

EVIDENCE FOR MEMBRANE-BOUND CARBONIC ANHYDRASE IN THE AIR BLADDER OF BOWFIN (*AMIA CALVA*), A PRIMITIVE AIR-BREATHING FISH

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Summary

The purpose of this study was to examine the subcellular distribution and isoenzyme characteristics of carbonic anhydrase from the gills and respiratory air bladder of bowfin *Amia calva*, a primitive air-breathing fish. Separation of subcellular fractions by differential centrifugation revealed that the vast majority of carbonic anhydrase from the gills of bowfin originated from the cytoplasmic fraction. Washing of the gill microsomal pellet also indicated that the carbonic anhydrase originally associated with this pellet was largely due to contamination from the cytoplasmic fraction. Experiments with a carbonic anhydrase inhibitor, sulphanilamide, and the plasma carbonic anhydrase inhibitor from this species confirmed that the bowfin gill probably contains only one carbonic anhydrase isoenzyme which had properties resembling those of CA II. In contrast to the situation in the gills, a relatively large percentage (27 %) of the total air bladder carbonic anhydrase was associated with the microsomal fraction. Washing of the air bladder microsomal pellet removed little of the carbonic anhydrase activity, indicating that most of the carbonic anhydrase in

the microsomal fraction was associated with the membranes. Like the mammalian pulmonary CA IV isoenzyme, microsomal carbonic anhydrase from the bowfin air bladder was less sensitive to the bowfin plasma carbonic anhydrase inhibitor, sodium dodecylsulphate (SDS) and sulphanilamide than was cytoplasmic carbonic anhydrase from the air bladder. Microsomal carbonic anhydrase from the bowfin air bladder also resembled CA IV in that it appears to be anchored to the membrane *via* a phosphatidylinositol–glycan linkage which could be cleaved by phosphatidylinositol-specific phospholipase C. Taken together, these results suggest that a membrane-bound carbonic anhydrase isoenzyme resembling mammalian CA IV in terms of inhibition characteristics and membrane attachment is present in the air-breathing organ of one of the most primitive air-breathing vertebrates.

Key words: carbonic anhydrase, isoenzyme, fish, bowfin, *Amia calva*, air breathing, air bladder, gill.

Introduction

Carbonic anhydrase is an enzyme that catalyses the reversible hydration/dehydration of $\text{CO}_2/\text{HCO}_3^-$. In addition to having an important function in the transport of CO_2 within the blood of vertebrates, carbonic anhydrase has been found to play a significant role in the homeostatic processes of a wide variety of tissues (Maren, 1967; Sly and Hu, 1995; Henry, 1996). In mammals, nine carbonic anhydrase isoenzymes have been identified in different tissues and subcellular fractions (Dodgson, 1991; Hewett-Emmett and Tashian, 1996). Among these are cytoplasmic (CA I, II, III), membrane-bound (CA IV) and mitochondrial (CA V) isoenzymes (Dodgson, 1991). The identification of carbonic anhydrase isoenzymes has been accomplished using a number of experimental approaches including molecular and kinetic characterization, subcellular fractionation and the use of specific inhibitors such as sulphonamides (Maren and Sanyal, 1983; Sanyal, 1984; Henry *et al.* 1986, 1993, 1997; Hewett-Emmett and Tashian, 1991). In comparison with mammals, much less is known about the

diversity of carbonic anhydrase isoenzymes and their functions in lower vertebrates.

Both cytoplasmic (CA I, II) and membrane-bound (CA IV) isoenzymes of carbonic anhydrase have been found in the lungs of mammals (Zhu and Sly, 1990; Nioka and Forster, 1991). Pulmonary CA IV is located on the extracellular luminal surface of capillary endothelial cells and is anchored through a phosphatidylinositol–glycan linkage (Zhu and Sly, 1990; Heming *et al.* 1993). Intravascular CA IV functions in establishing an equilibrium between CO_2 and plasma HCO_3^- to maintain postcapillary blood pH (Crandall and O'Brasky, 1978; Klocke, 1980; Bidani *et al.* 1983; Heming *et al.* 1993) and, to a limited extent, in dehydrating plasma HCO_3^- to CO_2 during pulmonary gas exchange (Bidani, 1991).

Carbonic anhydrase is also present in the gas-exchange organs of lower vertebrates. High levels of carbonic anhydrase activity occur in the gills of water-breathing vertebrates (Maren, 1967; Girard and Istin, 1975; Toews *et al.* 1978;

Conley and Mallatt, 1988; Henry *et al.* 1988, 1993). In the gills of lampreys and teleost fish, carbonic anhydrase appears to be restricted to the cytoplasm where it participates in acid–base and ionic regulation rather than in CO₂ excretion (Conley and Mallatt, 1988; Henry *et al.* 1988, 1993; Perry and Laurent, 1990). More recently, it was found that the gills of the dogfish shark *Squalus acanthias* contain both cytoplasmic carbonic anhydrase and a membrane-bound carbonic anhydrase which faces into the intravascular lumen (Swenson *et al.* 1995; Gilmour *et al.* 1997; Henry *et al.* 1997). It has been proposed that the primary function of membrane-bound carbonic anhydrase in dogfish gills is probably to equilibrate post-branchial CO₂ and H⁺ for chemoreceptors that influence ventilation rate (Gilmour *et al.* 1997; Henry *et al.* 1997), a role similar to that of CA IV in mammalian lungs. Significant carbonic anhydrase activity has also been measured in the air-breathing organ (ABO) of several air-breathing fish species (Burggren and Haswell, 1979; Heming and Watson, 1986). At present, however, virtually nothing is known about the subcellular distribution, isoenzyme characteristics or function of carbonic anhydrase in the ABOs of fish.

