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PURIFICATION AND PROPERTIES OF CYCLOSTOME CARBONIC ANHYDRASE FROM ERYTHROCYTES OF HAGFISH

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Summary

1. Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) has been purified from erythrocytes of hagfish (*Myxine glutinosa*). A single form with low specific CO_2 hydration activity was isolated.

The purified carbonic anhydrase appeared homogeneous judging from polyacrylamide gel electrophoresis and gel filtration experiments. The protein has a molecular weight of about 29 000, corresponding to about 260 amino acid residues. This molecular weight is in accordance with other vertebrate carbonic anhydrases with the exception of the elasmobranch enzymes, which have M_r 36 000—39 000.

2. The molecular weight obtained for hagfish carbonic anhydrase indicates that a carbonic anhydrase with M_r approx. 29 000 is the ancestral type of the vertebrate enzyme rather than, as in sharks, a heavier carbonic anhydrase molecule.

3. The circular dichroism spectrum may indicate a somewhat different structural arrangement of aromatic amino acid residues in this enzyme than in the mammalian carbonic anhydrases.

4. The enzyme is strongly inhibited by acetazolamide and also to a lesser extent by monovalent anions.

5. Zn^{2+}, which is essential for activity, appears, contrary to other characterized carbonic anhydrases, less strongly bound in the active site of the enzyme.

Introduction

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is widely distributed in nature and has been found in animals, plants and certain bacteria [1].

Most mammals exhibit a polymorphism of erythrocyte carbonic anhydrase

mainly due to the existence of two isoenzymes with distinctly different amino acid sequences [2,3] and specific activities [1]. The forms of the enzyme with high and low activities are most commonly designated C and B, respectively. The ruminants are, however, an exception to this rule and possess only a high-activity erythrocyte carbonic anhydrase [4—7].

The only submammalian vertebrate carbonic anhydrases that have been purified to homogeneity come from shark [8], tuna [9], eel [10], frog [11], and turkey [12], of which the shark enzyme represents the most ancient form. All of these nonmammalian carbonic anhydrases, except the eel enzyme, appear in the erythrocytes in a single form. They have approximately the same molecular weight as the mammalian enzymes, about 30 000. Elasmobranch carbonic anhydrases are, however, an exception having M_r 36 000—39 000 [8].

In order to obtain further information regarding the evolution of the enzyme we have purified and characterized the erythrocyte carbonic anhydrase from hagfish (*Myxine glutinosa*) belonging to the cyclostomes, which are considered to be the most primitive vertebrates.

Enzymic activity of carbonic anhydrase in erythrocytes and various tissues of hagfish has been reported [14], and kinetic measurements of the enzyme in hemolysate have been performed [15]. Our report is the first concerning a purified preparation of this enzyme.

Materials and Methods

Protein purification. Blood was obtained from a large venous cavity of freshly caught hagfish by using a syringe with heparin-treated needles. In this way, 50 ml blood were collected from 50 animals. Crude carbonic anhydrase was prepared using a modification of Method II of Keilin and Mann [16]. The erythrocytes were separated from whole blood in a refrigerated centrifuge at $2500 \times g$ for 20 min. They were then washed twice in an equal volume of cold 3.3% NaCl and subsequently hemolyzed by suspending the packed cells in a 5-fold volume excess of distilled water. Hemoglobin was removed, in a refrigerated room (5°C), by selective denaturation with 40 ml $CHCl_3$ and 80 ml 40% (v/v) C_2H_5OH/H_2O added to 100 ml hemolysate. The mixture was stirred for 20 min at 5°C. The precipitate and excess $CHCl_3$ were removed by centrifugation for 20 min at $1500 \times g$. The supernatant containing the carbonic anhydrase was filtered and dialyzed for 2 days against 5 mM Tris-HCl buffer, pH 8.9.

