

PROPERTIES OF A CARBONIC ANHYDRASE INHIBITOR PROTEIN IN FLOUNDER SERUM

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Summary

The blood serum of the European flounder *Platichthys flesus* strongly inhibits soluble erythrocytic carbonic anhydrase from the same species. The inhibition is of the uncompetitive type. Hence, the mechanism of the carbonic anhydrase inhibition is different from that of all other known carbonic anhydrase inhibitors. The serum showed no inhibitory effect on carbonic anhydrase from human and bovine red blood cells. By applying the ^{18}O exchange reaction, it could be demonstrated that the presence of the carbonic anhydrase inhibitor in the extracellular fluid has no effect on carbonic anhydrase in intact red blood cells. Thus, this carbonic anhydrase inhibitor seems to act only within the plasma space of the circulatory system.

However, the carbonic anhydrase inhibitor does appear to reduce the bicarbonate permeability of flounder red cells to approximately one-quarter of normal levels as measured by the ^{18}O exchange reaction. The 28 kDa carbonic anhydrase inhibitor was isolated from the serum by gel filtration. The isolated inhibitor was detected in acrylamide gels as a single band representing a 7 kDa protein. The denaturing conditions used in electrophoresis presumably led to a dissociation of the native protein into subunits.

Key words: blood CO_2 transport, fish, uncompetitive enzyme inhibition, red blood cell, ^{18}O exchange reaction, European flounder, *Platichthys flesus*.

Introduction

The enzyme carbonic anhydrase (CA) (EC 4.2.2.1.) is a catalyst of the reversible hydration of carbon dioxide to bicarbonate ions and protons (Maren, 1967). Carbonic anhydrase isozymes exhibit distinct differences in activity, tissue specificity and cellular localisation (Sly and Hu, 1995). Intraerythrocytic CA I and especially CA II are known to play an important role in the transport of CO_2 by blood (Klocke, 1988). Discussion continues as to whether CA IV, a membrane-bound carbonic anhydrase isozyme which, among other localizations, is present on the plasma face of vascular endothelium (Sender et al., 1998), significantly accelerates the efflux of CO_2 from tissue, e.g. from working skeletal muscle (Geers et al., 1997; Geers and Gros, 2000; Henry et al., 1997b) and, therefore, contributes to CO_2 transport within the body. Thus, it seems puzzling that both the plasma and serum of diverse vertebrate species including mammals, but not man and birds, have been shown to have an inhibitory effect on carbonic anhydrase (Booth, 1938; Haswell and Randall, 1976; Haswell et al., 1983; Henry et al., 1997a; Hill, 1986; Leiner et al., 1962; Maetz, 1956; Rispen et al., 1985).

We aimed to identify any inhibitor of carbonic anhydrase present in the plasma of the European flounder *Platichthys flesus* and to determine its inhibitory properties and its molecular mass. A major purpose of the study was to investigate whether the serum of the flounder reduces the

catalytic activity of carbonic anhydrase while the enzyme is enclosed in red blood cells.

Materials and methods

European flounder (*Platichthys flesus*) with a mean body mass of 200 g were caught in the Baltic Sea off Eckernförde, Germany. Live fish were brought to shore on the day of catch. Blood used for the preparation of serum and haemolysate was collected the same day, and red blood cell suspensions were prepared from blood samples taken from flounders that had been transported to our institute and kept in tanks filled with aerated artificial sea water. The continuously purified water had a temperature of 12–14 °C. The photoperiod was 12 h:12 h light:dark. The captive fish refused food. They were kept in the tanks for a maximum of 6 weeks before blood samples were taken.

Fish were anaesthetised in a solution of 0.02% (w/v) metacaine (3-aminobenzoic acid ethyl ester-methanesulphonate, Sigma) in sea water. Subsequently, each fish was given an intraperitoneal injection of 500 i.u. of heparin (Liquemin, Roche) and was then returned to the metacaine solution. After some minutes, animals were taken out again, sponged dry and bled by cutting off the caudal fin. One flounder yielded approximately 3 ml of blood.

Blood was collected in 1.5 ml Eppendorf tubes containing

20 µl of a heparin solution (23 mg ml⁻¹ ammonium heparin in phosphate buffer, pH 7.6). Plasma and erythrocytes were separated by centrifugation for 10 min at 8000g. The buffy coat was discarded. Plasma was pooled, frozen in liquid nitrogen and subsequently stored at -80 °C. Since thawed plasma always coagulated, it was filtered through a Buchner funnel prior to usage in our experiments. We therefore investigated serum rather than plasma.

