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

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

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

Introduction

Carbonic anhydrase (CA) is a zinc-containing enzyme that catalyzes the reversible hydration and dehydration reactions of CO₂. Since it was first discovered in vertebrate erythrocytes (Brinkman et al. 1932), CA has been found in many different tissues and has been implicated in a wide variety of physiological and biochemical processes (Maren 1967; Sly and Hu 1995; Henry 1996; Chegwidden and Carter 2000). All CAs characterized in the animal kingdom belong to a single gene family referred to as the α-carbonic anhydrases (α-CAs; Hewett-Emmett and Tashian 1996). To date, 14 different α-CA isozymes have been discovered in mammals (Chegwidden and Carter 2000). These CA isozymes have been identified using a number of different approaches including determination of their unique kinetic and inhibitor properties, sub-cellular distribution and/or molecular structure (Maren and Sanyal 1983; Sanyal 1984; Henry et al. 1986, 1993, 1997; Hewett-Emmett and Tashian 1991). In comparison to the situation in mammals, much less is known about the CA isozymes in non-mammalian vertebrates. There is also very little information about the evolution of the different vertebrate CA isozymes.

In most vertebrates, erythrocyte CA plays an important role in blood CO₂ transport and excretion. Since erythrocyte CA is relatively easy to obtain for experimental purposes, it is also probably the most widely studied CA isozyme. The erythrocytes of many mammalian species have been found to possess both low activity (CA I) and high activity (CA II) CA isozymes. In contrast, most early vertebrates are thought to possess only one cytoplasmic CA isozyme within their erythrocytes (Carlsson et al. 1980; Maren et al. 1980; Sanyal et al. 1982; Hall and Schraer 1983; Kim et al. 1983). In agnathans and elasmobranchs, erythrocyte CA resembles mammalian CA I in terms of both activity and sensitivity to inhibitors (Carlsson et al. 1980; Maren et al. 1980; Henry et al. 1993). In more recently evolved
teleost fish, however, the kinetic and inhibitor properties of erythrocyte CA are more similar to the mammalian CA II isozyme (Maren et al. 1980; Hall and Shraer 1983; Kim et al. 1983; Henry et al. 1993; Henry and Swenson 2000). It would therefore appear that the slow type I CA isozyme is probably the most ancient erythrocyte isozyme and that it was eventually replaced by a faster type II isozyme after the appearance of the elasmobranchs and prior to the evolution of the teleosts. A recent investigation of the kinetic and inhibitor properties of erythrocyte CA in the bowfin also seems to support this phylogenetic trend (Gervais and Tufts 1999). At present, however, there is very limited information about the properties of erythrocyte CA from other ancient fishes that are phylogenetically intermediate between elasmobranchs and teleosts.

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

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

### Materials and methods

**Animal preparation and blood collection**

Longnose gar, *Lepisosteus osseus*, were collected by commercial fishermen on the Bay of Quinte in southeastern Ontario. Gar were killed by a sharp blow to the head, and blood was collected by caudal puncture into a chilled round-bottom flask containing heparinized (40 µl ml–1) saline (in mmol l–1: 124 NaCl, 10 NaHCO₃, 5.5 glucose, 5 KCl, 1.1 CaCl₂, 0.5 MgCl₂). The erythrocytes were then washed three times in saline, lysed in 50 volumes of distilled water, and frozen for later measurement of CA activity.

**Measurement of erythrocyte CA activity**

Erythrocyte CA activity was measured using the electrometric ΔpH method (Henry 1991; Henry et al. 1993; Gervais and Tufts 1998). The reaction medium consisted of 10 ml buffer (in mmol l–1: 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.4 with 10% phosphoric acid) held at 4 °C by a circulating water bath attached to the reaction chamber. After the addition of the lysate, the reaction commenced with the addition of 400 µl CO₂ saturated distill water (~1 °C), delivered from a 1000-µl gas-tight Hamilton syringe. The reaction was then measured over a pH change of approximately 0.15 units using a GK2401C combined electrode (Radiometer) attached to a PHM64 research pH meter (Radiometer). To obtain the true catalyzed reaction rate in the chamber, the uncatalyzed rate (no lysate) was subtracted from the observed rate (lysate present) and the buffer capacity was taken into account to correct from pH units time–1 to mol H⁺ (CO₂) time–1. Buffer capacity of the reaction medium was determined by titrating the reaction buffer with known concentrations of strong acid (0.1 N HCl) in the absence of lysate.

### Series I. Inhibition and kinetic properties of CA

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

\[ I_r/i = K_i/(1 - i) + E_o \]

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

In order to determine the substrate affinity (Kₘₐₓ) of erythrocyte CA, its activity was measured against increasing concentrations of CO₂ and then fitted to a Lineweaver-Burk plot (Maren et al. 1980; Henry et al. 1993). The active site turnover rate, Kₖₐₜₜ, was then determined from the relationship Vₐₚₔ₝/Eₒ₋₀, where Vₐₚₔ₝ is the reciprocal of the y-intercept on the Lineweaver-Burk plot (Maren et al. 1980). Eₒ₋₀ was also used to estimate the concentration of CA in the erythrocytes. These analyses were also performed on mammalian CA I and CA II (Sigma) in order to provide a basis of comparison under identical experimental conditions.

### Series II. Determination of cDNA sequence for gar rbc CA

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