Ion-exchange chromatography. The subsequent purification was made on a DEAE-cellulose column (1.0 × 17 cm, Whatman DE-23) at 5°C, equilibrated with 5 mM Tris-HCl buffer, pH 8.9.

The pH of the enzyme solution was adjusted to correspond with that of the equilibration buffer by addition of Tris solution; conductivity was adjusted by dilution with distilled water. The protein was loaded on the column, and the column was washed with 100 ml equilibration buffer. Elution was performed with a linear concentration gradient of 300 ml each of 5 mM and 0.1 M Tris-HCl buffer, pH 8.9. Finally, the column was washed with 0.5 M Tris-HCl buffer, pH 8.9.

Enzyme assays. The CO_2 hydration activity was determined by the colorimetric method of Rickli et al. [17], and the definition of activity units of these authors was used, (activity units = $10(t_b - t_c)/t_c$, where t_b and t_c are the times for obtaining the color change of the indicator in the uncatalyzed and enzyme-catalyzed reactions, respectively. The reaction was followed from pH 8.2 to pH 6.5, and a color reference titrated to pH 6.5, was used.

The esterase activity was determined with *p*-nitrophenyl acetate as substrate according to Whitney et al. [18]. The assay medium contained 0.4 mM *p*-nitrophenyl acetate, 1% acetone, 0.55 μM enzyme and Tris-H_2SO_4 or 2,2-bis(hydroxymethyl)-2,2′,2″-nitriloethanol-H_2SO_4 buffers, all of ionic strength 0.1. k_{enz}, which is the apparent second-order rate constant defined by $v_0 = k_{enz}$ [E][S], was determined. v_0 is the initial catalytic rate ($M \cdot s^{-1}$) and [E] and [S] are the total enzyme and substrate concentrations (M), respectively.

Isoelectric focusing. Isoelectric focusing was carried out in an LKB Model 8101 focusing column with LKB ampholines as described by Vesterberg [19].

Gel filtration. Gel filtration was performed on a Sephadex G-100 (Pharmacia) column (2.1 × 110 cm) in 0.05 M Tris-HCl buffer, pH 7.5/0.1 M KCl. The column was calibrated with bovine serum albumin (M_r 67 000 [20]), human carbonic anhydrase B (M_r 28 850 [2]) and horse heart cytochrome *c* (M_r 12 400 [21]).

Using high molecular weight blue Dextran (Pharmacia), the void volume, V_v, was determined to be 138 ml. The calibration proteins were eluted at the following volumes: albumin, 1.36 V_v; human carbonic anhydrase B, 1.88 V_v; and cytochrome *c*, 2.44 V_v.

From these data a calibration curve, log (molecular weight) = *f* (elution volume) was constructed.

Polyacrylamide gel electrophoresis. This was carried out as described by Smith [22] except that spacer and sample gels were omitted. Gel concentrations of 7.5% were used and 0.095 M Tris-glycine buffer, pH 9.5 was used in both gel and electrode reservoirs. Gels were stained with amido black in 10% trichloroacetic acid overnight for proteins, and destained in 10% acetic acid.

Amino acid analyses. Amino acid analyses were performed according to Moore and Stein [23] on a Beckman Model 120 B automatic amino acid analyzer. Enzyme aliquots were hydrolyzed at 110°C in 6 M HCl for 24 h in tubes sealed in vacuo. Half-cystine was determined as cysteic acid after performic acid oxidation following the method of Hirs [24]. Tryptophan was estimated after hydrolysis in 3 M *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole according to the procedure of Lin and Chang [25].

Determination of the molar absorbance coefficient. The absorbance coefficient of the enzyme was estimated after amino acid analysis of an enzyme aliquot with known absorbance.

Spectral measurements. Absorbance was measured with a Beckman DB-GT spectrophotometer. Circular dichroism (CD) spectra were recorded on a Jasco J-41 A spectropolarimeter. From the obtained spectra the mean residue molecular ellipticities, θ, were calculated assuming a mean residue weight of 112.