Heming and Watson (1986) suggested that the relatively high carbonic anhydrase activity in the ABO of bowfin *Amia calva* may include a pool of intravascular membrane-bound carbonic anhydrase, as in the lungs of higher vertebrates. The bowfin is one of only two extant holostean fishes, both of which use a modified swimbladder for facultative aerial respiration (Johansen *et al.* 1970; Rahn *et al.* 1971; Randall *et al.* 1981). In the swimbladder of a more recent teleost, the eel *Anguilla anguilla*, a membrane-bound extracellular carbonic anhydrase has recently been found that is thought to be involved in the regulation of buoyancy (Pelster, 1995). Several indirect lines of evidence therefore suggest that the modified swimbladder (ABO) of early air-breathing species such as the bowfin may contain an intravascular membrane-bound carbonic anhydrase isoenzyme, which could be similar to the CA IV isoenzyme found in mammalian lungs. To date, however, this intriguing possibility has not been investigated further. The main purpose of the present study was therefore to determine the subcellular distribution and isoenzyme characteristics of carbonic anhydrase in the bowfin ABO. To provide a basis for comparison with the results from the ABO, and also to provide further information regarding carbonic anhydrase in lower vertebrate gills, the carbonic anhydrase from the gills of these animals was also examined for these characteristics. Characterization of carbonic anhydrase from bowfin was accomplished by differential centrifugation to determine subcellular distribution and by using pharmacological inhibitors as well as a recently discovered plasma inhibitor of carbonic anhydrase from this species (M. R. Gervais and B. L. Tufts, unpublished observations).

Materials and methods

Animal preparation and tissue collection

Bowfin *Amia calva* L. (1.5–3.0 kg) were collected in the

autumn and spring from the Bay of Quinte in southeastern Ontario. Fish were held in aerated dechlorinated fresh water (8–15 °C) and were fed a diet of crayfish and dead minnows. There were no visual signs of stress and no mortalities among the bowfin used in these experiments. Fish were not fed during the 2 week period prior to experimental use.

Individual fish were anaesthetized in aerated water containing 250 mg l⁻¹ tricaine methane sulphonate (MS-222; Sigma) buffered with 500 mg l⁻¹ NaHCO₃. Blood was collected by blind caudal puncture into a heparinized (40 i.u. ml⁻¹) syringe and then transferred to a flask containing heparinized (40 i.u. ml⁻¹) saline (in mmol l⁻¹: 124 NaCl, 10 NaHCO₃, 5.5 glucose, 5 KCl, 1.1 CaCl₂, 0.5 MgCl₂). Red blood cells (RBCs) were washed three times, lysed in 200 volumes of distilled water, and then frozen for later measurement of carbonic anhydrase activity.

Prior to harvesting, the gills and air bladder of bowfin were perfused with saline to remove RBC carbonic anhydrase. Access to the air bladder was through a 20 cm mid-ventral incision. Following cannulation of the right air bladder vessel (from aortic arch VI) with PE 50 tubing, saline (described above) containing 7 mmol l⁻¹ EDTA at pH 7.8 was perfused through the vessels of the air bladder with a 10 ml syringe. The left ductus Cuvieri (venous circulation) was cut to allow the perfusate (approximately 200 ml in total) to drain out of the air bladder vessels. The muscle lining the air bladder and the areas that were not perfused sufficiently were carefully dissected away. The bulbus arteriosus was cannulated with PE 200 tubing to perfuse the gills with approximately 200 ml of saline containing 7 mmol l⁻¹ EDTA. Pink gill tissue was carefully dissected away to remove any contamination from RBC carbonic anhydrase.

Gill and air bladder fractionation

Approximately 2.5 g of gill and air bladder tissue was added to 8 volumes of cold buffer (described below) and homogenized using a motor-driven Teflon–glass homogenizer. The resulting crude homogenates were analyzed for protein and carbonic anhydrase activity and then subjected to differential centrifugation (Henry *et al.* 1988, 1993). The fractionation scheme was as follows: (1) low speed (275 g for 20 min, Beckman J2-21M centrifuge) to produce a pellet containing intact cells, large cell fragments and nuclei; (2) superspeed (7500 g for 20 min, Beckman J2-21M centrifuge) to produce a pellet containing mitochondria; and (3) ultracentrifugation (100 000 g for 90 min, Beckman L8-55M ultracentrifuge) to produce a microsomal pellet and a cytoplasmic supernatant. Centrifugation was always performed at 4 °C. Pellets were then resuspended in 1–10 ml of cold buffer prior to analysis, and each fraction was assayed for carbonic anhydrase activity.