Erythrocytes were pooled and washed three times (by centrifugation for 10 min at 750g and 4 °C and subsequent resuspension) in 0.9% NaCl and then frozen in liquid nitrogen. After rethawing, the red cell lysate was centrifuged for 1 h at 100 000g to remove cell debris. The supernatant had a pH of 6.5. The pH was brought to 7.6 by addition of 0.1 mol l⁻¹ NaOH. The haemolysate was frozen in liquid nitrogen again and then stored at -80 °C. The lysate contained 5.7 g dl⁻¹ haemoglobin. When the haemolysate was used for mass spectrometric measurements, the detergent Triton X-100 was added to the reaction mixture to give a final concentration of 0.1% (w/v) in the solution.

To prepare red blood cell suspensions, the separated erythrocytes were washed four times, twice in a 125 mmol l⁻¹ NaCl solution containing 50 mmol l⁻¹ Hepes (pH 7.6) (Sigma) and then twice in unbuffered 0.9% NaCl. Finally, the cells were resuspended in 0.9% NaCl to obtain a suspension with a haematocrit of 0.15–0.20.

Identification of carbonic-anhydrase-inhibiting fractions of the serum

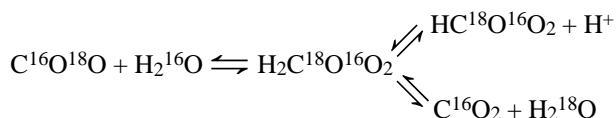
To identify the carbonic-anhydrase-inhibiting fractions of the serum, we used the comparatively simple and fast colorimetric micromethod of Maren (1960) modified as described by Bruns and Gros (1991).

The pH method of determining carbonic anhydrase activity

The principle of this method can be outlined as follows. After rapidly adding CO₂ to an initially alkaline, vigorously stirred buffer solution contained in a small air-tight and thermostatted vessel, the velocity of the CO₂ hydration process is followed using a pH electrode. The method is explained in detail by Wetzel and Gros (1998). We used a slightly modified buffer. To ensure exact measurements, the sample was prediluted if necessary to give a carbonic anhydrase activity of at most 4 in the assay. Carbonic anhydrase activity is defined as the factor by which CO₂ hydration is accelerated compared with uncatalyzed CO₂ hydration.

Mass spectrometry

The method is based on the mass spectrometric recording of the ¹⁸O exchange reaction.



A detailed explanation of the method, which was first

described by Itada and Forster (1977), is given by Peters et al. (2000). For experiments with material from flounder, the pH of the reaction solution was 7.6. The temperature of the fluid was kept at 15 °C. Recordings lasted for 15–30 min.

When the haemolysate was used as the sample, the carbonic anhydrase activity was calculated using the two different slopes of the logarithm of the mass spectrometric signal *versus* time recorded before and after the addition of the sample to the reaction solution. The ratio of the slopes gives the carbonic anhydrase activity in the assay. When experiments were carried out with intact red blood cells, the intracellular carbonic anhydrase activity (*A_i*) and the HCO₃⁻ permeability of the cell membrane (*P_{HCO₃}*) was calculated using the set of equations documented by Böllert et al. (1997) by fitting the theoretical expression to the recorded time course of the ratio of *m*46 to *m*44, where *m*46 and *m*44 are the mass/charge signals 46 (due to C¹⁸O¹⁶O) and 44 (due to C¹⁶O₂), respectively.

To calculate the mean surface-to-volume ratio, we assumed the oval flounder erythrocytes to be elliptical discs. The mean length and mean width of 20 red blood cells per preparation chosen at random were determined by light microscopy. The mean volume of a red blood cell was determined from the haematocrit and red blood cell count.