Denaturation. The stability of the hagfish carbonic anhydrase in guanidine-HCl in 0.1 M Tris-HCl, pH 7.5 was investigated by allowing the enzyme (0.06 mg/ml) to stand for 24 h in various concentrations of the reagent. The relative

specific enzymic activities in the CO_2 hydration reaction were then measured according to Carlsson et al. [26].

Zinc determination in blood serum. The zinc contents in blood serum were measured on a Perkin-Elmer atomic absorption spectrophotometer 300. A zinc calibration curve was constructed in the range 0.1—1.0 ppm by diluting a stock solution containing 1000 ppm zinc with 5% (v/v) glycerol to appropriate concentration levels.

Chemicals. Guanidine hydrochloride was prepared from guanidinium carbonate according to Nozaki [27]. All other chemicals were reagent grade and used without further purification.

Results

Enzyme purification

A summary of the yield of enzyme activity during the purification of hagfish carbonic anhydrase is shown in Table I. After dialysis of the crude chloroform-ethanol extract against Tris-HCl buffer precipitation of ultraviolet-absorbing material was noticed. Loss of enzyme activity in this step was, however, negligible (Table I). The dialysate was further purified on a DEAE-cellulose column. A typical elution pattern is shown in Fig. 1. Only a single carbonic anhydrase peak, with nearly constant specific activity, is observed. The fractions of this peak contained 75% of the original CO_2 hydration activity applied to the column, and no further activity was detected when the column finally was washed with 0.5 M buffer. The total yield of hagfish carbonic anhydrase from 50 ml of blood collected from 50 animals was 4.5 mg.

The eluate containing the CO_2 hydration activity was concentrated in vacuo in a collodion bag inserted in a filter flask. During this procedure the CO_2 hydration activity was reduced to 8% of the original. The activity of the concentrate was completely restorded by addition of the filtrate. Full reactivation was also achieved by incubation of equimolar amounts of Zn^{2+}. With Co^{2+}, the degree of reactivation was 53%, whereas no reactivation was obtained for Cu^{2+}. If the collodion bag was instead surrounded by buffer solution during the concentration, no inactivation occurred.

TABLE I

PURIFICATION OF HAGFISH ERYTHROCYTE CARBONIC ANHYDRASE

CO_2 hydration activity was measured according to Rickli et al. [17]. The data in this table are from one representative preparation from 54 ml of hemolysate.

Purification step	Total activity (units $\times 10^{-5}$)	Speicific activity (units/mg $\times 10^{-3}$)	Yield (%)
1 Hemolysate	2.07	0.04 *	100
2 Chloroform-ethanol extract	1.48	0.78 *	71
3 Dialysate	1.40	1.3 *	67
4 DEAE-cellulose chromatography	1.03	22.7 **	50

* Protein concentration was estimated according the method of Kalckar [41].

