of Taq polymerase (Invitrogen) in PCR buffer supplied with the enzyme. All PCR reactions involved an initial denaturation at 94°C for 3 min followed by 40 cycles of: 94°C for 30 s; annealing temperature for 60 s; 72°C for 60 s, and ending with a final extension for 10 min at 72°C. For gill tissue, the template for the PCR reactions consisted of 1 µL of gill cDNA, the annealing temperature was 49°C, and a degenerate primer pair was designed from regions of vertebrate CA IV sequences exhibiting a high degree of amino acid conservation; this pair consisted of the forward primer 5’-CAR WSI CCN ATH AAY ATH GT-3’ and reverse primer 5’-RTT IAC DAT RTG IAR YTC CAT-3’. Using this primer pair, a 290 bp cDNA fragment was amplified. In addition, a 361 bp cDNA fragment was amplified using as a template for the PCR reactions 1 µL of blood cDNA, an annealing temperature of 49.5°C, and a primer pair consisting of the forward and reverse primers 5’-CAG TTC CAY TTC CAY TGG G-3’ and 5’-RAC GAT CCA KGT GAC RCT CTC-3’, respectively; this primer pair was designed on the basis of consensus amino acid sequences for fish cytosolic CAs. These initial PCR products were ligated into PCR 2.1 vectors (TOPO-TA cloning kit, Invitrogen) and sequenced. A search of GenBank protein databases using BLASTX revealed that the 290 bp cDNA fragment cloned from gill tissue exhibited highest amino acid identity with known CA IV sequences, whereas the 361 bp cDNA fragment cloned from blood exhibited highest amino acid identity with known CA XIII and CA II sequences.

Based on the sequences of the cDNA fragments cloned, primers were designed to extend the initial fragment lengths by 3’ and 5’ rapid amplification of cDNA ends (RACE). For 3’
RACE, total gill or blood RNA was isolated using Trizol reagent (Invitrogen) and reverse-transcribed to cDNA using a 3’ RACE adapter primer (Gibco) and Superscript II reverse transcriptase (Invitrogen). Semi-nested PCR was then performed on the cDNA using abridged universal amplification primers (Gibco) with 3’ RACE primers. For blood, the first round primer 5’-AGC ACC TGA TGG TCT GGC TGT AA-3’ (60°C annealing temperature) was followed by a second round with primer 5’-GCT GCC TCC TTG TCT CAA T-3’ (59°C annealing temperature). For gill, additional rounds were required. Two initial rounds with primer 5’-TGA GGG CTA TGG TGA TCT TG-3’ were followed by a third round with primer 5’-GGT CAC TCG GTC CAG GTA A-3’. A final round of PCR with primer 5’-ACA AGG CAG TTC AGT TCC AC-3’ resulted in a product of the correct size that was extracted from a gel (PureLink Gel Extraction Kit; Invitrogen) prior to cloning and sequencing (see below). The procedures used for 5’ RACE differed for gill tissue and blood. cDNA for 5’ RACE was synthesized from total gill RNA using the FirstChoice RLM-RACE kit (Ambion), and PCR was carried out according to the kit instructions using the gene-specific primer 5’-CCA GGA TCC ACC TTT GTT C-3’ and an annealing temperature of 57°C. cDNA for 5’ RACE was synthesized from total blood RNA using an oligo dT primer and Superscript II reverse transcriptase (Invitrogen), purified using a PCR purification kit (Sigma), and then tailed with dCTP using a terminal transferase TdT (Invitrogen) with final reaction conditions 10 mmol L⁻¹ Tris-HCl (pH 8.4), 25 mmol L⁻¹ KCl, 1.5 mmol L⁻¹ MgCl₂, 200 μmol L⁻¹ dCTP, 1 μL cDNA and 1 μL TdT. The tailed cDNA was then used for two rounds of nested PCR. In the first round of PCR, the gene-specific primer 5’-TCC ATG TGA CGC TCT CAA AC-3’ was used with an abridged anchor primer (Invitrogen) at an annealing temperature of 63°C, while the second round utilized the gene-specific primer 5’-ATG CAG TCC AGA GAA GAA AGC-3’ with an abridged universal amplification primer
(Invitrogen) at an annealing temperature of 58°C. All RACE PCR products were cloned into pCR2.1 vectors (TOPO TA cloning kits; Invitrogen) and sequenced, and the sequenced RACE products were then confirmed by overlap with the appropriate initial cDNA fragment. Repeated bi-directional sequencing of RACE products together with multiple sequence alignment (DNAMAN; Lynnon Biosoft) permitted the construction of consensus sequences for dogfish CA IV (dfCA IV) and dogfish blood cytosolic CA (dfCAb).

Phylogenetic analyses

Dogfish CA deduced amino acid sequences were aligned with GenBank sequences of CA I, II, III, IV, VII, IX, XII, XIII, XIV and XV from selected vertebrates, as well as fish cytoplasmic CA sequences, using ClustalX version 1.83 (60) with penalties for gap opening and gap extension set to 30 and 0.75, respectively, for pairwise alignments, and 15 and 0.3, respectively, for multiple alignments. The PHYLIP package was then used to carry out neighbour-joining phylogenetic analysis (41) on a matrix of mean character distances, with a bootstrapping resampling option to assess the support for nodes (100 pseudoreplicates). In general, default parameters were used with the exceptions that, where the option to specify an outgroup existed, *Drosophila* CA was specified (9), and the input order of species was randomized where possible. The accession numbers for sequences used in the phylogenetic analysis are presented in the legend for Fig. 2.

Analysis of dCA IV mRNA expression by real-time PCR

For the quantification of dCA IV mRNA expression across a range of tissues by real-time PCR, total RNA was extracted from 30 mg aliquots of frozen tissue samples using Trizol
and treated with DNase I for 10 min at 37°C to ensure the removal of any remaining genomic DNA. Its quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf BioPhotometer; VWR International), and 2 µg of RNA was then used for cDNA synthesis by means of random hexamer primers and StrataScript reverse transcriptase (Stratagene; Cedarlane Laboratories).