Gel filtration

Gel filtration was performed at 4 °C using a gel filtration column (HiLoad-G 75, Pharmacia, Freiburg, Germany) suitable for fractionation in a molecular mass range of 3–70 kDa. Haemoglobin (64 kDa), ovalbumin (45 kDa), bovine CA II (30 kDa) and myoglobin (17 kDa) were used as marker proteins. Sample (1.9 ml) and Blue Dextran (0.1 ml) were loaded onto the column *via* a 2 ml sample loop and eluted with Hepes buffer (5 mmol l⁻¹ Hepes, 125 mmol l⁻¹ NaCl, pH 7.6). Immediately before the buffer was applied, dithiothreitol was added to give a concentration of 1 mmol l⁻¹. Elution was performed at a rate of 0.5 ml min⁻¹. The process was monitored by photometry at 280 nm; 2 ml fractions were collected. A first run on the gel filtration column led to the determination of the approximate molecular mass of the carbonic-anhydrase-inhibiting protein, which was 30 kDa. Knowing this, the rest of the serum was prepared for gel filtration by two ultrafiltration steps using membranes with molecular mass cut-offs of 100 kDa and 3 kDa (micro ultrafiltration system model 8MC, membrane types YM100 and YM3, Amicon, Witten, Germany).

After gel filtration, fractions that showed an inhibitory effect on the carbonic anhydrase activity of flounder red cell lysate were pooled and concentrated again by ultrafiltration using the YM3 membrane. The gel filtration was subsequently repeated.

Polyacrylamide gel electrophoresis

Protein contained in inhibitory fractions from the second gel filtration was concentrated again by ultrafiltration and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed with a vertical slab gel system (1.0 mm thick) (Biometra Minigel,

Pharmacia Biotech, Freiburg, Germany) at room temperature (22 °C) applying the method of Laemmli (1970).

Protein solutions were standardised to a concentration of 2 mg ml⁻¹. The running buffer (pH 8.5) contained 31.25 mmol l⁻¹ Tris, 0.24 mol l⁻¹ glycine, 4.8 mmol l⁻¹ SDS and 18.75 µmol l⁻¹ NaN₃. One volume of sample or molecular mass marker was mixed with two volumes of sample buffer (50 % glycine, 5 % mercaptoethanol, 2 % SDS, 1 % Bromophenol Blue and 0.125 mol l⁻¹ Tris, pH 6.8) and incubated for 3 min at 95 °C. The stacking gel contained 5 % acrylamide and the separating gel 18 % acrylamide. The gel was silver-stained using the method of Heukeshoven and Dernick (1985). The protein molecular mass marker kit was purchased from Pharmacia Biotech Europe, Freiburg, Germany.

Results

Inhibitory activity of flounder serum

Flounder serum has an inhibitory effect on the carbonic anhydrase activity of flounder haemolysate. The results of mass spectrometric experiments run with reaction mixtures containing serum at a concentration between 1 % (v/v) and 100 % (v/v) are documented in Fig. 1: 10 % (v/v) serum in the HCO₃⁻ solution caused almost complete inhibition of carbonic anhydrase. Even when ¹⁸O-labelled HCO₃⁻ was dissolved in pure serum, carbonic anhydrase activity was still present at a few per cent of the control value. In control experiments, flounder serum in the HCO₃⁻ solution at concentrations of 10 % (v/v) and 30 % (v/v) had no significant effect on the carbonic anhydrase activity of human haemolysate. The activity of bovine CA II was also not

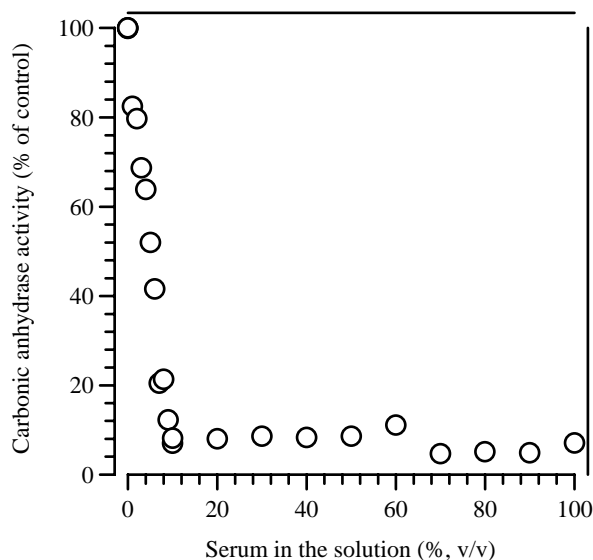


Fig. 1. Dependence of the inhibition of the carbonic anhydrase activity of flounder haemolysate determined by mass spectrometry on the concentration of flounder serum present in the bicarbonate solution. Carbonic anhydrase activity is given as a percentage of the control value.

