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Carbonic anhydrase activity in tissues of the icefish *Chionodraco hamatus* and of the red-blooded teleosts *Trematomus bernacchii* and *Anguilla anguilla*

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Summary

Carbonic anhydrase (CA) activity was measured in blood, intestine, kidney and gill of two Antarctic teleosts, the haemoglobinless *Chionodraco hamatus* and the red-blooded *Trematomus bernacchii*, and of the temperate teleost *Anguilla anguilla*. In all species, the highest CA activity was in the gills, with the greatest activity in *C. hamatus*. CA activity in the blood was highest in *A. anguilla*, but none was detected in the blood of *C. hamatus* despite the presence of plasma CA inhibitors. The enzyme was present but its activity was low in the intestine and kidney of all three species.

The existence of very high CA activity in *C. hamatus* gills compared with the red-blooded species was investigated further by isolating and characterising the branchial cytosolic CA isoforms. The turnover rate of the *C. hamatus* isoform was significantly higher than that of *T. bernacchii* and *A. anguilla*. The isoforms from both the Antarctic species exhibited lower apparent K_m ($K_{m,app}$) and heat stability than those from *A. anguilla*. Sensitivity to sulphonamides was similar in all species and was within the range of the mammalian CA II isoform. The branchial CA isoforms of *C. hamatus*, *T. bernacchii* and *A. anguilla* displayed relative molecular masses of 28.9, 29.9 and 31.2 kDa, respectively.

The results suggest that the hemoglobinless teleost possesses a different branchial cytosolic CA isoform from that of red-blooded teleosts.

Key words: Nothotheniodei, Chaenichthyidae, Antarctic teleost, haemoglobinless, pH homeostasis, carbonic anhydrase, blood, gills, *Chionodraco hamatus*, *Trematomus bernacchii*, *Anguilla anguilla*.

Introduction

Carbonic anhydrase (CA) is a zinc metalloenzyme that catalyses the reversible hydration of CO_2. Many CA isozymes have been characterized, and each has a different subcellular distribution: cytoplasmatic (CA I, II, III and VII), plasma membrane GPI-anchored (CA IV), mitochondrial (CA V) or secretory (CA VI). These isoforms also differ in kinetic characteristics, susceptibility to inhibitors and tissue distribution (Sly and Peiyi, 1995). Carbonic anhydrase plays a crucial role in the excretion of metabolic CO_2 in all vertebrates, including fish. In fish, CO_2 produced in the tissues is rapidly hydrated to HCO_3^- following diffusion into the blood, by the action of erythrocytic CA (Perry and Laurent, 1990; Henry and Swenson, 2000), and HCO_3^- represents almost 98 % of the total carbon dioxide stored and transported in the plasma. At the respiratory epithelium (gills or skin), erythrocytic CA catalyses the rapid dehydration of HCO_3^- to molecular CO_2, which then diffuses passively into the ventilatory water stream. Moreover, the CO_2/HCO_3^- system constitutes one of the most important physiological buffers for acid–base regulation (Heisler, 1984).

The Antarctic icefish of the family Channichthyidae (suborder Nothotheniodei) are a unique example of adult vertebrates lacking haemoglobin and functionally active erythrocytes, and possessing only a small number of erythrocyte-like cells (haematocrit=1–2 %) (MacDonald and Wells, 1991). Other significant characteristics of their circulatory system include a blood volume and a cardiac output up to sixfold higher than found in other teleosts (Hemmingsen and Douglas, 1970; Acierno et al., 1995). The absence of haemoglobin would represent, *per se*, a dramatic limitation to oxygen transport in the icefish. On the other hand, the very limited number of erythrocyte-like cells (and circulating CA) may compromise CO_2/HCO_3^- equilibria in the blood (Feller and Gerday, 1997). Owing to the elevated solubility of CO_2 in water at low temperatures, and the peculiar characteristics of its circulatory system, CO_2 excretion should not be a problem for the icefish. The absence of erythrocytic CA may, however, have an influence on icefish blood acid–base equilibria and pH regulation.

In teleosts, CA has been found in various tissues. It appears to be present in high concentrations in the gills (Rahim et al., 1988; Conley and Mallatt, 1987), where it plays an important role in osmoregulation, nitrogen (ammonia) excretion, acid–base balance and gas exchange (Henry and Heming, 1998). Specifically, cytoplasmic CA is believed to function in

support of ion transport, and membrane-associated CA is believed to function in facilitated CO_2 diffusion. A previous comparative study between two Antarctic species, an icefish *Channichthys rhinoceratus* and a red-blooded teleost *Notothenia magellanica*, revealed a much higher CA activity in gill homogenates of the haemoglobinless fish (Feller et al., 1981). It was hypothesised that this higher activity was related to particular osmoregulatory requirements. No information about its subcellular localisation or biochemical characteristics is, however, available to date. In contrast, in red-blooded teleosts from temperate zones the existence of branchial cytosolic CA isoforms with biochemical characteristics similar to those of mammalian CA II have been demonstrated (Henry et al., 1993; Sender et al., 1999).

A potential role for gut CA in acid–base regulation has been suggested in seawater-adapted rainbow trout *Oncorhynchus mykiss* (Wilson et al., 1996) and in the European eel *Anguilla anguilla* (Maffia et al., 1996b). In particular, two CA isoforms have been described in the eel intestine: one is located on the brush border membrane, and mediates bicarbonate absorption from the intestinal lumen, and one is located in the cytosol, and generates HCO_3^- from metabolic CO_2 (Maffia et al., 1996b).

In the current study, we compared CA activity and distribution in blood, gills, intestine and kidney of the haemoglobinless teleost *C. hamatus* with those of *T. bernacchii*, a red-blooded member of the same Antarctic sub-order that shares the same ecotype, and with those of *A. anguilla*, a teleost from temperate zones. The existence of plasma CA inhibitors in the icefish was also investigated. The cytosolic CA isoforms were purified from the gills of the three species, to compare their molecular mass, kinetic properties, heat stability and sensitivity to sulphonamides, and thereby to investigate potential adaptive mechanisms at a molecular level.

Materials and methods

Specimens of the Antarctic haemoglobinless teleost, *Chionodraco hamatus* Lomburg and of the red-blooded *Trematomus bernacchii* Boulenger (body mass 240–360 g) were caught by nets in Terranova Bay during the 'Fourteenth Italian Antarctic Expedition' (January–February, 1999) and kept in open circulating seawater (3.5 % salinity) aquaria at 0 °C. Specimens of the temperate teleost *Anguilla anguilla* L. at the yellow stage (200–250 g) were purchased from the commercial pond Acquatina Lagoon (Lecce, Italy) and kept in recirculating seawater (3.5 % salinity) aquaria at 18 °C. Animals were allowed at least 5 days to recover from the stress of capture and transport. All chemicals were of reagent grade purchased from Merck (Darmstadt, Germany), Sigma (St Louis, MO, USA) and Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Sample preparation

Blood samples were withdrawn from the caudal vein/artery and added to 0.5 volumes of a cold mixture of (in mmol l^{-1}): citric acid 19, sodium acetate 41, D-glucose 81. Blood cells were separated from plasma by centrifugation at 12 000 ***g*** for 5 min. In order to collect erythrocyte-free tissues, circulating blood was removed from gills, intestine and kidney by systemic perfusion with heparinised physiological saline for marine teleosts. Animals were killed by a sharp blow to the head and pithed; polyethylene cannulae were then inserted into ventral and dorsal aortas to start the systemic perfusion by means of a peristaltic pump (20 ml min^{-1} kg^{-1} body mass) until erythrocyte-free saline left the circulatory system. Tissues were then frozen in liquid nitrogen and stored at –80 °C until experiments. Samples of scraped intestinal mucosa, posterior kidney and gill filaments were dried for 12 h at 120 °C for determination of dry mass. Tissue (1 g) was homogenised with a Kinematica Polytron (5 min, maximum rate) in 30 ml of buffer A (in mmol l^{-1}): mannitol 300, Hepes–Tris 0.2, adjusted to pH 8.5 with KOH (at 0 °C).