Real-time PCR was then carried out on a Stratagene MX-4000 multiplex quantitative PCR system using a Fullvelocity QPCR Master Mix Kit (Stratagene; Cedarlane Laboratories). The PCR conditions for a 25 µL final reaction volume consisted of 0.5 µL cDNA template, 300 nmol L⁻¹ forward and reverse primers, 12 µL of 2x Master Mix and 1:30,000 final dilution of ROX, with annealing at 58°C for 30 s and extension at 72°C for 30 s over 40 cycles. The primer pair used to assess dCA IV mRNA levels was designed using Primer3 software and consisted of the forward primer 5’-CGT TTG CTG GCT TTG ATG A-3’ with the reverse primer 5’-CAA GCA CAG GAA GGG CAA A-3’. The specificity of the primers was verified by the cloning (TA 2.0 cloning kit; Invitrogen) and sequencing of amplified products. In addition, the PCR products of initial experiments were analysed by gel electrophoresis to ensure that SYBR green was not being incorporated into primer-dimers or non-specific amplicons, and single bands of the expected size were obtained in all cases. For every real-time PCR run, SYBR green dissociation curves were constructed after completion of 40 PCR cycles and revealed the presence of single amplicons for each primer pair. No template controls, in which reverse transcriptase was omitted during cDNA synthesis, were also included in every real-time PCR run to ensure that residual genomic DNA was not being amplified. Relative levels of dCA IV mRNA expression in different tissues were standardized to the amount of RNA present. Amplification efficiencies were determined from standard curves generated by serial dilutions of pooled RNA from all
tissues (N ≥ 3 fish per tissue), and β-actin was used as a control to ensure that all cDNA
syntheses were carried out with similar efficiencies (the primer pair used to assess dogfish β-
actin mRNA levels consisted of the forward primer 5’-ATG CCA ACA CTG TCC TGT C-3’
together with the reverse primer 5’-GAC AGG GAA GCC AGG AT-3’).

**Analysis of dCA IV mRNA localization by in situ hybridization**

*In situ* hybridization was used to localize dCA IV mRNA expression within the branchial
epithelium. An antisense riboprobe for dCA IV was generated from gill cDNA using a primer
pair designed to yield a PCR product of approximately 650 bp, specifically the forward primer
5’-ATG CAC TCA CTT ATT CTC TTG CT-3’ coupled with the reverse primer 5’-ATG AGC
CAT GAT AGC GGT AG-3’. The PCR product was cloned into the pCR2.0 vector (TA cloning
kit; Invitrogen) and the plasmid was sequenced to confirm the identity and orientation of the
product. To label the probe, first 2 µg of plasmid DNA was digested using Xba I (Invitrogen)
and the conditions recommended by the manufacturer. The resultant product was
phenol/chloroform purified and resuspended in 10 µL of DEPC H2O. Following verification of
the digest concentration by spectrophotometry (Eppendorf BioPhotometer; VWR International),
a probe labelling assay was carried out using T7 RNA polymerase (New England Biolabs) and a
digoxigenin (DIG) RNA labelling mix (Roche), as described by the manufacturer.

To prepare tissue sections for *in situ* hybridization, sections on slides were hydrated (2 x 15
min) in 1X phosphate-buffered saline containing 0.1% Tween 20 (PBST), then treated with 20
µg mL⁻¹ proteinase K (Gibco) in PBST for 20 min at room temperature. The tissues were rinsed
(2 x 10 min) in 1X PBST, re-fixed in 4% formaldehyde in PBS for 4 min, rinsed again (2 x 10
min; 1X PBST) and air-dried at 58°C for 15 min. To prepare the probe for hybridization, ~900
pg of probe was denatured for 3 min at 94°C in a solution containing 250 µg mL⁻¹ salmon sperm DNA and 250 µg Poly A topped to 12.5 µL with DEPC H₂O. Denaturation was halted by quickly chilling the probe on ice and centrifuging for 1 min at 7,500 g. Hybridization buffer [100 µL of 50% deionized formamide, 1X Denhardt’s, 0.2% SDS, 5% dextran sulphate, 0.75 mol L⁻¹ NaCl, 25 mmol L⁻¹ EDTA and 25 mmol L⁻¹ PIPES; 1X Denhardt’s consists of 0.1% Ficoll 400.000, 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin (BSA)] was then added to the probe and the solution was mixed by vortexing.

The probe (in hybridization buffer) was placed on the prepared tissue sections, which were then incubated overnight at 58°C in a humid chamber. Following hybridization, sections were thoroughly washed in 2X SSC (2 x 15 min, 58°C), 0.2X SSC (2 x 15 min, 58°C), 0.1X SSC (1 x 10 min, room temperature) and 0.1 mol L⁻¹ PBS (1 x 10 min, room temperature), where SSC consisted of 0.9 mol L⁻¹ NaCl and 0.09 mol L⁻¹ sodium citrate at pH 7.0. To detect hybridization signals, sections were first incubated for 1 h at room temperature with a blocking solution consisting of 1% goat serum and 2 mg mL⁻¹ BSA in 0.1 mol L⁻¹ PBS containing 0.3% Triton-X. The sections were then incubated overnight at 4°C with anti-DIG conjugated to alkaline phosphatase (Roche Molecular Biochemicals), diluted 1:1,000 in the blocking solution. Following a series of washes (2 x 15 min in 0.1 mol L⁻¹ PB at room temperature, brief rinse in water, 2 x 5 min at room temperature in coloration buffer consisting of 100 mmol L⁻¹ Tris at pH 9.5, 50 mmol L⁻¹ MgCl₂, 100 mmol L⁻¹ NaCl and 0.1% Tween-20), colour visualization was achieved by dissolving nitroblue tetrazolium and 5-bromocresyl-3-indolyl phosphate tablets (Sigma) in 10 mL of H₂O and layering this solution over the sections. Colour was allowed to develop for at least 4 h at room temperature in a dark, humid chamber. When satisfactory coloration was achieved, the slides were washed with 0.1 mol L⁻¹ PBS (2 x 15 min), mounted
with 60% glycerol and cover-slipped.