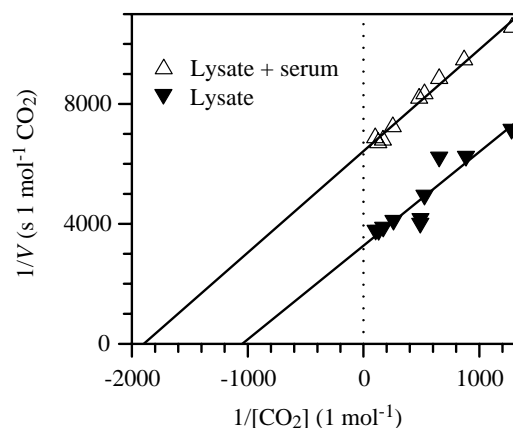


Fig. 2. Lineweaver-Burk plot summarizing the results of two series of experiments performed with lysate of flounder red blood cells using the pH method. For one series of experiments (open triangles), the buffer solution was mixed with flounder serum ($r^2=0.985$). For the other (filled triangles), no serum was present ($r^2=0.860$). The intersection of the regression lines with the dashed line gives $1/V_{\max}$, where V_{\max} is the maximal reaction velocity at high substrate concentration. At the abscissa intercept, $-1/K_m$ and $-1/K_{m,app} = -1/(1+[I]K_i^{-1})K_m^{-1}$ can be read, where K_m is the Michaelis constant, $K_{m,app}$ is the apparent Michaelis constant, K_i is the inhibition constant and $[I]$ is the concentration of the inhibitor.

reduced when flounder serum was present in the solution at a concentration of 20 % (v/v).

Type of carbonic anhydrase inhibition

Two series of assays of carbonic anhydrase activity were performed at various CO₂ concentrations using the pH method. In the first set of experiments, 2.5 µl of flounder haemolysate was used. For the second series, a mixture of 2.5 µl of haemolysate and 5 µl of serum was used. Fig. 2 illustrates the results of these measurements in the form of a Lineweaver-Burk plot. The parallel course of the two regression lines clearly indicates uncompetitive inhibition of the carbonic anhydrase by the flounder serum.

Effects of the serum on intact erythrocytes

The results of the mass spectrometric experiments are presented in Fig. 3. The experiments in which flounder serum was applied in comparatively high concentrations [≥ 50 % (v/v) in the HCO₃⁻ solution] resulted in values of A_i that were significantly lower than the corresponding control value obtained in the absence of serum. Experiments in which labelled HCO₃⁻ was dissolved in pure flounder serum resulted in a value of A_i that was approximately half that obtained in control experiments.

Calculated bicarbonate permeability (P_{HCO_3}) values showed considerable scatter; nevertheless, increasing the serum concentration in the reaction mixture led to a distinct decrease in P_{HCO_3} to approximately 25 % of the control value ($P < 0.001$, unpaired t -test). Because a high serum concentration elevates

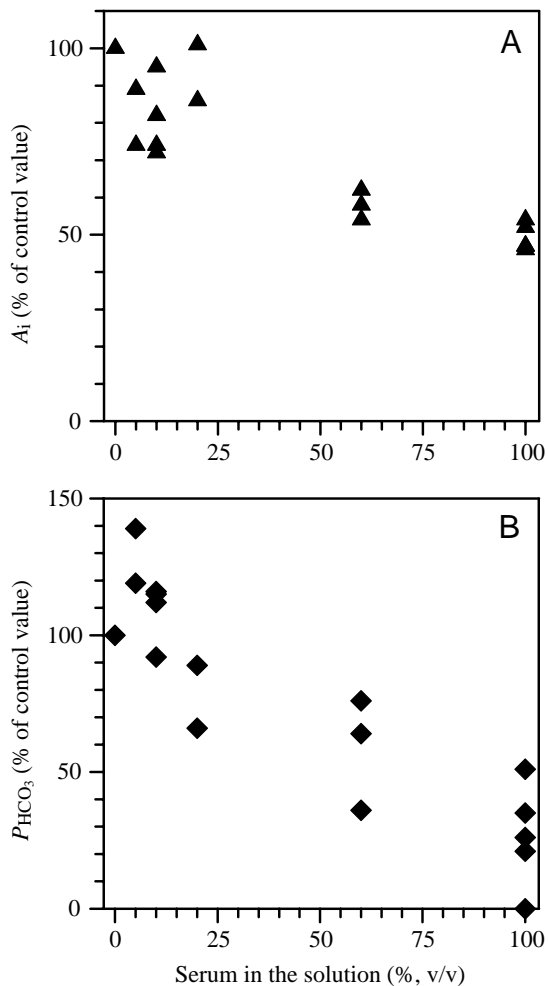


Fig. 3. Flounder blood serum in the bicarbonate solution had a concentration-dependent effect on (A) carbonic anhydrase activity (determined by mass spectrometry) within intact flounder red blood cells (A_i) and (B) the bicarbonate permeability (P_{HCO_3}) of the cytoplasmic membrane of flounder red blood cells calculated from the same mass spectrometric recordings.