Purification of soluble branchial CA

Gill filaments were separated from gill arches, weighed and homogenised in 10 ml of ice-cold phosphate-buffered saline (PBS; 0.92 % NaCl, 0.16 % $Na_2HPO_4{\cdot}2H_2O$, 0.02 % $NaH_2PO_4{\cdot}H_2O$) g^{-1} wet mass, using a Kinematica Polytron (5 min, maximum speed). The homogenate was centrifuged at 100 000 ***g*** for 1 h at 4 °C, yeilding a pellet containing cells and membrane fragments and a supernatant containing the cytosolic fraction. Cytosolic branchial CA was purified by affinity chromatography on *p*-aminomethylbenzene-sulphonamide immobilized on cyanogen-bromide-activated agarose gel (Sigma) (Whitney, 1974). The gel column (17 mm×225 mm) was equilibrated with (in mmol l^{-1}): Tris 25, Na_2SO_4 100, adjusted to pH 8.7 with HCl, and rinsed with Tris 25, $NaClO_4$ 300, adjusted to pH 8.7 with HCl; finally the enzyme was eluted in sodium acetate 100, $NaClO_4$ 500, pH 5.6. A Pharmacia LKB UV-cord SD was used to follow protein elution at 280 nm. The flow was maintained at 8 ml h^{-1} with a peristaltic pump (Pharmacia LKB Pump-1). The purification was carried out at 2 °C. Fractions containing CA activity were collected and concentrated by an ultra filtration cell (Amicon Corp, Lexington, USA) with membrane YM10, under nitrogen pressure (7×10^5 Pa).

Gel electrophoresis

To test the purity of the enzyme preparation, one-dimensional polyacrylamide (15 %) gel electrophoresis (Bio-Rad Mini-Protean II cell) was performed under denaturating conditions (Laemmli, 1970) in parallel with molecular mass standards (Bio-Rad SDS-Page standards – Broad Range) and stained with Coomassie Brilliant Blue R250. The molecular mass of different CA isoforms was calculated from a calibration curve obtained by plotting the relative mobility of standard proteins on the gel against the log of their respective molecular mass for five different SDS-gel electrophoreses.

Determination of CA activity

Electrometric method

CA activity was measured at 0 °C, by the electrometric ΔpH method previously described (Wilbur and Anderson, 1948) as

modified by Maffia et al. (1996b). Briefly, tissue homogenates (50–200 μg of protein) and purified CA fractions (0.5–2 μg of protein) were diluted in 8 ml of buffer A and added to 12 ml of buffer B (in mmol l^{-1}): Tris 9.7, Hepes 3.5, pH 8.65. The reaction was started by the addition of 10 ml of CO_2-saturated H_2O and gassing the assay medium with 5 % CO_2, 95 % O_2 (CO_2 concentration of 3.8 mmol l^{-1}). The enzymatic activity, expressed as μmol H^+ developed by the hydration of CO_2 in excess of a blank sample, was measured by multiplying the change in pH from 8.30 to 8.00 by 2.80 mmol kg^{-1} ΔpH^{-1} (buffer capacity of the incubation medium in that pH range) (Maffia et al., 1996b) and dividing by the time expressed in min. There were no significant differences in buffer capacity among the different tissues. Protein concentration was measured with Bio-Rad DC protein assay kit, using lyophilised bovine albumin as standard.

Radioactive method

The measurement of CA activity by the electrometric method is technically possible only at a temperature close to 0 °C, because of the masking effects of spontaneous CO_2 hydration at higher temperatures. Therefore, in order to compare the enzyme activity of the icefish and the eel at their appropriate environmental temperatures, a radioactive assay (Stemler, 1993) was employed to measure the CA activity of the purified branchial isoforms at 0 and 18 °C. Durapore membrane filters (GVHP 0.22 μm, Millipore Corporation, Bedford, MA, USA) were used as gas-permeable membranes, while Glass Fiber prefilters (AP25, Millipore Corporation, Bedford, MA, USA) wetted with 0.1 mol l^{-1} NaOH were used as a $^{14}CO_2$ trap. Purified branchial CA (200 ng) from *C. hamatus* and *A. anguilla* were used for each determination. The enzymatic reaction was started by adding $NaH^{14}CO_3$ to the assay mixture containing the enzyme. CA activity was measured at 0 and 18 °C for both species by quantifying the amount of $^{14}CO_2$ trapped within the fiberglass filter at 5, 10 and 20 s from the beginning of the enzymatic reaction. Controls were performed by measuring the $^{14}CO_2$ trapped within the fiberglass filter in the absence of enzyme.

Inhibition, kinetic analysis and heat stability of carbonic anhydrase

The presence of CA inhibitors in icefish plasma was investigated by adding plasma samples (15–400 μl) to the assay buffer. Sensitivity of branchial CA to acetazolamide (ACTZ) and sulphanilamide was examined by measuring CA activity in the presence of increasing concentrations of these inhibitors. Since both ACTZ and sulphanilamide are non-competitive reversible inhibitors, and ACTZ in particular inhibits CA very strongly at nanomolar concentrations, the inhibition constants were determined by plotting the data on an Easson–Stedman plot. The inhibition constant for ACTZ was calculated as the slope of the straight line described by the following equation:

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

where I_0 is the concentration of inhibitor, i is the fractional inhibition of enzyme activity at a given inhibitor concentration, and E_0 is the total concentration of free enzyme in the reaction chamber (Easson and Stedman, 1936). For the determination of kinetic parameters K_{cat} and apparent K_m ($K_{m,app}$), CA activity was measured by the electrometric method at increasing CO_2 concentrations (1, 3, 5, 10, 20, 30, 40, 55, 75 and 100 % CO_2, residue O_2). Experimental data were fitted to a Michaelis–Menten equation with a curve-fitting subroutine in the Graph-Pad software package. Heat stability was evaluated by pre-incubating the purified enzymes for 15 min at increasing temperatures (0–60 °C) and then measuring CA activity by the electrometric method.

Statistical analysis

All data in figures and tables are reported as means ± S.E.M. of at least four different tissue samples. Means were compared by Student's *t*-test while, for multiple comparisons, analysis of variance (ANOVA) was performed. $P<0.05$ was taken as the fiducial level for statistical significance.