Measurement of carbonic anhydrase activity and protein concentration

Carbonic anhydrase activity was measured by the electrometric ΔpH method (Henry, 1991; Henry *et al.* 1993). The reaction medium consisted of 10 ml of 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. After addition of the enzyme source, the reaction was started by the addition of 400 µl of CO₂-saturated distilled water, delivered from a 1000 µl gas-tight Hamilton syringe. The velocity of the reaction was measured over a change of 0.15 pH units. To obtain the true catalyzed rate in the chamber, the uncatalyzed rate was subtracted from the observed rate and the buffer capacity was taken into account to convert from pH units time⁻¹ to mol H⁺ time⁻¹. pH was measured using a Radiometer GK2401 C combined electrode connected to a Radiometer PHM64 research pH meter. Protein was measured using the bicinchoninic acid (BCA) method (Sigma) with bovine serum albumin (Sigma) as a standard.

Inhibition of carbonic anhydrase

To determine the isoenzyme characteristics of microsomal and cytoplasmic carbonic anhydrase, carbonic anhydrase activity was measured in the presence of various inhibitors. The sensitivity of carbonic anhydrase to sodium dodecylsulphate (SDS) was examined by measuring carbonic anhydrase activity in the presence of 0.005% SDS in the reaction chamber. Titrations of carbonic anhydrase were also carried out with increasing concentrations of sulphanilamide and the more potent inhibitor acetazolamide. The inhibition constant (K_i) for sulphanilamide was calculated according to the method of Dixon (1953). The inhibition constant for acetazolamide was calculated as the slope of the line with the following equation:

$$\frac{I_0}{i} = \frac{K_i}{1-i} + E_0, \quad (1)$$

where E_0 is the total concentration of free enzyme in the reaction chamber, I_0 is the concentration of inhibitor and i is the fractional inhibition of enzyme activity at a given inhibitor concentration (Easson and Stedman, 1937). The sensitivity of gill and air bladder carbonic anhydrase to the bowfin plasma inhibitor of carbonic anhydrase (M. R. Gervais and B. L. Tufts, unpublished results) was also determined.

Washing and cleaving

To determine whether the carbonic anhydrase activity observed in the microsomal fractions was due to membrane-bound carbonic anhydrase, microsomal pellets were washed three times to remove any remaining cytoplasmic carbonic anhydrase. Pellets were resuspended in the reaction buffer, agitated by mild sonication (5 W for 3 s) and then repelleted by ultracentrifugation as described above. The carbonic anhydrase activity of the pellet and supernatant was determined after each wash.

After the final wash, air bladder pellets were resuspended in the reaction buffer (described above) and incubated (37 °C for 90 min) with or without 1 unit of phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma) to determine whether carbonic anhydrase was anchored to the membrane *via* a phosphatidylinositol-glycan anchor (Cross, 1987; Low *et al.* 1988). PI-PLC will cleave phosphatidylinositolglycan-

anchored membrane-bound carbonic anhydrase and release it to the supernatant (Zhu and Sly, 1990; Bottcher *et al.* 1994). One unit of phosphatidylinositol-specific phospholipase C (PI-PLC) is defined as the amount that will liberate one unit of acetylcholinesterase per minute from a membrane-bound crude preparation at pH 7.4 at 30 °C. Following the 90 min incubation period, the air bladder samples were ultracentrifuged, and the carbonic anhydrase activities of the resulting pellet and supernatant were determined.

Results

Tissue carbonic anhydrase activity and distribution

Carbonic anhydrase activity was found in all three tissues examined (Table 1). Measured per milligram of protein, the carbonic anhydrase activity of the gills was approximately 2.5 times higher than that of the erythrocytes and 14 times higher than that of the air bladder.

Subcellular fractionation of the gills and air bladder produced different results. The majority (71%) of carbonic anhydrase activity in the gill was found in the cytoplasmic fraction (S_3 ; Fig. 1). The microsomal fraction (P_3) of the gill contained only 3% of the total gill activity. In contrast, carbonic anhydrase activity of the air bladder P_3 fraction contained 27% and the cytoplasmic fraction (S_3) accounted for only 39% of the total activity (Fig. 2). Measured per milligram of protein, the gill S_3 fraction had an activity of 252 µmol CO₂ min⁻¹ mg⁻¹ protein and the gill P_3 fraction had