the viscosity of the reaction mixture, control experiments were carried out in which we used a HCO_3^- solution containing 8.7 g dl^{-1} dextran (molecular mass 60–90 kDa). This solution caused a significant decrease in the calculated A_i , but did not cause a decrease in the calculated P_{HCO_3} (Fig. 4).

Molecular mass of the carbonic anhydrase inhibitor

Fig. 5 shows a typical chromatogram obtained by gel filtration of prefiltered flounder serum. The carbonic anhydrase activity of flounder haemolysate was inhibited exclusively by the fractions belonging to a small peak in the chromatogram, which represents a compound having a molecular mass of approximately 28 kDa. Refiltration of the fractions belonging to this peak yielded a chromatogram with two peaks. One peak again represented a 28 kDa protein. The second peak was caused by a significantly smaller protein, whose molecular mass, because of the separation characteristics of the column,

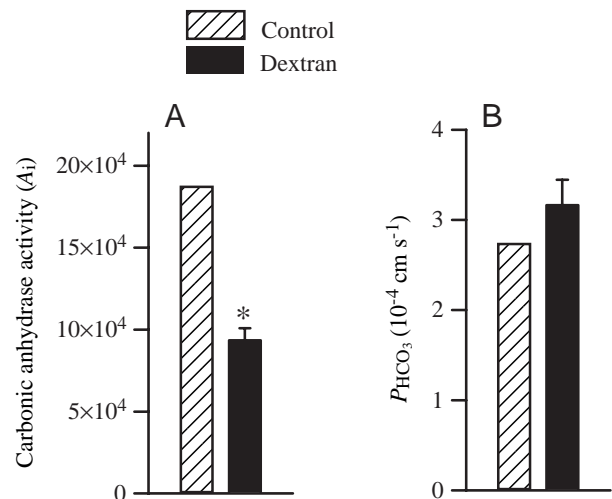


Fig. 4. When dextran was mixed with the solution of ^{18}O -labelled bicarbonate used for the mass spectrometry measurements, the calculation of the intracellular carbonic anhydrase activity (A_i) and the bicarbonate permeability (P_{HCO_3}) of the cell membrane from mass spectrometry recordings resulted in a significantly lower mean value of A_i but not in a significantly altered mean value of P_{HCO_3} compared with the respective mean values obtained in control experiments. Values are means \pm s.d., $N=4$. An asterisk indicates a value significantly different ($P<0.05$) from the control value.

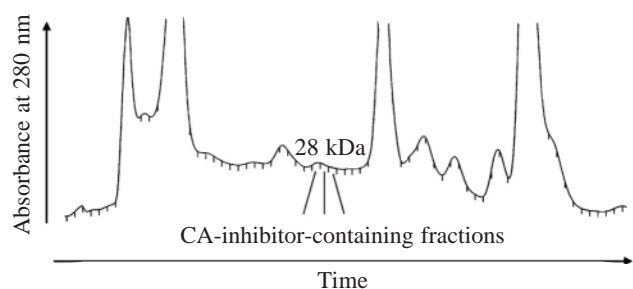


Fig. 5. The carbonic-anhydrase-inhibiting fractions obtained by gel filtration of the flounder serum could be assigned to a small peak in the chromatogram that was caused by a 28 kDa protein. CA, carbonic anhydrase.

could not be determined. Only fractions that contained the 28 kDa protein had an inhibitory effect on the carbonic anhydrase activity of flounder haemolysate. SDS-gel electrophoresis of the carbonic anhydrase inhibitor isolated by gel filtration revealed a single protein band. This band did not represent a protein with a molecular mass of 28 kDa, but one of approximately 7 kDa.