Results

CA activity in gills, intestine, kidney and blood

Given that one aim of this study was the comparison of total CA activity in different tissues, and that such tissues might be heterogeneous in terms of size and protein content, we calculated the protein mass (in mg g^{-1} of dry tissue), and the percentage tissue wet mass relative to body mass for gill filaments, intestinal mucosa and trunk kidney. Table 1 reveals that no significant difference was detectable for the relative gill masses of *C. hamatus*, *T. bernacchii* and *A. anguilla*, while the relative kidney mass was significantly higher in the icefish than in the red-blooded species. The relative amount of intestinal mucosa was significantly higher in both Antarctic species than in the temperate teleost. However, the mass of protein per mass of dry tissue was not significantly different among the three species.

Fig. 1 shows CA activities, reported as μmoles H^+ min^{-1} mg^{-1} protein, in gill filaments, intestinal mucosa, posterior kidney and blood of the three species. In all fishes the highest CA activity was detected in gill homogenates. In particular, gill CA activity was two- and eightfold higher in *C. hamatus* than in *T. bernacchii* and *A. anguilla*, respectively. In contrast, *C. hamatus* intestinal mucosa exhibited lower enzymatic activity than *T. bernacchii* and *A. anguilla* (Fig. 1B). Carbonic anhydrase activity in the trunk kidney of the temperate teleost was significantly lower than that measured in the two Antarctic species (Fig. 1C).

The radioactive method of measuring CA activity was used to confirm the existence of a temperature-dependent adaptation in the Antarctic fish (Fig. 2); moreover it appears that the CA activity of the Antarctic isoform is always markedly higher than that of the eel and that, more interestingly, the icefish gill CA activity at 0 °C is higher than that measured in the eel at 18 °C.

Table 1. *Ratios of protein mass to different tissue and body mass in Antarctic and temperate teleosts*

	Species					
	Chionodraco hamatus		*Trematomus bernacchii*		*Anguilla anguilla*	
Tissue	Protein (mg g^{-1} tissue dry mass) (*N*)	Tissue wet mass:body mass (*N*)	Protein (mg g^{-1} tissue dry mass) (*N*)	Tissue wet mass:body mass (*N*)	Protein (mg g^{-1} tissue dry mass) (*N*)	Tissue wet mass:body mass (*N*)
Gills	750.6±108.0 (5)	4.9±0.7 (4)	720.2±186.9 (5)	5.1±1.1 (3)	857.1±220.3 (15)	6.5±0.7 (15)
Kidney	838.1±237.8 (4)	7.8±1.4 (4)[a,b,**]	1144.3±294.4 (5)	2.5±0.3 (5)	818.0±116.5 (13)	3.5±0.3 (15)
Intestine	547.5±108.0 (3)	9.2±1.5 (3)[b,**]	997.3±170.2 (3)	10.0±2.5 (3)[c,**]	870.5±146.8 (13)	3.5±0.6 (15)
	Protein (mg ml^{-1} blood)	(ml blood:body mass) (%)	Protein (mg ml^{-1} blood)	(ml blood:body mass) (%)	Protein (mg ml^{-1} blood)	(ml blood:body mass) (%)
Blood	40.8±3.6[f]	7–8[d]	124.6±16.6	2.5[d]	118.9±1.0[f]	3.0–4.7[e]

Values are means ± S.E.M. of at least three different experiments. *N*, number of animals.

Values for the intestine refer to the mucosal component that was separated from the connectival and muscolaris layers; the posterior part of the kidney was used for the analysis.

An ANOVA followed by a *post-hoc* test was used. $^{**}P<0.01$; $^{*}P<0.05$. [a]Difference between *C. hamatus* and *T. bernacchii*; [b]difference between *C. hamatus* and *A. anguilla*; [c]difference between *T. bernacchii* and *A. anguilla.*

References: [d]Hemmingsen and Douglas, 1970; [e]Tesch, 1977; [f]Acierno et al., 1997.

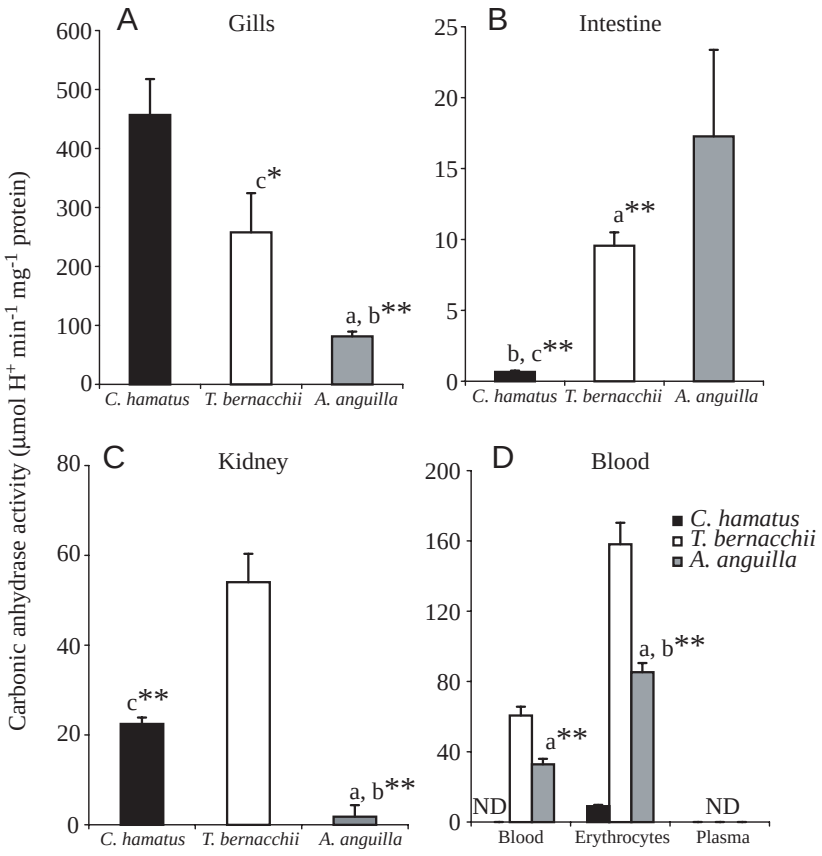

Fig. 1. Carbonic anhydrase specific activity in gills (A), intestine (B), kidney (C) and blood (D) of *C. hamatus*, *T. bernacchii* and *A. anguilla*. Values are expressed as means ± S.E.M. of 8 experiments. $^{**}P<0.01$, $^{*}P<0.05$ by ANOVA. [a]Difference between *C. hamatus* and *T. bernacchii*; [b]differences between *C. hamatus* and *A. anguilla*; [c]differences between *T. bernacchii* and *A. anguilla.*

CA activity in the blood of *T. bernacchii* was twice the level found in the blood of *A. anguilla*, and no CA activity was detected in plasma fractions of the three species (Fig. 1D). In *C. hamatus* blood, CA activity was not detectable but, if the erythrocyte-like cells were isolated from the plasma fraction by centrifugation (12000 ***g***, 3 min) and resuspended in physiological saline, a low level of activity was measurable (Fig. 1D). CA enzymatic activity in the erythrocyte-like cells of *C. hamatus* is inhibited by the presence of plasma in a dose-dependent manner (Fig. 3). Interestingly, the plasma is also able to inhibit the branchial CA of the icefish, but was ineffective against bovine erythrocyte CA.