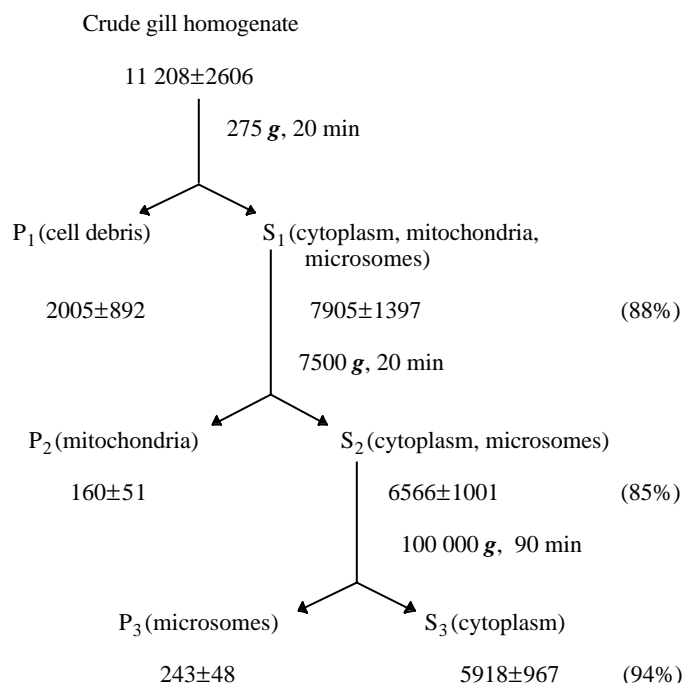


Fig. 1. Cellular fractionation of bowfin gill homogenate *via* differential centrifugation. Total carbonic anhydrase activity for each fraction is reported as µmol CO₂ min⁻¹. Values are mean ± S.E.M. ($N=6$). Percentage recovery for each step is indicated in parentheses.

Table 1. Carbonic anhydrase activity of the gills, erythrocytes and air bladder of bowfin

Tissue	Carbonic anhydrase ($\mu\text{mol CO}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$)
Gill	206.8 \pm 59.6
Erythrocytes	82.1 \pm 7.6
Air bladder	14.9 \pm 2.2

Values are means \pm S.E.M. (N=6).

an activity of $54 \mu\text{mol CO}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$, whereas the air bladder S_3 fraction had an activity of $20 \mu\text{mol CO}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$ and the air bladder P_3 fraction had an activity of $92 \mu\text{mol CO}_2 \text{ min}^{-1} \text{ mg}^{-1} \text{ protein}$.

Inhibition of microsomal and cytoplasmic carbonic anhydrase

The response to 0.005% SDS was essentially the same for both the gills and the air bladder. Both the gill and air bladder cytoplasmic carbonic anhydrase were sensitive to 0.005% SDS, but the microsomal carbonic anhydrase activity from these tissues was not significantly inhibited by this treatment (Fig. 3). Carbonic anhydrase from both tissues was also sensitive to the inhibitor sulphanilamide (Fig. 4). Carbonic anhydrase from the S_3 and P_3 fractions of the gills had similar inhibition constants (K_i); however, carbonic anhydrase from the S_3 fraction of the bladder was 2.5 times more sensitive to sulphanilamide than carbonic anhydrase from the P_3 of the

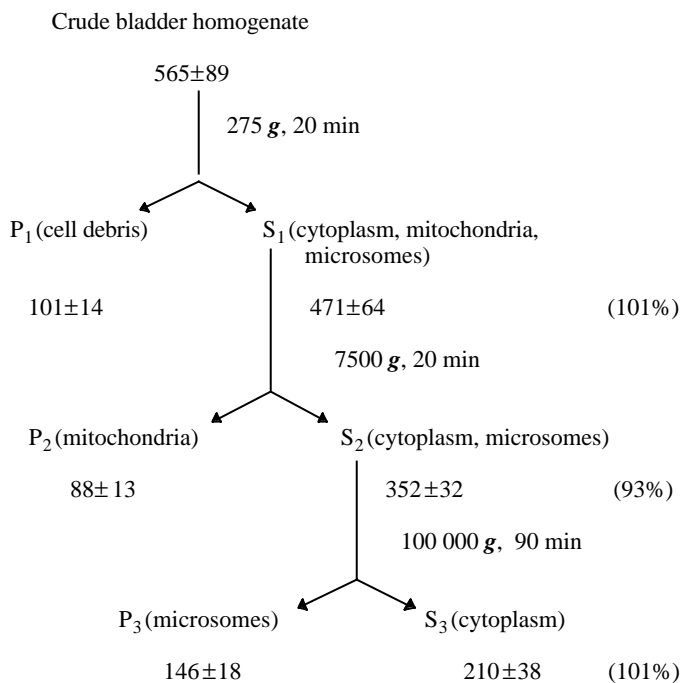


Fig. 2. Cellular fractionation of the bowfin air bladder homogenate via differential centrifugation. Total carbonic anhydrase activity for each fraction is reported as $\mu\text{mol CO}_2 \text{ min}^{-1}$. Values are mean \pm S.E.M. (N=6). Percentage recovery for each step is indicated in parentheses.

Table 2. Sulphanilamide, blood plasma and acetazolamide inhibition of carbonic anhydrase from the gill and air bladder of bowfin

Fraction	K_i Sulph ($\mu\text{mol l}^{-1}$)	I_{50} plasma (μl)	K_i Az (nmol l^{-1})
Gill S_3	0.41 \pm 0.03	150 \pm 32	1.46 \pm 0.30
Gill P_3	0.54 \pm 0.21	246 \pm 48	—
Bladder S_3	0.18 \pm 0.06	225 \pm 16	0.42 \pm 0.28
Bladder P_3	0.46 \pm 0.08	900 \pm 155	—

Values are mean \pm S.E.M. (N=4).