Discussion

The experiments revealed that blood serum of seawater-adapted flounder contains a protein that inhibits the erythrocytic carbonic anhydrase of this euryhaline species. Inhibition of carbonic anhydrase activity by the serum of

several freshwater-adapted fish species (Dimberg, 1994; Maetz, 1956) and by the plasma of freshwater- and seawater-adapted eel (*Anguilla anguilla*) (Haswell et al., 1983) has also been reported, so the inhibitory action of the serum on the erythrocytic carbonic anhydrase of the same species is obviously a common feature of at least a group of teleost species from both freshwater and seawater habitats, as it is in a number of mammalian species.

The reversibility of the carbonic anhydrase inhibition has been demonstrated for a carbonic anhydrase inhibitor isolated from porcine plasma (Roush and Fierke, 1992). We find that inhibition of erythrocytic flounder carbonic anhydrase by the serum of the same species is uncompetitive. The inhibitor (I) binds to the enzyme–substrate complex (ES), forming an ESI complex. An uncompetitive inhibitor lowers both the Michaelis constant (K_m) and the maximal reaction velocity (V_{max}). This type of inhibition of carbonic anhydrase has not been observed for any other inhibitor.

A serum concentration of only 5% (v/v) in the assay blocked half the carbonic anhydrase activity of the haemolysate, as detected by mass spectrometry. The inhibitory serum protein constitutes only a minor fraction of the serum proteins, so a very small concentration of the inhibitor results in substantial inhibition of the carbonic anhydrase activity. It is not clear why flounder serum does not completely inhibit carbonic anhydrase activity, as measured by ^{18}O exchange, in the haemolysate of the same species. Apparently, the velocity of the ^{18}O exchange reaction in this haemolysate is increased not only by carbonic anhydrase but also by another reaction, which cannot be blocked by the serum. Proteins other than carbonic anhydrase and/or phosphates in the haemolysate may be responsible (Dennard and Williams, 1966; Matsumoto et al., 1984). Booth (1938) noted that the serum of several species exerts a catalytic effect on CO_2 hydration despite its inhibitory effect on added carbonic anhydrase.

The carbonic anhydrase inhibitor of the flounder has a distinct effect on erythrocytic carbonic anhydrase of the same species but not on erythrocytic carbonic anhydrase of the two mammalian species tested. Such a specificity of the carbonic anhydrase inhibition by blood serum from different fish species has already been reported (Maetz, 1956). The carbonic anhydrase inhibitor from sheep has little or no effect on the activity of carbonic anhydrase originating from different fish species (Leiner et al., 1962). In contrast, well-known carbonic anhydrase inhibitors, such as sulphonamides and monovalent inorganic anions, have similar effects on carbonic anhydrase isozymes from various origins.

Roush and Fierke (1992) concluded from their findings that the carbonic anhydrase inhibitor protein of porcine plasma may bind to amino acid residues on the surface of the CAII molecule that are completely conserved only in mammalian CAII. Binding at these residues may cover the active site cavity and prevent diffusion of substrate to the catalytic zinc. This is consistent with the observation that the K_i of the porcine carbonic anhydrase inhibitor towards mammalian CAII depends only slightly on the mammalian species from which

the CAII is extracted (Roush and Fierke, 1992). In contrast, erythrocytic carbonic anhydrase of a given teleost is significantly more strongly inhibited by plasma of the same species than by plasma of other teleost species (Henry et al., 1997a), so the binding domain on the carbonic anhydrase must be species-specific in fishes.

At first glance, the results of the mass spectrometric experiments carried out with intact erythrocytes seem to prove that serum inhibits carbonic anhydrase activity inside the red blood cells. However, control experiments performed with dextran-containing solutions reveal that the lower calculated A_1 seems to be the consequence of the elevated viscosity of the HCO_3^- solution. Addition of either serum or dextran reduces the amplitude of the first steep part of the biphasic time course of the reaction compared with the results of control experiments. In this connection, we point out that for the calculations it is assumed that the diffusion of CO_2 from the external solution to the cytosol is not a rate-limiting step for the ^{18}O exchange reaction (Itada and Forster, 1977). This prerequisite may not be fulfilled when an elevated viscosity gives rise to a substantial unstirred layer on the surface of the red blood cells, which impairs diffusion of CO_2 into the cell (Wunder and Gros, 1998). The use of dextran in the HCO_3^- solution did not result in a decrease in the calculated P_{HCO_3} of the red blood cell membrane, so the question remains of how serum exerts its influence on P_{HCO_3} . It is possible that an interaction between a serum component and the anion exchanger in the red blood cell membrane reduces the P_{HCO_3} of the membrane or that the serum interferes in some way with the mass spectrometric experiment.