** Protein concentration was determined using an $\epsilon_{280\ nm} = 46\ 000\ M^{-1} \cdot cm^{-1}$.

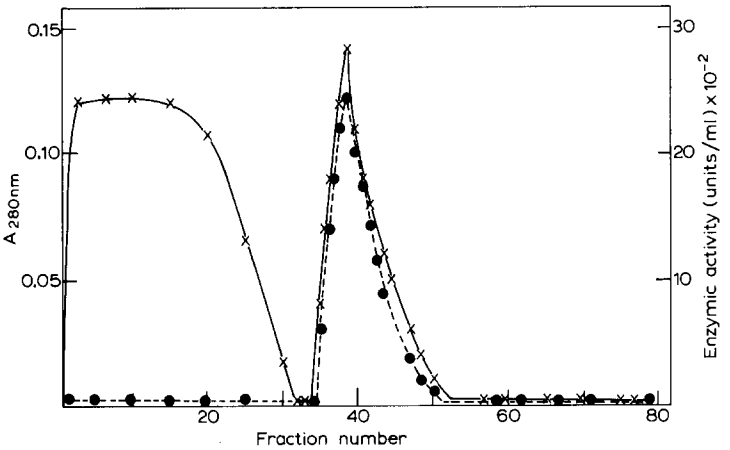

Fig. 1. Chromatography of a crude chloroform-ethanol extract of hagfish carbonic anhydrase on a DEAE-cellulose column (1.0 X 17 cm) equilibrated with 5 mM Tris-HCl buffer, pH 8.9. A linear concentration gradient (600 ml of 5 mM—0.10 M Tris-HCl buffer, pH 8.9) was used to elute the enzyme from fraction 27 (11-ml fractions, flow rate 5 ml/h). The temperature was 4°C. X———X, A_{280nm}; •------•, CO_2 hydration activity in units/ml. Fractions 34—48 were pooled.

Homogeneity and some physical properties of the purified enzyme

The enzyme solution from the final step of the purification procedure above has a near-ultraviolet absorption spectrum, which is typical for proteins. The ratio of absorbance at 280 nm and 260 nm was 1.5, suggesting that contamination of nucleic acids is negligible [28]. The molar absorbance coefficient at 280 nm (ϵ_{280nm}) and some other physical properties are shown in Table II.

Polyacrylamide gel electrophoresis of the concentrated enzyme from the DEAE-cellulose chromatography revealed only a single band. Isoelectric focusing of the purified material in the pH range 3—10 was also performed (Fig. 2). Only a single form was detected and appeared in a narrow, symmetrical band of activity. The isoelectric point is given in Table II.

To examine whether any form of carbonic anhydrase was lost during the preparation, the chloroform-ethanol supernatant from the initial purification step was analyzed by isoelectric focusing in a pH gradient of 3—10. However, no additional CO_2 hydration activity peak was observed.

The purified enzyme was further analysed by chromatography on a Sepha-

TABLE II

PHYSICAL PROPERTIES OF CARBONIC ANHYDRASES FROM HAGFISH AND SHARK

	Hagfish	Bull shark *	Tiger shark *
Molecular weight	29 200 **	35 900	38 600
$\epsilon_{280\ nm}$ ($M^{-1} \cdot cm^{-1}$)	46 000 ***	75 000	63 000
Isoelectric point	7.0	4.5	

* Values taken from Maynard and Coleman [8].

** Calculated from animo acid composition.

*** Calculated using M_r 28 500.

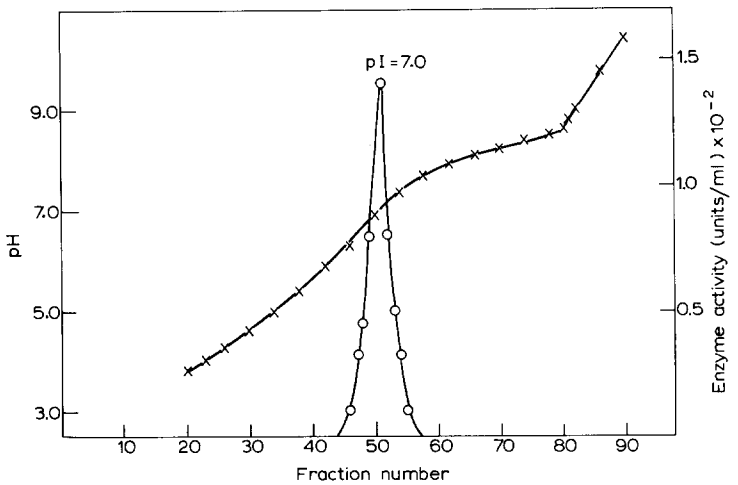

Fig. 2. Isoelectric focusing of hagfish carbonic anhydrase obtained from DEAE-cellulose chromatography. The pH gradient was 3—10. The time for the focusing was 51 h, and the temperature was held at 10° C. A peristaltic pump was used to collect 1-ml fractions. o———o, CO_2 hydration activity in units/ml; X———X, pH of the fractions.

dex G-100 column. The hagfish carbonic anhydrase was eluted as a single symmetrical peak. The molecular weight of the enzyme was determined to be 28 500 ± 300, based on three gel filtrations, where the enzyme appeared after 1.89 ± 0.01 void vols.