K_i Sulph and K_i Az are the sulphanilamide and acetazolamide inhibition constants, respectively.

Plasma I_{50} is the approximate volume of plasma required to cause 50% inhibition.

S_3 and P_3 are the cytoplasmic and microsomal fractions, respectively.

bladder and from both gill fractions (Table 2). Carbonic anhydrase from the S_3 fraction of the bladder was also more sensitive to acetazolamide than that of the S_3 fraction of the gills (Table 2). The sensitivity of carbonic anhydrase to blood plasma was similar for both gill fractions, but varied between

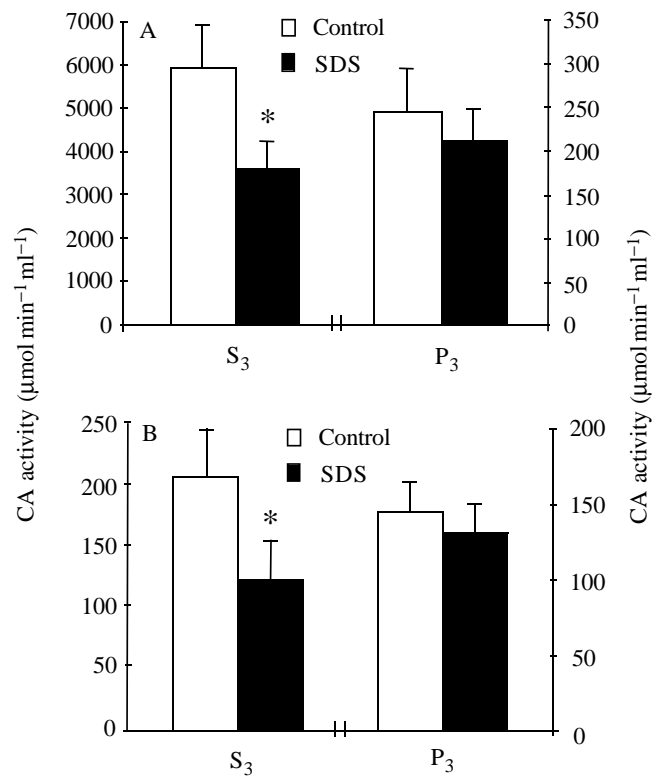


Fig. 3. Effect of 0.005% SDS on carbonic anhydrase (CA) from the cytoplasmic (S_3) and microsomal (P_3) fractions of the gills (A) and air bladder (B) of bowfin (means \pm S.E.M., N=6). Note that carbonic anhydrase activities were multiplied by the fraction volumes (ml) to account for the dilution of each fraction. A significant difference from the respective control value is indicated by an asterisk (paired t -test; $P < 0.05$).

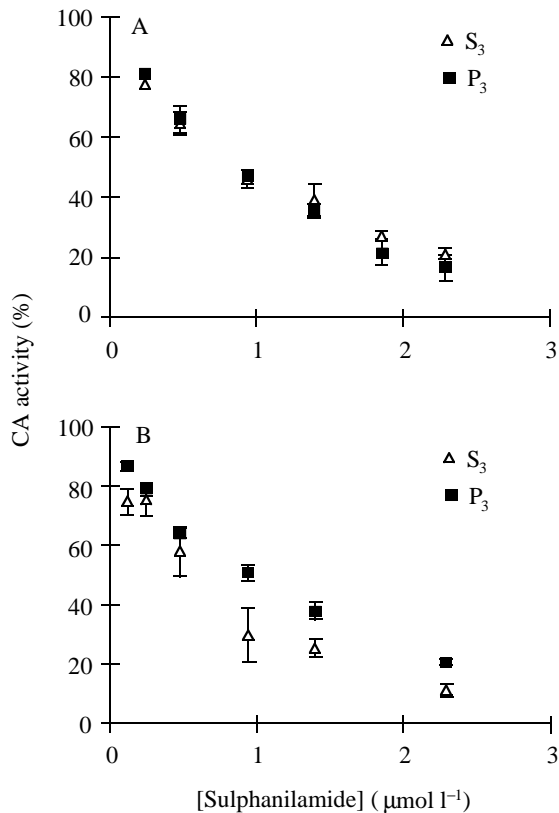


Fig. 4. Sulphanilamide inhibition of carbonic anhydrase (CA) from the cytoplasmic (S₃) and microsomal (P₃) fractions of the gills (A) and air bladder (B) of bowfin (means \pm S.E.M., $N=4$).

the bladder P₃ and S₃ fractions (Fig. 5). As indicated by their I_{50} values (Table 2), carbonic anhydrase from the bladder P₃ fraction was four times less sensitive to plasma than carbonic anhydrase from the bladder S₃ fraction.