CA distribution in intestine, kidney, gills and blood of the three species was estimated by calculating the total enzymatic activity present in each tissue (CA specific activity × total tissue protein and organ mass per body mass, see Table 1) and in the blood (enzymatic activity ml^{-1} blood × blood volume using values from the published literature, see Table 1) (Fig. 4). In the temperate red-blooded teleost, the highest amount of total CA activity appeared in blood, although gill filaments also contained significant activity. The posterior kidney and intestinal mucosa had only small proportions of the total activity. By contrast, in the Antarctic teleosts the highest proportion of total CA activity was in gill filaments. A significant proportion of enzymatic activity was also present

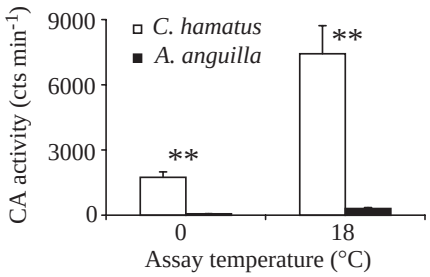

Fig. 2. Carbonic anhydrase activity purified from gills of *C. hamatus* and *A. anguilla*, measured at the environmental temperatures of the two species. Values for CO_2 trapped (cts min^{-1}) are means ± S.E.M. of 4 experiments. All reported values were significantly different (**P<0.01, ANOVA).

in the kidney and in the intestinal mucosa of *C. hamatus* and *T. bernacchii*. When considering all three species, the amount of total blood CA enzymatic activity was directly related to the hematocrit. If the total amount of CA activity of all the tissues and organs studied is arbitrarily considered as 100 %, it appears that in the eel more than 80 % of enzyme activity is localised in blood, whereas organ-specific total CA activity in the Antarctic fish gills is 67 % in *T. bernacchii* and 90 % in *C. hamatus*.

Subcellular distribution and molecular mass of branchial CA isoforms

90 % of the total CA activity in the gills of the three species was derived from the soluble (cytosolic) isoform, while a low, but not insignificant amount (10 % of total activity found in the homogenate) appeared to be associated with membrane-bound CA isoforms. Purified CA fractions showed a specific activity that was approximately 100-fold higher with respect to the initial homogenates (Table 2). The effectiveness of purification is the result of a yield in excess of 64 %. Purity of isolated CA was confirmed by SDS-polyacrylamide (15 %) gel electrophoresis under denaturing conditions (Fig. 5). A comparison of the relative molecular masses of branchial CA isoforms from *C. hamatus*, *T. bernacchii* and *A. anguilla* revealed that the molecular mass of the isoform from *C. hamatus* was significantly less than that from *A. anguilla* (Table 3).

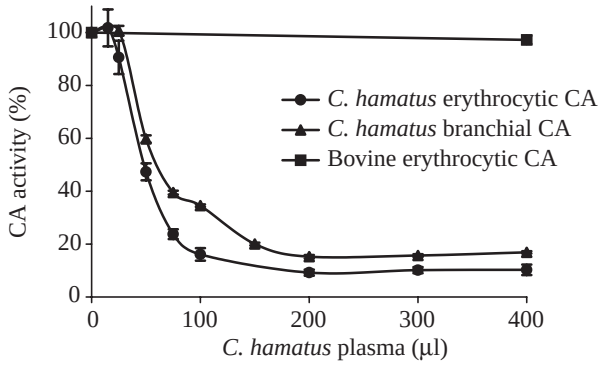

Fig. 3. Inhibitory effect of *C. hamatus* plasma on the activity of different CA isoforms. CA activity was measured as described in Materials and methods. Values are means ± S.E.M. for 3 different determinations.

Kinetic parameters, inhibition and heat stability of branchial CA isoforms

Fig. 6 shows the kinetic analysis of the three isolated branchial isoforms, using the Michaelis–Menten equation. Kinetic parameters, as derived by a non-linear regression analysis (see Materials and methods), are reported in Table 3. Values of $K_{m,app}$ for CA purified from the Antarctic species were half the value measured in the temperate fish. Furthermore, the catalytic rates (K_{cat}) of *C. hamatus* and *T. bernacchii* branchial CA isoforms were higher than that found in the eel, with the highest value in the icefish.

The sensitivity of gill CA isoforms to acetazolamide (ACTZ) and sulphanilamide was similar in the three species. The inhibition constants (K_i) for ACTZ ranged from 3×10^{-10}

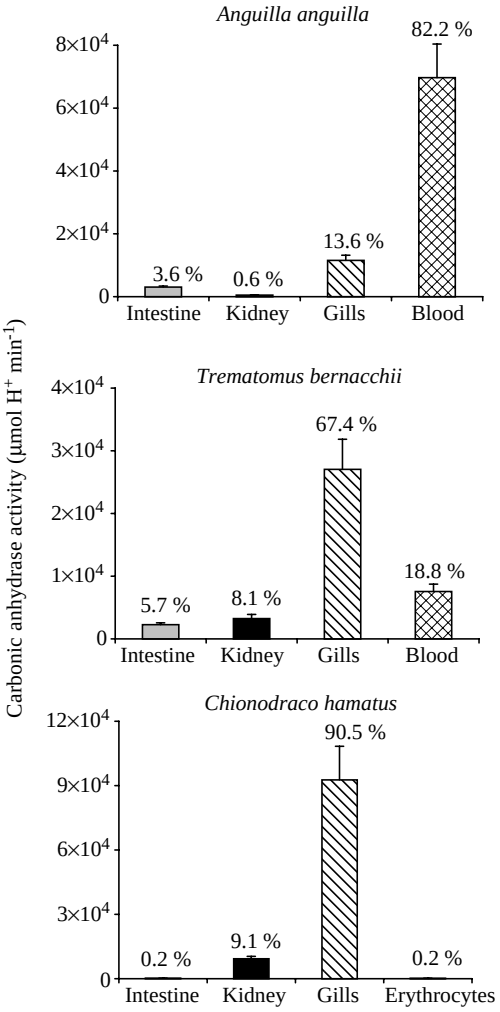

Fig. 4. Distribution of total CA activity in gills, kidney, intestine and blood of *C. hamatus*, *T. bernacchii* and *A. anguilla*. CA activity was expressed both as total activity and as the percentage of the sum total of enzymatic activity measured in all of the tissues (see Results). Values are means ± S.E.M. of 8 experiments.

Table 2. *Specific activity of carbonic anhydrase isoforms isolated from the gills of Antarctic and temperate teleosts*

	Branchial CA activity (μmol H^+ min^{-1} mg^{-1} protein)	*N*	Enrichment factor	Yield (%)
Chionodraco hamatus	52 412±8 641	18	129	64.9
Trematomus bernacchii	25 776±3 615	7	124	92.82
Anguilla anguilla	7 313±1 121	13	119	86.46

CA, carbonic anhydrase.
Values are means ± S.E.M. of at least 7 different preparations.
Enrichment factor is the ratio of the specific activity in isolated CA fractions compared with specific activity in homogenates.
Yield indicates the percentage amount of enzymatic activity measured in isolated CA fractions with respect to the homogenates.