The circular dichroism spectrum of hagfish carbonic anhydrase is illustrated in Fig. 3.

Amino acid composition

The amino acid composition of hagfish carbonic anhydrase is given in Table

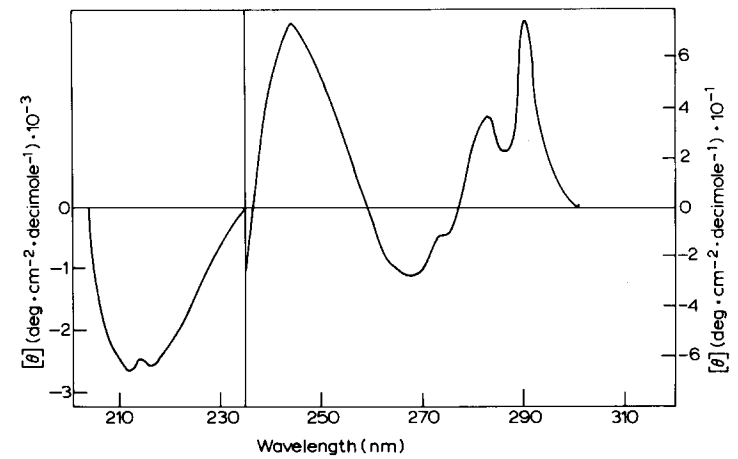

Fig. 3. CD spectra of hagfish carbonic anhydrase. At wavelengths above 235 nm a 1 cm cell was used, while a 1 mm cell was employed at lower wavelengths. Enzyme concentration was 0.41 mg/ml in 0.025 M Tris-HCl, pH 8.9. The temperature was 25° C. Note the difference in scales for the spectral ranges 200—235 and 235—320 nm.

TABLE III

AMINO ACID COMPOSITION FROM HAGFISH, MAN AND SHARK

Residue	Hagfish		Human B *	Bull shark **	Tiger shark **
	Observed	Integer			
Trp	6.0	6	6	5	7
Lys	15.9	16	18	26	29
His	12.6	13	11	9	14
Arg	10.7	11	7	10	13
Asp	33.1	33	31	33	40
Thr	11.9	12	14	11	13
Ser	27.2	27	30	29	31
Glu	24.7	25	22	41	40
Pro	14.7	15	17	24	21
Gly	18.2	18	16	30	32
Ala	15.3	15	19	19	23
Val	12.7	13	17	10	13
Met	3.3	3	2	2	3
Ile	6.9	7	10	12	16
Leu	26.2	26	20	32	34
Tyr	7.3	7	8	8	9
Phe	9.8	10	11	12	18
Cys	4.1	4	1	25	18
Total		261	261	338	374

* Values according to Andersson et al. [2].
** Values taken from Maynard and Coleman [8].

III. The compositions of the human B enzyme and the two shark enzymes are given for comparison.

Denaturation

The concentration of guanidine-HCl giving 50% inactivation in 24 h is approx. 0.8 M for the hagfish carbonic anhydrase.

Enzyme activity and inhibition

The specific CO_2 hydration activity, measured according to Rickli et al.

TABLE IV

EFFECT OF INHIBITORS ON THE CO_2 HYDRATION ACTIVITY OF HAGFISH AND HUMAN CARBONIC ANHYDRASES

The activity was measured according to Rickli et al. [17]. The enzyme and inhibitor were incubated in the presence of buffer for 1 min to ensure equilibrium before the reaction was started by addition of the CO_2 solution. The corresponding data for the human enzymes obtained in the same assay are also included for means of comparisons.