Washing and treatment with PI-PLC

Washing of the microsomal pellets from the gill and air bladder also produced different results (Fig. 6). The first wash transferred approximately half of the gill pellet activity to the supernatant. In contrast, washing the bladder microsomal pellet did not significantly reduce the activity of the pellet until the final wash. After the final wash, 79% of the initial air bladder carbonic anhydrase activity remained in the pellet, whereas only 32% remained in the gill pellet. At least 83% of the activity removed from the pellets by the first two washes was recovered in the supernatant for both the gill and the bladder. After the third wash, this recovery was reduced to 50%, indicating that the washing procedure itself probably caused some reduction in total carbonic anhydrase activity. Following the washing, air bladder pellets were incubated with 1 unit of phosphatidylinositol-specific phospholipase C (PI-PLC). Treatment with PI-PLC transferred 84% of the pellet carbonic anhydrase from the air bladder to the supernatant (Fig. 7). In control non-treated samples, only 13% of the carbonic anhydrase activity was transferred to the supernatant.

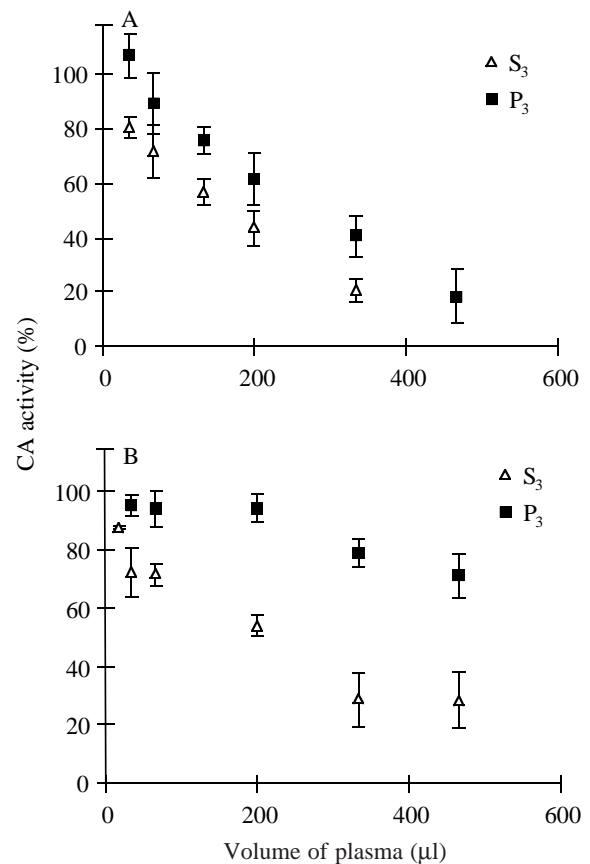


Fig. 5. Effect of blood plasma on carbonic anhydrase (CA) from the cytoplasmic (S₃) and microsomal (P₃) fractions of the gills (A) and air bladder (B) of bowfin (means \pm S.E.M., $N=4$).

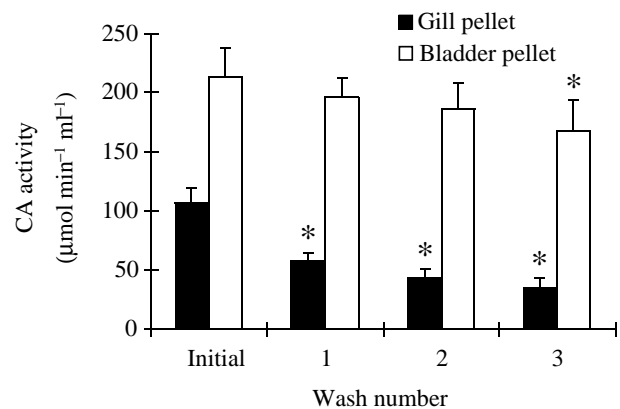


Fig. 6. Effect of washing by mild sonication on carbonic anhydrase (CA) activity of the bowfin gill and air bladder microsomal pellets (means \pm S.E.M., $N=4$). Note that carbonic anhydrase activities were multiplied by the fraction volumes (ml) to account for the dilution of each fraction. A significant difference from the initial value is indicated by an asterisk (Scheffe F -test; $P < 0.05$).

Discussion

Substantial carbonic anhydrase activity was measured in the gills, erythrocytes and air bladder of the bowfin. Measured per

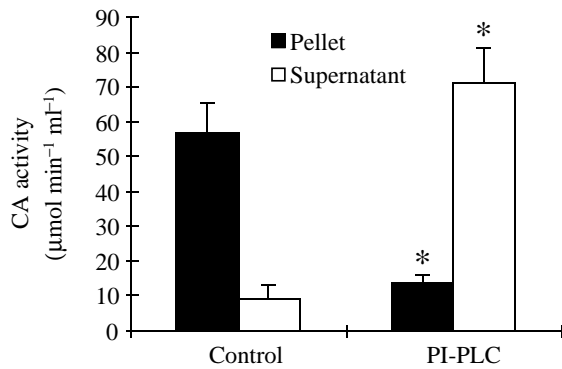


Fig. 7. Effect of phosphatidylinositol-specific phospholipase C (PI-PLC) on the carbonic anhydrase (CA) activity of the microsomal pellet of the bowfin air bladder (means + S.E.M., $N=4$). Note that carbonic anhydrase activities were multiplied by the fraction volumes (ml) to account for the dilution of each fraction. A significant difference from the respective control value is indicated by an asterisk (paired t -test; $P<0.05$).