Sections (9 – 12 sections per fish for each of 3 fish) were viewed using a Zeiss Axiophot light microscope equipped with a Hamamatsu C5985 chilled CCD camera, and images were captured using the Metamorph v4.01 imaging system. To assess the specificity of hybridization, probe was omitted from the hybridization buffer or sections were pre-treated with excess unlabelled probe. For the latter, sections were incubated, prior to hybridization, for 3 h at 58°C with 5X unlabelled probe in hybridization buffer, and then hybridized with ~900 pg of probe together with 5X unlabelled probe according to the protocol outlined above.

Analysis of dCA IV protein localization by immunohistochemistry

Localization of dCA IV within the branchial epithelium of dogfish was accomplished using a custom rabbit polyclonal antibody (Abgent) raised against a synthetic peptide antigen conjugated to keyhole limpet protein. The antiserum was purified by protein G affinity chromatography followed by peptide affinity purification (Abgent). The peptide TRRTLPDERLTPFTFTGY used to raise the antibody corresponded to amino acids 57 – 74 of the rainbow trout CA IV protein sequence (GenBank accession AAR99330); the corresponding region of the dogfish CA IV amino acid sequence, TRNAQSNVHLTPIIFEGY (amino acids 56 – 73), was identical to the trout CA IV peptide at 8 of 18 amino acids.

Western analysis was used to test the ability of the trout CA IV antibody to detect dogfish CA IV. Proteins were prepared from frozen dogfish gill tissue samples by first grinding the tissue under liquid N₂ with a pre-cooled mortar and pestle, and then homogenizing the powdered tissue in RIPA buffer (50 mmol L⁻¹ Tris-Cl pH = 8.0, 150 mmol L⁻¹ NaCl, 1% NP-40 substitute, 0.1% SDS, 0.5% sodium deoxycholate) containing protease inhibitors (complete™ Mini
protease inhibitor cocktail tablets; Roche Molecular Biochemicals) and 2 µg mL⁻¹ pepstatin A (Sigma) by passing the suspension through a needle and syringe a number of times. Samples were stored on ice for 15 min, centrifuged at 7500 g for 10 min at 4°C, and the supernatant was then flash-frozen and stored at -80°C until subsequent analysis of the soluble proteins. Total protein concentration was assayed using the bicinchoninic acid method (Pierce Biotechnology Micro BCA protein assay; Fisher Scientific) with BSA as the standard. Samples containing 120 µg of protein were then separated by SDS-PAGE using 10.5% tris-tricine polyacrylamide gels. Proteins were then transferred to 0.45 µm nitrocellulose membranes (Bio-Rad) using a wet transfer unit. Membranes were blocked in 5% PBST-milk for 1 h at room temperature. One membrane was then probed with a 1:1000 dilution of the rabbit anti-trout CA IV for 1 h at 37°C, while a second blot was incubated simultaneously with the tCA IV antibody in the presence of an excess (20 µg) of the peptide against which the antibody was raised. Both membranes were then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Life Sciences) for 1 h at room temperature. After several washes, the proteins were visualized using the Western Lightning Chemiluminescence Reagent Plus Kit (PerkinElmer, Inc.). The protein size marker used was obtained from Fermentas Life Sciences.

To localize dCA IV by immunohistochemistry, a hydrophobic barrier was created around each gill tissue section on a slide with a PAP pen (Electron microscopy suppliers). The sections were first incubated (3 x 5 min) in a blocking buffer containing 2% normal goat serum, 0.1 mol L⁻¹ PB, 0.9% Triton-X, 1% gelatin and 2% BSA, and were then incubated for 2 h at room temperature in a humidified chamber with primary antibody diluted in blocking buffer. The primary antibody was the rabbit anti-trout tCA IV (1:200), or this antibody together with α5 (1:100), a mouse monoclonal antibody against the α1 subunit of chicken Na⁺,K⁺-ATPase. The
α5 antibody developed by D.M. Fambrough was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA. The α5 antibody has been used to localize Na⁺,K⁺-ATPase in a wide range of tissues and organisms, including gill tissue of elasmobranch fish (38). Negative control sections were incubated with blocking buffer from which primary antibodies had been omitted, or with the rabbit anti-trout tCA IV antibody in the presence of an excess (20 µg) of the peptide against which the antibody was raised. Detection was accomplished using Alexa 488-coupled goat anti-rabbit IgG (Fisher) for tCA IV or Alexa 546-coupled goat anti-mouse IgG (Fisher) for α5. Following incubation with the primary antibody, sections were washed in 0.1 mol L⁻¹ PB (3 x 5 min), and incubated for 1 h at room temperature in a humidified chamber with a 1:400 dilution in 0.1 mol L⁻¹ PB of one or both secondary antibodies, as appropriate. The sections were again washed (3 x 5 min in 0.1 mol L⁻¹ PB), then mounted using a medium (Vector Laboratories) containing 4′,6′-diamidino-2-phenylindole (DAPI) for the visualization of nuclei. A conventional epifluorescence microscope (Zeiss Axiophot) fitted with a CCD camera (Hamamatsu C5985) was used to view the sections (at least 9 – 12 sections per fish for each of 3 fish), and images were captured using Metamorph v4.01 imaging software. In some instances, sections were also examined using a confocal microscope (Olympus Fluoview BX50W1).
Results