Inhibitor	K_I (M)		
	Hagfish	Human B	Human C
CNO^-	$3 \cdot 10^{-6}$	$8 \cdot 10^{-7}$	$4 \cdot 10^{-6}$
N_3^-	$1 \cdot 10^{-3}$	$6 \cdot 10^{-5}$	$4 \cdot 10^{-4}$
Cl^-	0.3	0.02	0.2
Acetazolamide	$9 \cdot 10^{-9}$	$6 \cdot 10^{-8}$	$7 \cdot 10^{-9}$

[17], was $22.7 \cdot 10^3$ units/mg (Table I).

The pH-dependence of the esterase activity, with *p*-nitrophenyl acetate as substrate, was studied. The activity-linked pK_a of the reaction is 7.1, and the apparent second-order rate constant, k_{enz}, approaches 430 $M^{-1} \cdot s^{-1}$ at alkaline pH, which is about 40% of the esterase activity of human carbonic anhydrase B.

The effects of various inhibitors on the CO_2 hydration activity is presented in Table IV and are compared to the corresponding K_I values obtained for the human carbonic anhydrases in the same assay.

Discussion

Judging from the DEAE-cellulose chromatography, only a single form of carbonic anhydrase is present in the erythrocytes of hagfish (Fig. 1). This is also supported by isoelectric focusing (Fig. 2) and polyacrylamide gel electrophoresis of the pooled fractions from chromatography.

Interestingly, the molecular weight of hagfish carbonic anhydrase is about 29 000, which is similar to all other characterized vertebrate carbonic anhydrases, with the exception of elasmobranch carbonic anhydrases, of which molecular weights in the range 36 000—39 000 have been obtained for the bull sharks (*Carcharhinus leucas*) and the tiger shark (*Galeocerdo cuvieri*) [8]. By gel filtration analysis we have also obtained a higher molecular weight (34 000) for the carbonic anhydrase from the shark *Squalus acanthias* (spiny dogfish).

Another conspicuous structural difference between the shark and the hagfish carbonic anhydrases is the relatively low content of half-cystine residues in the hagfish enzyme. Judging from amino acid composition data (Table III) and molecular weights of carbonic anhydrases, the hagfish enzyme seems to be related more closely to the carbonic anhydrases of mammals than to those of the elasmobranchs, despite the relationship between the cyclostomes and the elasmobranchs.

Our results from structural analysis of the hagfish enzyme may, contrary to what has been proposed [29], indicate that the ancestral vertebrate carbonic anhydrase had M_r approx. 29 000 and was not, as in sharks, a heavier carbonic anhydrase molecule whose polypeptide chain had been shortened during the course of evolution. The elasmobranch carbonic anhydrases have probably undergone a marked change after the divergence of the elasmobranchs and the teleosts (bony fish). The examined sharks are, from an evolutionary point of view, modern species. It would therefore be interesting to investigate the carbonic anhydrases from shark species that are considered to be of a more primitive type.

Analysis of the amino acid compositions of the mammalian carbonic anhydrase B and C has revealed a consistent difference in serine content in the two forms. In all species studied the B-type has a considerably higher serine content (28—33 residues per molecule [1]) than the C-type (16—22 residues per molecule [1]). The hagfish carbonic anhydrase thereby resembles the B enzymes (Table III).

As shown in Table I the specific CO_2 hydration activity of the hagfish carbonic anhydrase is 22 000 units/mg. The corresponding values for the human B

and C enzymes were determined to be 20 000 and 55 000 units/mg, respectively. For further comparisons see Ashworth et al. [6]. Apparently, the hagfish carbonic anhydrase has a CO_2 hydration activity of the same magnitude as the low activity (B-type) carbonic anhydrases. Kinetic work carried out on hagfish hemolysate, combined with titration of enzyme activity with sulfonamides, also indicate that the enzyme is of the low activity type [15].