milligram of protein, the carbonic anhydrase activity ratio of bowfin gill:erythrocyte:air bladder found in this study (14:6:1, Table 1) is within the range reported by Heming and Watson (1986) for bowfin (9:3:1). The ratio of bowfin air bladder:erythrocyte carbonic anhydrase activity (1:6) is also similar to the carbonic anhydrase activity ratio (ABO:erythrocytes) of air-breathing teleosts, blue gourami (1:4) and walking catfish (1:5; Burggren and Haswell, 1979), but is approximately 13 times greater than the rat lung:erythrocyte ratio (1:80; Maren, 1967).

Subcellular fractionation of the bowfin air bladder revealed that the microsomal fraction, which consists of both the external and internal membranes of cells, contained 27% of the total carbonic anhydrase activity (Fig. 2). This value is much higher than the percentage of carbonic anhydrase activity associated with the microsomal fraction in the gas-exchange organs of other vertebrates, rat lung (7%; Henry *et al.* 1986) and the gills of dogfish shark (1%; Henry *et al.* 1997). Nonetheless, the cytoplasmic fraction of the bowfin air bladder still accounted for the largest portion of the total carbonic anhydrase activity (39%; Fig. 2). This finding is in agreement with the results of Henry *et al.* (1986), who found that cytoplasmic carbonic anhydrase accounted for most of the carbonic anhydrase activity in the rat lung (67%). In the present study, it is possible that some of the carbonic anhydrase in the cytoplasmic fraction of the air bladder homogenate originated from minor erythrocyte contamination of the samples. If this is indeed the case, it is possible that an even greater percentage of the total air bladder carbonic anhydrase is membrane-bound.

In contrast to the air bladder, the vast majority of carbonic anhydrase activity in the gills of bowfin appears to originate from the cytosol. Microsomal carbonic anhydrase accounted for only 3% of the total activity of the gill (Fig. 1). Moreover, while the majority (79%) of the air bladder microsomal carbonic anhydrase remained in the membrane fraction after washing, less than half (32%) of the gill microsomal carbonic anhydrase

remained after washing (Fig. 6). This indicates that most of the gill microsomal carbonic anhydrase was probably only loosely associated with the membranes, rather than membrane-bound. Thus, on the basis of the relative activities of the various fractions, it appears that little, if any, membrane-bound carbonic anhydrase exists in the gills of bowfin. A similar conclusion has been reached for the gills of lamprey, trout and catfish (Henry *et al.* 1988, 1993, 1997; Rahim *et al.* 1988).

In addition to subcellular localization, carbonic anhydrase isoenzymes can be differentiated on the basis of their sensitivity to specific carbonic anhydrase inhibitors. The inhibition properties of carbonic anhydrase isoenzymes from the mammalian lung have been extensively studied (Zhu and Sly, 1990; Heming *et al.* 1993; Maren *et al.* 1993; Fleming *et al.* 1994), but little is known about the types of carbonic anhydrase isoenzymes that are present in the respiratory organs of lower vertebrates. Another objective of this study was, therefore, to examine the carbonic anhydrase isoenzyme characteristics in the gills and air bladder of bowfin. This kinetic characterization was also useful to evaluate whether carbonic anhydrase associated with the microsomal tissue fraction indeed represented a different carbonic anhydrase isoenzyme from that present in the cytoplasmic fraction from either the gill or air bladder. On the basis of past studies, there are several characteristics of mammalian pulmonary CA IV that distinguish it from the cytosolic isoenzyme CA II. These include the fact that mammalian CA IV is (1) resistant to denaturation by SDS, (2) less susceptible to sulphonamide inhibition, (3) less susceptible to the endogenous plasma carbonic anhydrase inhibitor, and (4) partially anchored by a phosphatidylinositol-glycan linkage (Whitney and Briggie, 1982; Zhu and Sly, 1990; Maren *et al.* 1993; Heming *et al.* 1993). Each of these four characteristics that distinguish the membrane-bound CA IV isoenzyme from mammalian lungs was investigated for bowfin carbonic anhydrase.

The response to 0.005% SDS was similar for both the bowfin gill and air bladder subcellular fractions. Cytoplasmic carbonic anhydrase activity was significantly inhibited in both tissues by treatment with SDS (Fig. 3). This result provided further confirmation that carbonic anhydrase from the supernatant fraction of both tissues was indeed cytoplasmic. The fact that microsomal carbonic anhydrase from these tissues was not significantly inhibited by SDS could be viewed as evidence that both the gill and air bladder microsomal carbonic anhydrase, like CA IV, are more stabilized by intramolecular disulphide bonds than is the cytoplasmic carbonic anhydrase (Whitney and Briggie, 1982; Waheed *et al.* 1996). It is important to note, however, that sensitivity to SDS is not always effective in differentiating membrane-bound from cytoplasmic carbonic anhydrase in non-mammalian animals (Bottcher *et al.* 1994).