*Molecular cloning of dogfish CA isoforms*

Using homology cloning strategies, two dogfish CA isoforms were cloned. From dogfish gill tissue, an 1127 base pair cDNA was assembled (GenBank accession DQ092628). This cDNA product included a complete coding sequence for a deduced protein of 306 amino acids (Fig. 1A) that most closely resembled CA IV, sharing 40% identity and 57% similarity to human CA IV (GenBank accession NP_000708), and 44-51% identity and 57-62% similarity to CA IV sequences for other fish species (pufferfish, *Tetraodon nigroviridis*, GenBank accession CAG08972 and CAF98532; zebrafish, *Danio rerio*, XP_682741 and AAH78387; rainbow trout, *Oncorhynchus mykiss*, AAR99330). Web-based protein prediction tools suggested the existence of an N-terminal signal peptide 19 amino acids in length that, together with a C-terminal GPI modification site at amino acid 282 or 283 (of the immature protein), would yield a mature GPI-linked protein of ~263 amino acids (Fig. 1A). In addition, this predicted amino acid sequence included four cysteine residues (at positions 5, 13, 25 and 209 of the mature protein) that were deemed likely to form two disulphide linkages (cys 5 – cys 13 and cys 25 – cys 209), and a single N-glycosylation site (Fig. 1A). The presumed active site of this dogfish sequence strongly resembled those of known or putative vertebrate CA IVs, particularly those of other fish species and *Xenopus* (Table 1). Notably, all residues thought to constitute the active site itself were conserved between the dogfish sequence and those of *Xenopus* and other fish species, and all but one between the dogfish sequence and mammalian CA IVs (Table 1). In addition, phylogenetic analysis of vertebrate CA sequences grouped this dogfish gene with other vertebrate CA IVs, within a broad grouping of membrane-associated CA isoforms that was distinct from the
cytoplasmic CA grouping (Fig. 2). Hence, this dogfish gene was tentatively identified as CA IV (dfCA IV).

A second cDNA, of 1107 base pairs, was assembled from dogfish blood (dfCAb) and included a complete coding sequence for a deduced protein of 260 amino acids (Fig. 1B). The deduced protein was most similar both to fish CAs, sharing 57-60% identity and 70-73% similarity with fish CAs cloned largely from blood (gar, *Lepisosteus osseus*, GenBank accession AAM94169; tilapia, *Oreochromis mossambicus*, AAQ89896; lamprey, *Petromyzon marinus*, AAZ83742; Japanese dace, *Tribolodon hakonensis*, BAB83090; and rainbow trout, AAP73748), and mammalian CA XIII, sharing 57-58% identity and 73-74% similarity with mouse, rat and human CA XIII (mouse, GenBank accession NP_078771; rat XP_574890; human NP_940986).

The presumed active site of this dogfish sequence was comparable to those of fish cytosolic CAs, as well as those of consensus mammalian CA VII (5 aa differences), CA XIII (7 aa differences), CA II (9 aa differences) and CA I (11 aa differences) sequences. Unlike virtually all other CA sequences examined, however, a serine rather than a histidine residue was present as the proton shuttling ligand (Tables 1 and 2). In addition, the dogfish sequence was unusual in exhibiting a tyrosine rather than a tryptophan residue at position 192 (Table 2). In phylogenetic analyses, this dogfish gene joined the broad cytoplasmic CA grouping (Fig. 2), a grouping distinct from that of membrane-associated CA isoforms, and including CA VII sequences, as well as tetrapod CA I, II, III and XIII, and fish cytoplasmic CAs. However, within the broad cytoplasmic CA grouping, the dogfish blood sequence did not join the monophyletic clade of fish blood-specific and cytoplasmic CA isoforms (Fig. 2). Rather, the dogfish sequence was basal (although note the low bootstrap value) to both the fish cytoplasmic CA clade and the tetrapod CA I, II, III and XIII isoforms. The dogfish blood CA sequence was in this respect similar to lamprey CA, although
lamprey CA groups more closely with CA VII sequences (10), and the dogfish blood CA sequence was clearly distinguishable from this grouping (Fig. 2); notably, the CA VII grouping includes a partial sequence for dogfish CA VII (GenBank accession CX196604).

*Tissue distribution and localization in gill tissue of dfCA IV*

Using real-time PCR, dfCA IV mRNA expression was assessed across a range of tissues. This analysis revealed gill to be the site of highest expression, closely followed by kidney (Fig. 3). Lower levels of dfCA IV mRNA expression were present in intestine, rectal gland and brain, with the remaining tissues exhibiting negligible expression.

Dogfish CA IV mRNA and protein expression patterns in the gill were examined using *in situ* hybridization and immunohistochemistry, respectively. Positive hybridization signals were observed in cells that, on the basis of their location, were considered to be pillar cells (Fig. 4A, B). Certain cells of the lamellar epithelium, primarily those flanking the pillar cells, also exhibited positive hybridization signals. These positive signals were eliminated when gill tissue was pre-treated with excess unlabelled probe (Fig. 4C) or incubated in the absence of probe (negative control; data not shown).

A trout CA IV antibody (12) was used to probe the pattern of dfCA IV protein expression in dogfish gill tissue (Fig. 5). Use of the trout CA IV antibody for this purpose was validated by western analysis of dogfish gill tissue, which revealed that the trout CA IV antibody detected a protein of 40 kDa mass, slightly higher than the predicted molecular mass for dfCA IV of 35 kDa (Fig. 5B). This immunoreactive band was eliminated by preabsorption of the antibody with an excess of the peptide against which it was raised (blocking peptide, Fig. 5B), confirming the specificity of the trout CA IV antibody for dfCA IV. The pattern of dfCA IV protein expression
in the dogfish gill was similar to that of its mRNA expression (Fig. 5A). Cells exhibiting immunoreactivity were again identified as pillar cells on the basis of their location (Fig. 5A, panels a, b and c). Frequently, but not universally, the lamellar epithelial cells flanking the pillar cells were immunoreactive for CA IV, and occasionally this CA IV protein was co-localized with Na⁺,K⁺-ATPase (Fig. 5Ab). CA IV immunofluorescence was prevented by omission of the primary (tCA IV) antibody (Fig. 5Ad) or when sections were pre-incubated with an excess of the peptide against which the antibody was raised (Fig. 5Ae). The membrane localization of CA IV immunofluorescence was evident when sections were examined by confocal microscopy (Fig. 6; supplementary data).
Discussion

Previous work provided indirect physiological and biochemical evidence to support the presence in dogfish gills of a type IV CA that is accessible to blood CO₂ reactions (15, 16, 71). In the present study, a type IV CA was successfully cloned from dogfish gill. Moreover, the dogfish CA IV was demonstrated to be present within the dogfish gill in a location consistent with it being available to catalyze blood CO₂ reactions, i.e. associated with the plasma membrane of pillar cells (Fig. 7). This new information, in conjunction with the results of previous work in which extracellular CA was demonstrated to contribute to CO₂ excretion in dogfish (15, 16), offers convincing support for a model in which dfCA IV-catalyzed dehydration of plasma HCO₃⁻ makes a substantial contribution to CO₂ excretion in dogfish.