A comparison of the inhibitory effects that various inhibitors of carbonic anhydrases have on hagfish carbonic anhydrase shows that the hagfish enzyme resembles human carbonic anhydrase C more than human carbonic anhydrase B (Table IV).

It is evidently difficult to make an unambiguous determination of the relationship between hagfish carbonic anhydrase and the enzymes of the phylogenetically distant mammals based on kinetic and limited structure information. It has previously been tentatively suggested by Tashian et al. [30], that the C-type carbonic anhydrase is the most ancient form. A definite conclusion as to the origin of the B and C enzymes must wait until the amino acid sequence of a submammalian carbonic anhydrase is known.

The CD spectrum of hagfish carbonic anhydrase (Fig. 3) is, as are spectra of other carbonic anhydrases, rather complex. In the near-ultraviolet region, the spectrum of the hagfish carbonic anhydrase, like the bacterial carbonic anhydrase from *Neisseria sicca* [31], has ellipticity bands of positive magnitude, whereas, in this wavelength region, negative ellipticities have been observed for mammalian and elasmobranch carbonic anhydrases [7,8,32,33]. The spectral differences might be due to the absence and/or lower intensity of some negative bands in the CD spectrum of hagfish carbonic anhydrase present in the spectra of mammalian carbonic anhydrases.

The complex CD pattern in the near-ultraviolet range have proposed to arise partly from aromatic chromophores asymmetrically packed in aromatic clusters [1,32]. From amino acid sequence work [2,3,34—36] and X-ray crystallographic analyses [37], this aromatic structure seems to be well conserved in the mammalian carbonic anhydrases.

The observed differences in the near-ultraviolet region of the CD spectrum of hagfish carbonic anhydrase might therefore indicate a somewhat different arrangement of some aromatic amino acid residues in this enzyme.

In the far-ultraviolet region, the CD spectrum resembles other recorded spectra of carbonic anhydrases with extrema near 210 nm and 217 nm. A negative band at 217 nm has been assigned to antiparallel β-structure [38], and the CD spectrum of hagfish carbonic anhydrase is in agreement with the presence of this type of secondary structure, which is a predominant feature in the human carbonic anhydrases [1].

The stability of the native three-dimensional conformation with regards to denaturation in guanidine-hydrochloride, is comparable to that of the human C and bovine B carbonic anhydrases [26,39].

The hagfish carbonic anhydrase appears to be functionally similar to the mammalian carbonic anhydrases. Thus, the basic form of the enzyme, with a typical pK_a value of about 7.0, is the catalytically active form. The enzyme is also characteristically inhibited by monovalent anions and the aromatic sulfonamide acetazoleamide (Table IV). For an in vivo function of the hagfish car-

bonic anhydrase, it is not surprising that the only erythrocyte form of the enzyme present is, like human carbonic anhydrase C, relatively insensitive to anion inhibition, since Maren et al. [40] have shown that the CO_2 hydration activity of the anion-sensitive human carbonic anhydrase B is almost abolished by the normal concentrations of chloride and bicarbonate in the erythrocytes.

The zinc ion, which is essential for activity, and which is normally firmly coordinated to the active site of the enzyme, appears, as was mentioned in the Results sections, less strongly bound in the hagfish carbonic anhydrase. In order to see whether the zinc concentration of hagfish blood is higher than in other animals, we measured the zinc concentration in hagfish blood plasma and obtained a value of 49 μg/ml. A parallel analysis of human blood plasma showed a typical zinc concentration of 0.8 μg/ml. The large supply of Zn^{2+} in the hagfish blood might reduce the necessity for the active site of hagfish carbonic anhydrase to form a strong metal ion complex. For higher vertebrates, one could speculate that an active site capable of stronger coordination of Zn^{2+} has evolved in order to compensate for the decreased availability of this metal ion in the blood.

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