Isolation of the carbonic anhydrase inhibitor from flounder serum by gel filtration provided evidence that the inhibitor is a molecule with a molecular mass of 28 kDa. The fact that this molecular mass could not be confirmed by SDS–PAGE is probably due to the denaturing and reducing conditions present during the SDS–PAGE, which may have led to dissociation of the 28 kDa protein into 7 kDa subunits. The molecular mass found by gel filtration is in agreement with the finding of Haswell et al. (1983), who reported that the plasma of the eel contained a carbonic anhydrase inhibitor of size 10–30 kDa. Dimberg (1994), however, using gel filtration, isolated an inhibitory protein from trout (*Oncorhynchus mykiss*) plasma with a molecular mass of approximately 7 kDa. Thus, the size of the carbonic-anhydrase-inhibiting plasma protein may differ among fish species. It is noted that all molecular masses reported for carbonic anhydrase inhibitors isolated from fish plasma are distinctly smaller than 79 kDa, the mass of the carbonic anhydrase inhibitor isolated from porcine plasma (Roush and Fierke, 1992). It may be worth investigating whether the carbonic anhydrase inhibitors isolated from the plasma of different fish species also show extensive sequence homology with transferrins, as does the carbonic anhydrase inhibitor isolated from pig plasma (Wuebbens et al., 1997).

Since we could not substantiate an inhibitory effect of flounder serum on intracellularly located carbonic anhydrase,

it is remarkable that the addition of a purified carbonic anhydrase inhibitor protein to trout blood *in vitro* has been reported to decrease the Root effect, as does the addition of acetazolamide, a carbonic-anhydrase-inhibiting sulphonamide (Dimberg, 1994). A reduction in bicarbonate transport across the red cell membrane by the carbonic anhydrase inhibitor protein (or possibly other serum constituents), as suggested by our results (Fig. 3), should not decrease the Root effect when blood is equilibrated with gas for 10 min, as performed by Dimberg (1994). However, a decrease in bicarbonate permeability of the red cell membrane may impede the uptake of CO₂ by the blood or the delivery of CO₂ from the blood during the short capillary transit. The significance and mechanism of the observed reduction in the bicarbonate permeability are open to further investigation.

Any physiological function of binding of the inhibitor protein to carbonic anhydrase is probably based on an interaction with carbonic anhydrase present in plasma, e.g. carbonic anhydrase from lysed red blood cells, or with CA IV located on the apical membrane of the vascular endothelium. It has been reported that a carbonic anhydrase inhibitor isolated from porcine plasma inhibits CA II of the pig much more effectively than it inhibits CA IV, CA III and CA I of this species (Roush and Fierke, 1992). Heming et al. (1993) found evidence for a moderate inhibitory effect of the porcine carbonic anhydrase inhibitor on vascular carbonic anhydrase activity in an isolated saline-perfused rat lung preparation. To date, there is only one report of differential effects of a carbonic anhydrase inhibitor isolated from the blood plasma of a fish, the rainbow trout (*Oncorhynchus mykiss*), on carbonic anhydrases purified from the gill and blood of the same species (Dimberg, 1994). However, the question of whether the gill carbonic anhydrase preparation contained any membrane-bound enzyme or only cytoplasmic carbonic anhydrase remains unsettled. Several reports suggest that there is no substantial activity of membrane-bound carbonic anhydrase in fish gills (Henry et al., 1988, 1993; Sender et al., 1999; Swenson et al., 1996). The question of whether inhibition of carbonic anhydrase released into the blood plasma from leaky red blood cells or an effect of the inhibitor on CA IV on the vascular endothelium impairs or improves the transport of CO₂ remains to be pursued by *in vivo* experiments. Such experiments should also address a possible *in vivo* effect of the carbonic anhydrase inhibitor on oxygen transport. It is possible that the carbonic-anhydrase-inhibiting action of the protein is only a secondary phenomenon whose primary function could be the prevention of the loss of the zinc-containing carbonic anhydrase from the plasma *via* the kidney. This hypothesis is based on the findings of Appelgren et al. (1989) and Öjteg and Wistrand (1994), who investigated the distribution of intravenously injected ¹²⁵I-labelled carbonic anhydrase isozymes in rat and the kinetics of their renal elimination from blood plasma, and on the discovery of an extensive sequence homology between transferrin and a carbonic anhydrase inhibitor isolated from pig plasma (Wuebbens et al., 1997).

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