Type IV CA is distinguished from other CA isoforms by a collection of characteristics that were present in dfCA IV, contributing to its tentative identification as a CA IV isozyme. CA IV belongs to the grouping of membrane-associated isozymes (IV, IX, XII, XIV and XV), and specifically to the sub-group (IV and XV) that is bound to the plasma membrane by a GPI anchor (reviewed by 9, 47). These groupings are well-supported and clearly visible in the phylogenetic tree generated in the present study (Fig. 2), as is a less well-supported separation of the CA IV clade into mammalian, amphibian and fish divisions. As with other GPI-anchored proteins, post-translational modification occurs in the endoplasmic reticulum prior to the delivery of the mature protein to the plasma membrane. Specifically, an 18-20 residue N-terminal signal peptide that functions in membrane targeting, and a C-terminal hydrophobic domain are removed by endoproteolytic cleavage (32, 33). Removal of the C-terminal hydrophobic domain allows attachment of the GPI anchor to a highly conserved serine residue. In keeping with these key
characteristics of CA IV, prediction software suggested that the dfCA IV sequence contains a 19 residue N-terminal leader peptide together with a 25 residue C-terminal hydrophobic domain that would result in attachment of the GPI anchor to the conserved serine residue (Fig. 1). Most CA IVs (human CA IV is an exception) contain carbohydrate (67), and the dfCA IV sequence exhibits a potential glycosylation site. Human CA IV exhibits two disulphide linkages (cys 6 – cys 11 and cys 23 – cys 203) that act to stabilize the mature conformation of the protein, and in particular, an active site loop (48, 49), thereby conferring upon CA IV its unusual resistance to solubilization by the detergent SDS (66, 69). In the dfCA IV sequence, cysteine residues are present at the appropriate locations and are predicted to form two disulphide bridges (Fig. 1). Finally, CA IV is a high activity isoform (1). Although no catalytic data for dfCA IV are available, the similarity of the active site pocket of dfCA IV to those of vertebrate CA IV sequences of known high activity (Table 1) suggests that dfCA IV is also a high activity isozyme.

In mammals, CA IV has been localized to endothelial surfaces of capillary beds (e.g. lung - 11, 69) and/or epithelial cell plasma membrane surfaces (e.g. kidney – 4, 72) in a variety of tissues, including the gas exchange surface (lung), kidney, brain, gastrointestinal tract, skeletal muscle and heart (35, 47). CA IV in these locations has an apical or luminal orientation, and is thought to play roles in both local and systemic acid-base regulation, primarily through its impact on buffering and/or HCO_3^- ion reabsorption (e.g. 35, 42, 44, 51, 68). The tissue distribution of dfCA IV mRNA expression was consistent with patterns of CA IV distribution in tetrapods, with the gas exchange surface (gill), kidney, intestine and brain standing out as sites of dfCA IV mRNA expression (Fig. 3). Interestingly, dogfish rectal gland was also included in this group (Fig. 3). The elasmobranch rectal gland is a specialized salt secreting tissue (45).
Although current models do not implicate CA in rectal gland salt secretion mechanisms (46), CA activity is present in the gland (25), where it is thought to facilitate the elimination of metabolically-produced CO₂ (54). Using histochemical approaches, Lacy (25) reported CA localization along basolateral membranes of rectal gland epithelial cells, a location that would be consistent with CA IV expression.

Western analysis corroborated the real-time PCR data for dfCA IV mRNA expression in gill tissue by demonstrating dfCA IV protein expression (Fig. 5B). The presence of dfCA IV protein in gill tissue is in agreement with the findings of earlier studies on dogfish gills of a membrane-linked CA activity that could be released from its membrane association by PI-PLC (16, 19). The available biochemical evidence suggests that the gills of other chondrichthyan fish may also possess a type IV CA activity (17). By contrast, most biochemical evidence (e.g. 13, 16, 17, 19, 20, 22, 43; but see 65) as well as the available molecular data (12) suggest that the gills of teleost fish lack CA IV-like activity. An explanation for this difference between cartilaginous and teleost fish remains elusive.

The localization of dfCA IV within dogfish gill tissue was examined using in situ hybridization (for mRNA expression) and immunohistochemistry (for protein expression). Similar distribution patterns of positive hybridization signals (Fig. 4) and immunoreactivity (Fig. 5) were apparent, supporting the localization of dfCA IV to the plasma membranes (Fig. 6) of pillar cells (Fig. 7) and, to a lesser extent, the lamellar epithelial cells flanking the pillar cells. Pillar cells consist of two parts; a nucleated body that acts as a post to separate the two epithelial layers forming the lamella, and cytoplasmic processes or flanges that spread out to meet the flanges of adjacent pillar cells, thereby delimiting and lining the blood space (26, 34, 70). Although pillar cells are unique to fish gills, they are in some respects the functional equivalents
of the endothelial cells that line the pulmonary vasculature of tetrapods (70). Thus, localization of dfCA IV to pillar cell membranes is consistent with the capillary endothelial location of pulmonary CA IV in mammals (11), and suggests that dfCA IV activity would be accessible to plasma CO2 reactions. Underlying the pillar cells is a basal lamina upon which sits the lamellar epithelium. The lamellar epithelium, in turn, is made up largely of squamous pavement cells typically organized into one to three cell layers (27, 70). Dogfish CA IV localized to the lamellar epithelial cells flanking pillar cells could conceivably be oriented towards either the interstitial space or the external environment. Because CO2 reactions in water flowing over the gills of dogfish do not appear to have access to CA activity (37), it is likely that dfCA IV localized to the lamellar epithelium is oriented towards the interstitial space.