Table 3. *Properties of gill carbonic anhydrase isoforms in Antarctic and temperate teleosts*

	Chionodraco hamatus	*Trematomus bernacchii*	*Anguilla anguilla*
Molecular mass (kDa)	28.9±0.7[b,*]	29.9±1.4	31.2±0.6
Apparent K_m (mmol l^{-1})	10.41±2.63[b,*]	11.04±1.79[c,*]	21.72±2.68
K_{cat} (min^{-1})	$40\times10^5\pm3.0\times10^5$ [b,**]	$33\times10^5\pm2.0\times10^5$ [c,**]	$15\times10^5\pm2.0\times10^5$
K_i (mol l^{-1}) ACTZ	$3.0\times10^{-10}\pm0.3\times10^{-10}$	$3.5\times10^{-10}\pm0.5\times10^{-10}$	$8.1\times10^{-10}\pm0.9\times10^{-10}$
Sulphanilamide	$4.0\times10^{-7}\pm0.8\times10^{-7}$	$1.6\times10^{-6}\pm0.2\times10^{-6}$	$1.7\times10^{-6}\pm0.3\times10^{-6}$
Heat stability (approx. °C)	30	30	40

ACTZ, acetazolamide.
Values are expressed as means ± S.E.M. of at least five different preparations.
Molecular masses were determined by SDS-PAGE, using standard proteins as molecular mass markers.
Kinetic parameters and K_i for sulphonamides were obtained as described in Materials and methods.
An ANOVA followed by a *post-hoc* test was used. **$P<0.01$; *$P<0.05$; [a]difference between *C. hamatus* and *T. bernacchii*; [b]difference between *C. hamatus* and *A. anguilla*; [c]difference between *T. bernacchii* and *A. anguilla.*

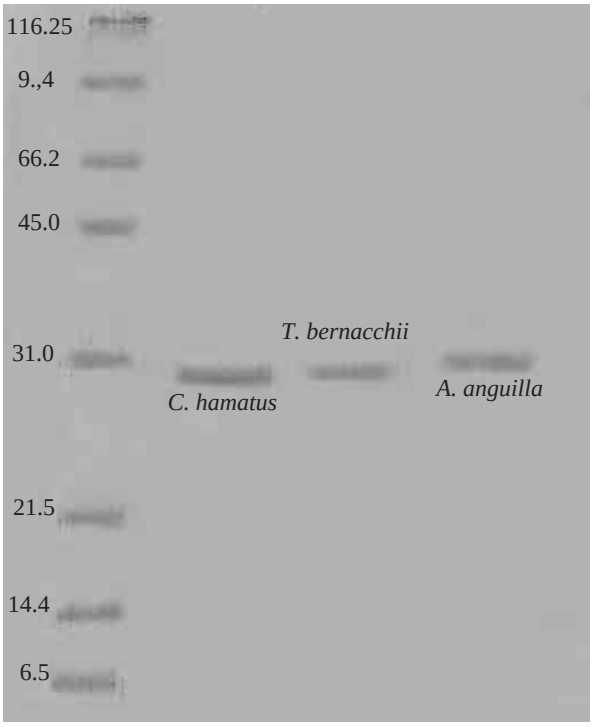

Fig. 5. SDS-PAGE of CA isoforms from *C. hamatus*, *T. bernacchii* and *A. anguilla* gills. The isoforms each migrated as single protein bands of 28.9, 29.9 and 31.2 kDa respectively, as determined from 5 different experiments. The positions of molecular marker proteins are shown.

to 8.1×10^{-10} moles l^{-1} (Table 3), while for sulphonamide values between 4×10^{-7} and 1.7×10^{-6} moles l^{-1} were found. The mean dose–response effects of the two inhibitors on icefish CA activity are shown in Fig. 7, together with the Easson–Stedman plot from which the K_i values were calculated.

The CA isoforms from the cold-adapted species have a significantly lower heat stability than that of the temperate teleost (Fig. 8). The catalytic properties of the CA isoforms from both the Antarctic species were stable up to 30–32 °C, and the activity of the isoform from the temperate species was not adversely affected by temperatures up to 40 °C.

Discussion

pH homeostasis in haemoglobinless teleosts

Haemoglobin, plasma proteins and HCO_3^- are the main acid–base buffers in vertebrate blood. The high content of membrane-bound carbonic anhydrase, coupled with an HCO_3^- transporter, make the erythrocyte a powerful unit for blood pH regulation, because of its capacity for rapid interconversion between gaseous CO_2 and HCO_3^-. Antarctic icefish (Channichthyidae) lack haemoglobin and have a drastically reduced hematocrit, so they are likely to have developed alternative mechanisms for maintaining blood acid–base homeostasis. One of these could certainly be the increase in plasma volume per unit of body mass, a characteristic that not only increases the O_2 content of the blood (Feller and Gerday,

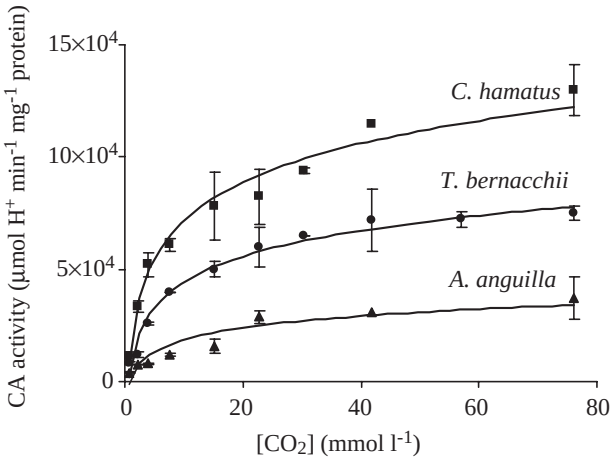

Fig. 6. Dependence of CA activity on increasing CO_2 concentrations, as fitted to a Michaelis–Menten equation. Values are means ± S.E.M. of 3 determinations.

1997) but also allows the circulating plasma to dilute protons produced by the respiring tissues. Furthermore, it has already been reported that, notwithstanding the absence of haemoglobin, the titratable blood buffer capacity (β) of the icefish *C. hamatus* is not significantly lower than that measured in other Antarctic and temperate red-blooded teleosts (Acierno et al., 1997; Wells et al., 1988), a finding that appears to be related to the considerably higher content of inorganic phosphates (Acierno et al., 1997) and proteins rich in imidazole-based histidyl residues (Feller et al., 1994) in icefish plasma.