Carbonic anhydrase from the microsomal fraction of the bowfin air bladder was three times less sensitive to the sulphonamide inhibitor sulphonilamide than was cytoplasmic carbonic anhydrase (Fig. 4B; Table 2). This result provides further evidence that carbonic anhydrase from the microsomal fraction of the air bladder is a distinct isoenzyme from

cytoplasmic carbonic anhydrase. Both mammalian CA IV and membrane-bound carbonic anhydrase of lower vertebrates have also been shown to be relatively less sensitive than cytoplasmic carbonic anhydrase to sulphonilamide (Zhu and Sly, 1990; Maren *et al.* 1993; Maffia *et al.* 1996). In terms of absolute values of K_i , however, the K_i for the microsomal carbonic anhydrase of the bowfin air bladder is considerably lower than that of mammalian CA IV (Maren *et al.* 1993). In contrast to the air bladder, the gill microsomal fraction from the bowfin had a sulphonilamide K_i similar to that of the cytoplasmic carbonic anhydrase (Table 2). This result is therefore consistent with the fractionation experiment, which indicated that the vast majority of bowfin gill carbonic anhydrase probably originates from the cytoplasm.

To our knowledge, no previous studies have attempted to characterize the isoenzyme properties of carbonic anhydrase in the gills of fish. On the basis of the inhibition properties, bowfin gill carbonic anhydrase most closely resembles the CA II isoenzyme of mammals (Sanyal, 1984; Maren *et al.* 1993). Carbonic anhydrase from the bowfin gill cytoplasmic fraction was 2–3 times less sensitive to sulphanilamide and acetazolamide than cytoplasmic carbonic anhydrase from the air bladder (Table 2). These results suggest that carbonic anhydrase from the cytoplasmic fraction of the gills and air bladder may also be different isoenzymes, but further investigation is required to confirm this.

An endogenous carbonic anhydrase inhibitor has been found in the blood plasma of mammals (Roush and Fierke, 1992; Wuebbens *et al.* 1997) and fish (Haswell *et al.* 1983; Dimberg, 1994; Henry *et al.* 1997). Heming *et al.* (1993) noted that mammalian CA IV is approximately three times less sensitive to the porcine plasma inhibitor than is mammalian cytoplasmic CA II. In contrast to Heming and Watson (1986), we have recently found that bowfin also have a plasma inhibitor (M. R. Gervais and B. L. Tufts, unpublished data), but that it is present at a much lower concentration than in most other fish examined (Haswell *et al.* 1983; Dimberg, 1994; Henry *et al.* 1997). Nonetheless, the sensitivity of bowfin carbonic anhydrase to the plasma inhibitor could therefore be used to differentiate between carbonic anhydrase isoenzymes (Roush and Fierke, 1992; Heming *et al.* 1993). In bowfin, carbonic anhydrase from the cytoplasmic and microsomal fractions of the gill, and from the cytoplasmic fraction of the air bladder, was 4–5 times more sensitive to plasma than was the microsomal carbonic anhydrase from the air bladder (Table 2). These results again provide strong evidence that carbonic anhydrase from the microsomal fraction of the bowfin air bladder is a different isoenzyme from that in the cytoplasm of the air bladder. In contrast, our combined results suggest that the gills appear to have only one carbonic anhydrase isoenzyme.

Since the results from our initial experiments provided strong evidence that the bowfin air bladder contains a membrane-bound carbonic anhydrase, we investigated whether the enzyme was anchored to the membrane by a glycosylphosphatidylinositol (GPI) anchor (Zhu and Sly, 1990). The results from these experiments indicated that the majority of the bowfin air bladder

microsomal carbonic anhydrase appears to be GPI-anchored since treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) released most of this membrane-associated carbonic anhydrase to the supernatant (Fig. 7). A similar result was obtained for membrane-bound CA IV from the human lung (Zhu and Sly, 1990).

In conclusion, the results of this study indicate the presence of a unique membrane-bound carbonic anhydrase isoenzyme in the air bladder of bowfin which resembles mammalian CA IV in terms of its inhibition characteristics and membrane attachment. In contrast, the gills of bowfin appear to contain a single cytoplasmic isoenzyme which is similar to mammalian CA II in terms of its inhibition characteristics. Since bowfin are members of the primitive fish order Holostei and are therefore among the most primitive air-breathing vertebrates, these findings suggest that a CA-IV-like isoenzyme was probably present in some of the earliest ABOs. The function of this membrane-bound carbonic anhydrase isoenzyme in the bowfin air bladder may be similar to that of the mammalian lung CA IV or, as suggested by Heming and Watson (1986), it may be important for CO₂ excretion across the air bladder during air-breathing. Conversely, since the bowfin ABO is a modified swimbladder, it is also possible that the primary function of this membrane-bound carbonic anhydrase isoenzyme is simply buoyancy regulation, as suggested for the membrane-bound carbonic anhydrase in the swimbladder of a more recent water-breathing teleost, the eel (Pelster, 1995). Further study will be necessary to determine the primary function and evolutionary significance of membrane-bound carbonic anhydrase in the bowfin ABO.

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