The role of dfCA IV that has emerged from studies employing physiological approaches is to contribute to CO2 excretion by catalyzing the dehydration of plasma HCO3− ions (15, 16), and the pillar cell localization of dfCA IV is certainly consistent with this function (Fig. 7). Although pulmonary capillary endothelial CA IV in mammals also appears well placed to contribute to CO2 excretion by catalyzing plasma HCO3− dehydration, neither model simulations of lung gas exchange (2, 3, 6) nor experimental measurements of CO2 excretion following CA inhibition (5, 52, 56) support a significant role for extracellular CA activity in CO2 excretion (reviewed by 21, 51). Pulmonary capillary CA IV may be limited in its contribution to CO2 excretion by two key factors, namely the 100-fold greater CA activity and 10-fold greater buffering capacity of the red cell environment over that of plasma (21, 51). Higher levels of CA activity coupled with greater proton availability for HCO3− dehydration tend to drive the majority of HCO3− dehydration in mammals through the red cell, minimizing the contribution of extracellular CA to CO2 excretion. By contrast, several factors may conspire to increase the
importance of plasma HCO₃⁻ dehydration in dogfish. Whereas plasma buffer capacity in most mammals contributes little to whole blood buffering (e.g. 22% in humans; 28), plasma buffering in elasmobranch fish accounts for 40-70% of whole blood buffer values (17). Coupled with the absence of a Haldane effect (73), the relatively high plasma buffer capacity in dogfish will tend to diminish differences between the plasma and red cell compartments in proton availability for HCO₃⁻ dehydration (16). In addition, dogfish lack an endogenous plasma CA inhibitor (19). The presence of endogenous CA inhibitors in the plasma of some mammalian species (24, 39, 40) may lower the effective activity of CA IV, thereby contributing to limitations on the involvement of CA IV-catalyzed HCO₃⁻ dehydration in CO₂ excretion (18). Finally, red cell CA activity in dogfish is low relative to that of teleost fish or mammals (19, 31). The low red cell CA activity of dogfish contrasts with the availability of a high activity CA IV isozyme to catalyze plasma CO₂ reactions, and will again serve to diminish differences between the plasma and red cell compartments. Moreover, while both mammalian CA II and CA IV are considered to be high activity isozymes, human CA IV is markedly more efficient than CA II in HCO₃⁻ dehydration (1). A similar or even more marked disparity between dfCAb and dfCA IV would further level out differences between the red cell and plasma compartments, accounting for the enhanced role of plasma HCO₃⁻ dehydration in CO₂ excretion in dogfish.

The cloning of a dogfish blood CA isoform in the present study provided some insight into the low red cell CA activity reported for dogfish (19, 31). Analysis of the active site pocket of dfCAb revealed the substitution of a serine residue for histidine at the proton shuttling ligand (Table 2). In the catalytic mechanism of CA (reviewed by 30, 36, 48, 50), a captive CO₂ molecule is attacked by a highly reactive Zn-bound hydroxide ion, resulting in the formation of a HCO₃⁻ ion that is coordinated to the Zn. The HCO₃⁻ ion is then replaced by a water molecule.
To regenerate the reactive Zn-bound hydroxide, a proton must be transferred from the active site to the environment. It is this function that is carried out by the proton shuttle, which is typically a histidine residue for efficient proton transfer. Proton transfer is the rate-limiting step in the catalytic mechanism, and enzyme activity can be greatly reduced by replacement of the histidine with residues that cannot transfer protons (30, 48, 63). Thus, the substitution of serine for histidine as the proton shuttle would be expected to lower the catalytic activity of dfCAb, and could account for the low turnover number \( k_{cat} \) reported for dogfish red cell CA (31), a turnover number that is approximately an order of magnitude lower than values reported for teleost species (8, 31). In addition, Esbaugh and Tufts (10) pointed out that the determination of turnover number using red cell lysates is influenced by the sensitivity of the CA to the sulphonamide acetazolamide, since this inhibitor is used to estimate enzyme concentration, and dogfish red cell CA was reported to exhibit an unusually low sensitivity to acetazolamide (31). As the elements of the active site pocket with which acetazolamide interacts (29) are conserved in dfCAb, an explanation for the high resistance of dogfish red cell CA to acetazolamide inhibition remains to be determined. Interestingly, the inhibition constant against acetazolamide of a cytoplasmic CA in dogfish gills was reported to be similar to that of other fish species and hence much lower than that of dogfish red cell CA (16), implying the existence of more than one cytoplasmic CA isoform in dogfish.

The low activity of dogfish red cell CA (or presumed low activity of dfCAb) contrasts with the high activity of other cytoplasmic CA isozymes from fish (or presumed high activity based on active site comparisons), including that of the lamprey (10). The existence of a high activity red cell CA isoform in lamprey, together with the placement of lamprey red cell CA in phylogenetic analyses (Fig. 2; 10), implies the appearance early in vertebrate evolution of a high
activity CA, the catalytic structure of which was then strongly conserved throughout the vertebrate lineage. Given this context, the apparent loss of high activity in dogfish blood CA is puzzling and warrants further investigation. In particular, the potential existence of more than one red cell CA isoform, as is found in trout (8) and many mammals (51), should be explored.
Acknowledgements

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Figure legends

**Figure 1.** (A) The deduced amino acid sequence of dogfish (*Squalus acanthias*) carbonic anhydrase type IV (dfCA IV). An N-terminal signal peptide typical of CA IV isoforms is underlined (predicted using the web software SignalP 3.0 which is available via the ExPASy Proteomics site at [http://ca.expasy.org/tools/](http://ca.expasy.org/tools/), together with the other web-based software tools listed here), while double underlining indicates a likely C-terminal hydrophobic domain that is cleaved at a GPI modification site, also characteristic of CA IV isoforms (DGPI and big-PI Predictor). Cysteine residues likely to form two disulphide bridges (cys 5 – cys 13 and cys 25 – cys 209; Disulfind, available at [http://www.predictprotein.org/about.html](http://www.predictprotein.org/about.html)) are shaded. A potential glycosylation site is underlined with a dotted line (NetNGlyc). (B) The nucleotide and deduced amino acid sequences of the CA cloned from dogfish blood (dfCAb). Only the coding region of the sequence is presented, from the start codon (underlined) to the stop codon (asterisk).