CA activity in blood

Information about blood CO_2/HCO_3^- equilibria in this taxonomic group is scarce, however. In the erythrocytes of red-blooded teleosts, protons derived from CA-dependent CO_2 hydration are instantly buffered by haemoglobin (inducing the well known 'Bohr effect'). As a consequence, in the red-blooded species the function of erythrocyte CA is strictly related to the presence of haemoglobin. Our study revealed that CA activity is present in the erythrocyte-like cells of *C. hamatus*, albeit at much lower levels than that found in the erythrocytes of both the red-blooded species *T. bernacchii* and *A. anguilla*, but comparable to that detected in the icefish *Channichthys rhinoceratus* (Feller et al., 1981). Interestingly, the icefish *C. hamatus* appears to have preserved a considerable amount of efficient CA inhibitors in its plasma, and these are commonly found in all red-blooded teleosts (Henry et al., 1997). The possible physiological role of these inhibitors in the blood of a teleost with a very low level of circulating CA is unknown at present, although they may simply be an evolutionary relic. In any case, we consider that no physiologically significant CA activity is detectable in icefish plasma.

CA activity in the gills

Despite the fact that the haemoglobinless fish seem to possess little capacity for anaerobic metabolism, plasma lactic acid concentrations can be similar to other teleosts. That is, owing to the extremely high blood volume, there can be a high total lactic acid content (Feller and Gerday, 1997). It might be

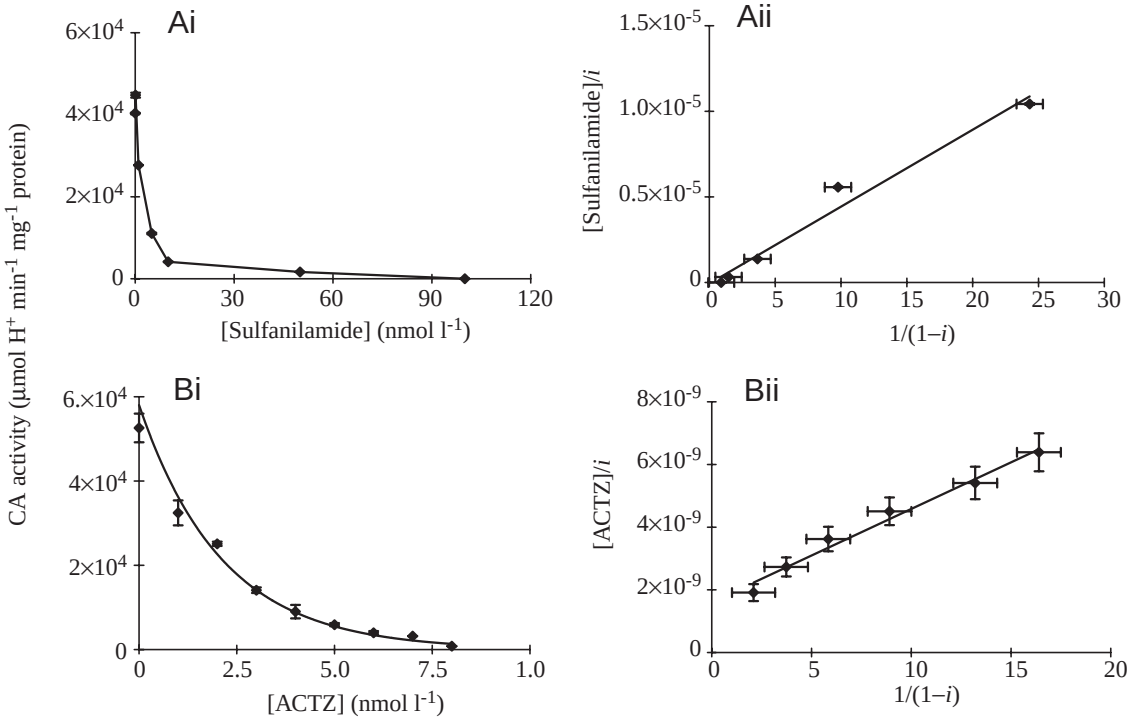

Fig. 7. Effects of sulphanilamide (A) and acetazolamide (ACTZ) (B) on icefish CA activity. (Ai,Bi) Dose–response curves; (Aii,Bii) Easson–Stedman plots. Values are means ± S.E.M. of 3 determinations. For details, see Materials and methods.

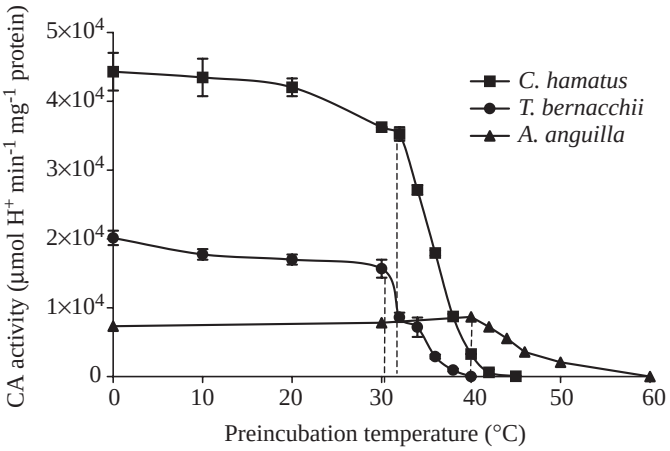

Fig. 8. Effect of preincubation temperature (15 min) on the activity of branchial CA isoforms of *C. hamatus*, *T. bernacchii* and *A. anguilla* measured by the electrometric method at 0 °C. Values are means ± S.E.M. of 3 experiments. For details, see Materials and methods. The dashed lines indicate the temperatures above which the activity of each isoform is markedly reduced.

hypothesised that, in haemoglobinless teleosts, CA localised on absorptive or secretory epithelia such as the intestine, the kidney and, in particular, the gills, could rapidly supply the blood with the HCO_3^- required to buffer metabolic acidoses elicited by anaerobic exercise. Indeed, this study revealed a markedly higher CA activity in gills of *C. hamatus* when compared to the red-blooded species (*T. bernacchii* and *A. anguilla*), which confirms previous comparative studies between another icefish species, *C. rhinoceratus*, and the Antarctic red-blooded species *Notothenia rossii* (Feller et al., 1981). We would hypothesise that, in the haemoglobinless teleosts, this epithelium could substitute at least partially for the physiological role of red blood cells in acid–base regulation, by providing a supply of HCO_3^- to the blood. The higher CA activity in the gills of the icefish cannot, however, be ascribed only to the provision of HCO_3^- to the blood, because CA is a multifunctional enzyme that is thought to play a role in diverse physiological functions of the fish gill epithelium, including in osmoregulation (Evans et al., 1982), clearance of the waste products of nitrogen metabolism (Evans and Cameron, 1986), gas exchange (Randall and Daxboeck, 1984) and acid–base balance (Heisler, 1984). Nonetheless, there are good reasons to believe that there are few differences between haemoglobinless and red-blooded teleosts in osmotic water loss or passive salt influx across the gill epithelium. All species that we have considered are seawater animals and the two Antarctic species share the same ecosystem. Indeed, morphometric and physiological studies (Rankin and Tuurala, 1998) suggest that the only difference in gill structure between the icefish and the red-blooded teleosts is a markedly larger diameter of branchial arteries and marginal channels in the former, which are correlated with the much higher blood flow. Furthermore, since all teleosts under investigation are carnivorous species, nitrogen metabolism should also be somewhat similar, even if the metabolic rates in relation to protein catabolism can be different in temperate compared with Antarctic species.

There is no strong evidence to date for any role of branchial CA in CO_2 elimination in teleosts. In red-blooded teleosts, it is generally accepted that erythrocytic CA is the primary and possibly the only site of HCO_3^- dehydration in the proximity of the respiratory epithelium. A recent review (Henry and Heming, 1998) indicates that gill-membrane-bound CA isoforms could contribute to facilitate CO_2 diffusion while soluble (cytoplasmic) CA isoforms should be involved in ion transport. Unfortunately, although our data indicate the presence of a significant amount of membrane-bound CA, we were unable to determine the exact cellular distribution of this isoform. In any case, at low temperatures CO_2 solubility in water is significantly increased and, owing to its very large blood volume and cardiac output, icefish could efficiently eliminate the CO_2 by storing and transporting it as molecular CO_2 in the blood. As a consequence the lack of circulating CA would have no influence on the rates of CO_2 excretion at the gill or skin.

For these reasons we hypothesise that the high branchial CA activity found in the gills of *C. hamatus*, which is mainly from the cytoplasmic isoform, may be related to systemic acid–base regulation. In teleosts, gill CA activity is localised to both pavement and chloride cells (Rahim, 1988; Sender et al., 1999) where it can rapidly hydrate metabolic CO_2 to form HCO_3^- and H^+. HCO_3^- and a proportion of the protons can be secreted into the blood *via* basolateral transport systems such as the Cl^-/HCO_3^- and the Na^+/H^+ exchangers. At the apical side, where several teleosts appear to exhibit significant CA activity (Sender et al., 1999), transporters such as the H^+-ATPase and/or the Na^+/H^+ exchanger could excrete excess protons into the respiratory water. These mechanisms in the icefish gill epithelium would provide it with a means of manipulating plasma bicarbonate levels and buffering blood acid–base disturbances in the absence of erythrocytic CA.

Branchial CA isoforms

Data presented in this study indicate that the putative novel function for CA in the gills of *C. hamatus* could be performed by an enzymatic isoform with a high turnover rate. Indeed, the catalytic rate (K_{cat}) measured by the electrometric method (at 0 °C) in the branchial *C. hamatus* isoform was the highest of the investigated species (Table 3). The assay temperature of 0 °C is close to the environmental temperature of the Antarctic fish (–1.9 °C), but is very much lower than the optimal environmental temperature of the European eel. Under such conditions the CA activity measured for the eel gill could, therefore, be significantly underestimated. We used a radiometric method to measure activity at the respective environmental temperatures of the Antartic and temperate species, and confirmed that the activity of the branchial CA in the haemoglobinless teleost at 0 °C was higher than that of the eel at 18 °C. In terms of activity, there was a significant difference between the CA isoforms isolated from *C. hamatus* and *T. bernacchii*, although both displayed a similar substrate

affinity, heat stability and molecular mass. At the same time, both the kinetic properties (Fig. 6; Table 3) and the molecular weight (Fig. 5; Table 3) of CA purified from the gills of the Antarctic teleosts were significantly different from those of the enzyme purified from *A. anguilla*. The CAs purified from *C. hamatus* and *T. bernacchii* showed a K_{cat} value at 0 °C that was 2.7- or twofold greater than that found in *A. anguilla*, respectively (Table 3), a result that is consistent with previous studies comparing homologous enzymes from cold- and warm-adapted species (Somero, 1995). This characteristic may offset the effects of low temperature on the CA activity of Antarctic teleosts as well as sustaining the potentially important role of gill CA in the haemoglobinless fish. The maintenance of an adequate enzyme-substrate affinity could also be an evolutionary strategy to obtain the maximal responsiveness of the enzyme at low temperatures. That the apparent K_m for CO_2 of the Antarctic teleosts CA isoforms at 0 °C was twofold lower than that found in the European eel (Fig. 6; Table 3) agrees with previous results obtained in a comparative analysis of two enzymes, leucine-aminopeptidase and alkaline phosphatase of *T. bernacchii* and *A. anguilla* intestine (Maffia et al., 1993). The sensitivity of inhibition of CA activity to acetazolamide (ACTZ) and sulphanilamide was not significantly different in the three teleost species (Table 3) and was comparable to that measured in the gills of *Platichthys flesus* (Sender et al., 1999), rainbow trout *Salmo gairdneri* (Henry et al., 1993) and human CAII (Sanyal et al., 1981). In light of the high sensitivity to sulphonamides and the cytoplasmic localization, the branchial CA isoform of the three teleost species investigated in this study can be considered comparable to the mammalian CAII isoform.

Heat-stability of the Antarctic fish CA isoforms was about 10 °C lower than that of the temperate species (Fig. 8). A low heat stability has been observed for trypsin from the Antarctic teleost *Pazanothothenia magellanica* (Genicot et al., 1988) and for both alkaline phosphatase and Na^+-D-glucose cotransporters of *T. bernacchii* (Maffia et al., 1993; 1996a). This behaviour could be ascribed to a reduction in the proportion and/or strength of hydrophobic interactions for the Antarctic fish CA isoform.

An increase in enzyme flexibility resulting from a few amino acid substitutions could be, at least partially, responsible for the very high catalytic rate and substrate affinity of the *C. hamatus* branchial CA at 0 °C. This hypothesis is supported by a partial protein sequence (data not shown) revealing some amino acid substitutions with respect to CA isoforms from Wistar rat brain and the zebrafish *Danio rerio*, in regions distant from the active site, which seem to be highly conserved in all species.

A dissimilar protein structure or glycosylation could cause the different electrophoretic migration of the enzymes from *C. hamatus* and *A. anguilla* (Fig. 5). As determined by SDS-PAGE, the molecular mass of approx. 29 kDa of the *C. hamatus* isoform indicates that a novel enzyme variant with high turnover rate is located in the respiratory epithelium of the icefish.

In conclusion, comparing the three species, it appears that *C. hamatus* branchial CA displays two different aspects of evolutionary adaptation; the first is related to 'cold-adaption' and the second is associated with the absence of erythrocytes. The possible role of this enzyme in rapidly supplying blood with bicarbonate may be a compensatory mechanism for blood pH regulation in the absence of erythrocytic CA.

Detailed information on the cell expression of this cytosolic CA isoform and eventually of other membrane-bound CAs, as well as their exact localization in the different cell types of the gill epithelium, could give a more complete picture of the role of this enzyme in icefish gill, clarifying the overall function of the respiratory epithelium in these teleosts. Finally, sequence analysis of the novel CA isoform, comparative studies with other enzyme isoforms, and site-directed mutagenesis experiments, could give us information on the molecular basis of the evolved adaptation mechanisms to survival in a cold environment.

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References

Acierno, R., Macdonald, J. A., Agnisola, C. and Tota, B. (1995). Blood volume in the haemoglobinless Antarctic teleost *Chionodraco hamatus* (Lonnberg). *J. Exp. Zool.* **272**, 407–409.

Acierno, R., Maffia, M., Rollo, M. and Storelli, C. (1997). Buffer capacity in the blood of the hemoglobinless Antarctic fish *Chionodraco hamatus. Comp. Biochem. Physiol*. **118A**, 989–992.