**Figure 2.** A phylogenetic tree to illustrate the relationships between the two dogfish (*Squalus acanthias*) carbonic anhydrase (CA) isoforms cloned in the present study (and which are highlighted in black), dfCA IV (dogfish IV, GenBank protein accession AAZ03744) and dfCAb (dogfish CAb), and selected vertebrate cytoplasmic and membrane-associated CA isoforms. The phylogenetic tree was constructed using neighbour-joining analysis (see Materials and Methods for more detail). The tree was ordered using *Drosophila* CA (AAY56645) as a monophyletic outgroup. Horizontal branch lengths are scaled to represent the relative number of amino acid substitutions occurring along a branch, and support values
at the nodes are indicated as a percentage from bootstrap analysis using 100 pseudoreplicates. GenBank protein accession numbers for the sequences used in the tree were as follows: CA I, human AAH27890, mouse AAH11223, rat XP_226922; CA II, human AAA51909, mouse AAA37357, rat CAA41227, Xenopus CAJ83242; CA III, human AAA52293, mouse NP_031632, rat AAA40846; CA IV, rainbow trout AAR99330, zebrafish AAH78387, fugu (Tetraodon nigroviridis) CAG08972, Xenopus AAH54242, human NP_000708, mouse AAC52569, rat NP_062047; CA VII, dogfish CX196604 (this GenBank nucleotide accession number was used to deduce the amino acid sequence), zebrafish NP_957107, Xenopus CAJ83128, human AAL78167, mouse AAG16230, rat XP_226204; CA IX, human CAI13455, mouse AAL14193, rat XP_233380; CA XII, Xenopus CAJ81489, human AAP35302, mouse AAH31385, rat AAX50191; CA XIII, human NP_940986, mouse NP_078771, rat XP_222295; CA XIV, human Q9ULX7, mouse Q9WVT6, rat XP_342298; CA XV, mouse AAK16671; fish cytoplasmic CAs, lamprey AAZ83742, gar AAM94169, tilapia AAQ89896, rainbow trout blood isoform (CAb) AAP73748, rainbow trout cytoplasmic isoform (CAC) AAR99329, zebrafish NP_571185 (denoted CAb as it grouped with other blood-specific isoforms) and NP_954685 (denoted CAC as it grouped with other cytoplasmic isoforms), Japanese dace BAB83090, carp AAZ83743, salmon BG933892 (this GenBank nucleotide accession number was used to deduce the amino acid sequence).

**Figure 3.** Tissue distribution of dogfish (Squalus acanthias) CA IV mRNA expression as determined by real-time PCR. Data were compared to gill, which was given a relative value of 1. Values are means ± 1 SEM with N = 4.
Figure 4. Representative light micrographs illustrating the localization by in situ hybridization of dogfish type IV carbonic anhydrase (dfCA IV) mRNA expression in the gills of dogfish (Squalus acanthias). Panels A and B present two different regions of gill lamellae viewed at medium and high magnification, respectively. Strong signals for dfCA IV are evident in what appear to be pillar cells, as well as the lamellar epithelial cells flanking the pillar cells. These signals were eliminated by incubation of sections in the presence of excess unlabelled probe (C). Scale bar = 5 µm.

Figure 5. (A) Localization by immunohistochemistry of dogfish type IV carbonic anhydrase (dfCA IV) protein in the gills of dogfish (Squalus acanthias). The images are overlays of images collected individually for dfCA IV immunoreactivity (green), α5 (Na+,K+-ATPase) immunoreactivity (red) and nuclei visualization (blue). Areas of overlap of dfCA IV and α5 immunoreactivity are indicated in yellow/orange. Nuclei were visualized using 4’,6’-diamidino-2-phenylindole. Medium (panel a) and high (b,c) magnification images representative of the sections examined indicate that cells tentatively identified as pillar cells on the basis of their location consistently displayed immunoreactivity for only dfCA IV. The cells of the lamellar epithelium that displayed immunoreactivity for dfCA IV typically were found flanking the pillar cells. Co-localization with α5 occurred to a variable extent in some of these cells (arrows in b); in other cases, cells expressed only the α5 signal. Omission of primary antibodies eliminated all immunofluorescence (d). Pre-absorption of the CA IV antibody using the peptide against which the antibody was raised eliminated dfCA IV immunoreactivity without affecting α5 immunoreactivity (e). Scale bars = 5 µm. (B) Western blot of dogfish gill tissue depicting an immunoreactive band at ~40 kDa (control).
that was eliminated after preabsorbing the primary antibody with excess peptide antigen (blocking antibody).

**Figure 6.** Immunohistochemical localization of dogfish type IV carbonic anhydrase (dfCA IV) protein to the plasma membrane of pillar cells in the gills of dogfish (*Squalus acanthias*). The images are individual optical sections, 1.2 µm apart, collected by confocal microscopy and displayed sequentially from (A) to (I). Note in particular the immunofluorescence associated with the flanges of the pillar cell in the centre of the images (arrows). Scale bar = 20 µm.