Conley, D. M. and Mallatt, J. (1987). Histochemical localization of Na^+-K^+ ATPase and carbonic anhydrase activity in gills of 17 fish species. *Can. J. Zool*. **66,** 2398–2405.

Easson, L. H. and Stedman, E. (1936). The absolute activity of choline-esterase. *Proc. R. Soc. Lond. B* **121**, 142–164.

Evans, D. H. and Cameron, J. N. (1986). Gill ammonia transport. *J. Exp. Zool*. **239**, 17–23.

Evans, D. H., Claiborne, J. B., Farmer, L., Mallery, C. and Krasny, E. K. (1982). Fish gill ionic transport: methods and models. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **163**, 108–130.

Feller, G. and Gerday, C. (1997). Adaptations of the haemoglobinless antarctic icefish (*Channichthyidae)* to hypoxic tolerance. *Comp. Biochem. Physiol*. **118A**, 981–987.

Feller, G., Pequeux, A. and Hamoir, G. (1981). La presence d'anhydrase carbonique chez 2 poissons de l'archipel des Kerguelen, *Channichthys rhynoceratus* exempt d'hemoglobine et *Notothenia magellanica* de formule sanguine normale. *CR Acad. Sci. Paris* **293**, 395–397.

Feller, G., Poncin, A., Aittaleb, M., Schyns, R. and Gerday, C. (1994). The blood proteins of the Antarctic icefish *Channichthys rhinoceratus*: biological significance and purification of the two main components. *Comp. Biochem. Physiol.* **109B**, 89–97.

Genicot, S., Feller, G. and Gerday, C. H. (1988). Trypsin from Antarctic fish (*Pazanothothenia magellanica* Forster) as compared with trout (*Salmo gairdneri)* trypsin. *Comp. Biochem. Physiol.* **90B**, 601–609.

Heisler, N. (1984). Acid–base regulation in fishes. In *Fish Physiology*, vol. XA (ed. W. S. Hoar and D. J. Randall), pp. 315–401. New York: Academic Press.

Hemmingsen, E. A. and Douglas, E. L. (1970). Respiratory characteristics of the haemoglobin-free fish *Chaenocephalus aceratus. Comp. Biochem. Physiol*. **39**, 773–742.

Henry, R. P. and Heming, T. A. (1998). Carbonic anhydrase and respiratory gas exchange. In *Fish Physiology Haemoglobin and Respiration*, vol. 17 (ed. S. F. Perry and B. L. Tufts), pp. 75–111. San Diego: Academic Press.

Henry, R. P. and Swenson, E. R. (2000). The distribution and physiological

significance of carbonic anhydrase in vertebrate gas exchange organs. *Resp. Physiol.* **121**, 1–12.

Henry, R. P., Gilmour, K. M., Wood, C. M. and Perry, S. F. (1997). Extracellular carbonic anhydrase activity and carbonic anhydrase inhibitors in the circulatory system of fish. *Physiol. Zool.* **70**, 650–659.

Henry, R. P., Tufts, B. L. and Boutilier, R. G. (1993). The distribution of carbonic anhydrase type I and II isozymes in lamprey and trout: possible co-evolution with erythrocyte chloride/bicarbonate exchange. *J. Comp. Physiol.* **163B**, 380–388.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

MacDonald, J. A. and Wells, R. M. G. (1991). Viscosity of body fluids from Antarctic Notothenioid fish. In *The Biology of Antarctic Fishes* (ed. G. di Prisco, B. Maresca and B. Tota), pp. 163–178. Berlin: Springer Verlag.

Maffia, M., Acierno, R., Cillo, E. and Storelli, C. (1996a). Na^+-D-glucose cotransport by intestinal BBMVs of the Antarctic fish *Trematomus bernacchii*. *Am. J. Physiol.* **271**, R1576–R1583.

Maffia, M., Acierno, R., Deceglie, G., Vilella, S. and Storelli, C. (1993). Adaptation of intestinal cell membrane enzymes to low temperature in the Antarctic teleost *Pagothenia bernacchii*. *J. Comp. Physiol.* **163B**, 265–270.

Maffia, M., Trischitta, F., Lionetto, M. G., Storelli, C. and Schettino, T. (1996b). Bicarbonate absorption in eel intestine: evidence for the presence of membrane-bound carbonic ahydrase on the brush border membranes of the enterocyte. *J. Exp. Zool.* **275**, 365–373.

Perry, S. F. and Laurent, P. (1990). The role of carbonic anhydrase in carbon dioxide excretion, acid–base balance and ionic regulation in aquatic gill breathers. In *Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative and Environmental Aspects*, vol VI, *Comparative Physiology* (ed. J. P. Truchot and B. Lahlou), pp. 39–57. Basel: Karger.

Rahim, S. M., Delaunoy, J. P. and Laurent, P. (1988). Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. *Histochemistry* **89**, 451–459.

Randall, D. J. and Daxboeck, C. (1984). Oxygen and carbon dioxide transfer across fish gills. In *Fish Physiology*, vol. XA (ed. W. S. Hoar and D. J. Randall), pp. 263–314. New York: Academic Press.

Rankin, J. C. and Tuurala, H. (1998). Gills of Antarctic fish. *Comp. Biochem. Physiol*. **119A**, 149–163.

Sanyal, G., Pessah, N. I. and Maren, T. H. (1981). Kinetics and inhibition of membrane bound carbonic anhydrase from canine renal cortex. *Biochem. Biophys. Acta* **657**, 128–137.

Sender, S., Bottcher, K., Cetin, Y. and Gros, G. (1999). Carbonic anhydrase in the gills of seawater and freshwater-acclimated flounders *Platichthys flesus*: purification, characterization, and immunohistochemical localization. *J. Histochem. Cytochem*. **47**, 43–50.

Sly, W. S. and Peiyi, Y. H. (1995). Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu. Rev. Biochem.* **64**, 375–401.

Somero, G. N. (1995). Proteins and temperature. *Annual Rev. Physiol.* **57**, 43–68.

Stemler, A. (1993). An assay for carbonic anhydrase activity and reactions that produce radiolabeled gases or small uncharged molecules. *Anal. Biochem.* **210**, 328–331.

Tesch, F. W. (1977). Blood. In *The Eel* (ed. P. H. Greenwood), pp. 38–41. London: Chapman and Hall.

Wells, R. M. G., Summers, G., Beard, L. A. and Grigg, G. C. (1988). Ecological and behavioural correlates of intracellular buffering capacity in the muscles of Antarctic fishes. *Polar. Biol*. **8**, 321–325.

Whitney, P. L. (1974). Affinity chromatography of carbonic anhydrase. *Anal. Biochem.* **57**, 467–476.

Wilbur, K. M. and Anderson, N. G. (1948). Electrometric and colorimetric determination of carbonic anhydrase. *J. Biol. Chem*. **176**, 147–154.

Wilson, R. W., Gilmour, K. M., Henry, R. P. and Wood, C. M. (1996). Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid–base balance? *J. Exp. Biol.* **199**, 2331–2343.