**Figure 7.** Schematic representations depicting the location and presumed function of dfCA IV in the dogfish gill. In (A), a schematic cross-section through the lamella is presented. Pillar cells (PC) define the blood space (indicated by the shaded arrow representing the oxygenation of blood). Pillar cells rest on a basement membrane (BM), with the water-facing surfaces of the lamella being made up largely of pavement cells (PVC). The grey shading indicates the blood-facing surfaces of the pillar cells where dfCA IV is thought to be expressed; note the absence of dfCA IV from the cytoplasm of the pillar cells, and the intimate contact possible between dfCA IV and blood. Modelled after Olson (34). The area marked by the dotted box is presented in panel (B) to illustrate the presumed role of dfCA IV in catalyzing the dehydration of plasma HCO$_3^-$ . CA is also present in the plasma (see text) and in the red blood cell (RBC). RBC CA activity (presumably dfCAb) also contributes to CO$_2$ excretion. CA activity within lamellar epithelial cells catalyzes the hydration of CO$_2$ to HCO$_3^-$ and H$^+$; these ions can then be excreted by mechanisms that remain to be specified for
purposes of acid-base regulation (61, 62).

**Supplementary data.** Immunohistochemical localization by confocal microscopy of dogfish type IV carbonic anhydrase (dfCA IV) protein to the plasma membrane of pillar cells in the gills of dogfish (*Squalus acanthias*). The images are animated three-dimensional reconstructions of 22 individual optical sections; two different rotations of the same section are presented. To run the animation, choose the “slide show” option in MS Powerpoint.
Table 1. A comparison of the amino acid residues comprising the presumed active site pocket across vertebrate CA IV (and/or putative CA IV) sequences.

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CA IV sequences were individually aligned against human CA I (GenBank accession AAH27890) to identify the residues of the active site cavity; “residue number” refers to the human CA I sequence, while “z” indicates histidine residues that bind the zinc ion, “p” indicates the proton shuttling ligand, and residues thought to constitute the active site itself are indicated by an asterisk (23, 58, 59). Grey shading has been used to designate residues that are largely invariant across all CA IV sequences examined, while black shading denotes residues that are conserved across fish and amphibian sequences and differ from those
Table 2. A comparison of the amino acid residues comprising the presumed active site pocket across fish blood or cytoplasmic CA sequences, and mammalian CA I, II, VII and XIII.

| Residue number | 7 | 29 | 61 | 64 | 65 | 66 | 67 | 69 | 91 | 92 | 94 | 96 | 106 | 107 | 117 | 121 | 131 | 141 | 143 | 145 | 192 | 194 | 198 | 199 | 200 | 201 | 202 | 204 | 206 | 207 | 209 | 211 | 244 | 246 |
|----------------|---|----|----|----|----|----|----|----|----|----|----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **CA I**       | Y  | S  | N  | V  | H  | S  | F  | H  | N  | F  | Q  | H  | H  | E  | E  | H  | E  | H  | V  | L  | L  | I  | G  | W  | Y  | L  | T  | H  | P  | P  | H  | S  | V  | W  | I  | N  | R  |
| **CA II**      | Y  | S  | N  | N  | H  | S  | F  | N  | E  | I  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | L  | C  | V  | W  | V  | N  | R  |
| **CA VII**     | Y  | S  | N  | N  | H  | S  | F  | Q  | D  | K  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | F  | S  | V  | W  | V  | N  | R  |
| **dfCAb**      | Y  | S  | N  | T  | S  | S  | V  | Q  | D  | R  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | F  | S  | V  | W  | V  | N  | R  |
| **Gar**        | Y  | S  | N  | N  | H  | S  | F  | Q  | D  | R  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | L  | S  | V  | W  | V  | N  | R  |
| **Carp**       | Y  | S  | N  | N  | H  | S  | F  | Q  | D  | R  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | L  | S  | V  | W  | V  | N  | R  |
| **Zebrafish1** | Y  | S  | N  | N  | H  | S  | F  | Q  | S  | K  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | L  | S  | V  | W  | V  | N  | R  |
| **Zebrafish2** | Y  | S  | N  | N  | H  | S  | F  | Q  | G  | K  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | L  | S  | V  | W  | V  | N  | R  |
| **tCAb**       | Y  | S  | N  | N  | H  | S  | F  | Q  | G  | K  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | L  | S  | V  | W  | V  | N  | R  |
| **tCAc**       | Y  | S  | N  | N  | H  | S  | F  | Q  | T  | K  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | Y  | S  | V  | W  | V  | N  | R  |
| **Tilapia**    | Y  | S  | N  | N  | H  | S  | V  | Q  | T  | K  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | Y  | S  | V  | W  | V  | N  | R  |
| **Dace**       | Y  | S  | N  | N  | H  | S  | F  | Q  | T  | K  | Q  | H  | H  | E  | E  | H  | V  | F  | L  | V  | G  | W  | Y  | L  | T  | T  | P  | P  | Y  | S  | V  | W  | V  | N  | R  |

CA sequences were individually aligned against human CA I (GenBank accession AAH27890) to identify the residues of the active site cavity; “residue number” refers to the human CA I sequence, while “z” indicates histidine residues that bind the zinc ion, “p” indicates the proton shuttling ligand, and residues thought to constitute the active site itself are indicated by an asterisk (23, 58, 59). Grey shading has been used to designate residues that are largely invariant across all of the cytosolic CA sequences.
examined. CA II represents the consensus sequence reported by Tashian et al. (59), CA XIII represents the consensus sequence among human (GenBank accession NP_940986), mouse (NP_078771) and rat (XP_222295), and human CA VII (AAL78167) was used for CA VII. dfCAb represents the CA sequence cloned from dogfish blood. GenBank accession numbers for the remaining sequences were as follows: Lamprey, *Petromyzon marinus* AAZ83742; Gar, *Lepisosteus osseus* AAM94169; Carp, *Cyprinus carpio* AAZ83743; Zebrafish, *Danio rerio* ¹NP_571185 and ²NP_954685; Rainbow trout, *Oncorhynchus mykiss* tCAb AAP73748 and tCAc AAR99329; Tilapia, *Oreochromis mossambicus* AAQ89896; and Japanese dace, *Tribolodon hakonensis* BAB83090.
Figure 1
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