Elasmobranch Carbonic Anhydrase

PURIFICATION AND PROPERTIES OF THE ENZYME FROM TWO SPECIES OF SHARK*

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SUMMARY

Homogeneous carbonic anhydrase has been prepared from erythrocytes of the bull and tiger shark. Molecular and equivalence weight determinations indicate molecular weights in the range of 36,000 to 40,000 depending on the method employed. Amino acid compositions are similar to those reported for mammalian carbonic anhydrases with several notable exceptions. The bull and tiger shark enzymes contain ~25 and 18 half-cystine residues per molecule, respectively. These residues are apparently present as disulfide cross-links, since neither the native nor denatured molecules react with sulfhydryl reagents, but do react after reduction with mercaptoethanol. There are high contents of glutamic and aspartic acid compatible with the very acidic nature of these proteins. The isoelectric point of the bull shark enzyme is ~pH 4.5. The shark enzymes catalyze both the hydration of CO₂ and the hydrolysis of certain aromatic esters. Maximal catalytic rates are comparable to the high activity forms of mammalian carbonic anhydrases. Aromatic sulfonamides are powerful inhibitors. Circular dichroic spectra in the ultraviolet region indicate the presence of many structural features similar to those found in the mammalian enzymes.

The shark enzymes contain 1 g atom of zinc per mole of enzyme. A stable metal-free, inactive apoenzyme can be prepared which is completely reactivated by Zn(IΙ) or Co(IΙ) ions. The Co(IΙ) derivatives have intense visible absorption maxima at 510, 555, 618, and 645 nm. These spectra, as well as the spectra of sulfonamide complexes of the Co(IΙ) enzymes, are almost identical with those of the Co(IΙ) derivatives of mammalian carbonic anhydrases, and suggest that similar coordination geometries exist at the active centers of the elasmobranch and mammalian enzymes. Since fossil morphological evidence suggests that present day sharks species have not evolved much since the Devonian period, this particular type of metal complex would appear to have been a constant feature of carbonic anhydrase over 400 million years of animal evolution.

Carbonic anhydrases isolated from mammalian erythrocytes have a number of similar structural features. The enzyme isolated in homogeneous form from human (1, 2), bovine (3), monkey (4), horse (5), and dog (6) erythrocytes has a molecular weight near 30,000 and contains 1 Zn(IΙ) ion per molecule, the latter an absolute requirement for activity. All of these mammalian carbonic anhydrases are specifically inhibited by aromatic sulfonamides, as well as a number of metal binding anions. Mammalian carbonic anhydrases catalyze the reversible hydration of carbon dioxide (7), the hydration of aldehydes (8, 9), and the hydrolysis of several aromatic esters (10, 11). The amino acid compositions of these carbonic anhydrases show numerous similarities (12). There are no disulfide bridges in most mammalian carbonic anhydrases investigated since they contain either no cysteine or only 1 residue per molecule. The only exception appears to be the horse B isozyme which has 2 cysteine residues (5).

The ultraviolet optical rotatory dispersion and circular dichroic spectra of mammalian carbonic anhydrases show optically active absorption bands in the region of the near ultraviolet aromatic chromophores, apparently reflecting clusters of aromatic residues in highly specific arrays (13). In addition, the far ultraviolet optical rotatory dispersion and CD spectra exhibit features unique to carbonic anhydrases (14–16), and the spectra are not easily fitted by any combination of the optical rotatory dispersion or CD spectra observed for the common conformations of synthetic polypeptide models (17).

The Zn(IΙ) of native mammalian carbonic anhydrases has been replaced by a number of other transition metal ions (18, 19). The Co(IΙ)-substituted carbonic anhydrases are the only non-zinc carbonic anhydrases possessing significant catalytic activity (18). The visible spectra of Co(IΙ) carbonic anhydrases are all quite similar, indicating a common metal coordination geometry.

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† The abbreviation used is: CD, circular dichroism.
It has been suggested that the metal coordination geometry is either a distorted tetrahedron or a distorted square-plane (14, 20-22). Several species exhibit isozymes differing in specific activity and amino acid composition (12). Particularly striking are the two isozymes isolated from primate erythrocytes including man and monkey (4).

On the basis of the physicochemical studies summarized above, it has been suggested that the mammalian erythrocyte carbonic anhydrase molecule evolved as a stable molecular structure before the divergence of the mammalian classes approximately 50 million years ago (23). Sharks evolved approximately 400 million years ago and fossil evidence suggests that sharks have undergone little evolutionary change since that time (24). A detailed physicochemical study of the carbonic anhydrase from this most primitive of large animals should provide further evidence on the evolution of carbonic anhydrase as well as reveal invariant chemical features required of the metalloprotein catalyzing CO₂ hydration. Detailed studies on the pure elasmobranch enzyme are also of physiological interest, since a number of kinetic and inhibition studies have been carried out on the carbonic anhydrase from the spiny dogfish (25-27). Carbonic anhydrases from elasmobranchs or other marine animals have not been extensively purified, although some properties of a partially purified shark carbonic anhydrase have been reported by Leiner, Beck, and Eckert (27).

Studies of a partially purified carbonic anhydrase from the squid (Sepia officinalis) have also been reported (28). The shark enzyme is the first carbonic anhydrase from a nonmammalian animal source to be obtained in homogeneous form. Preparations have been obtained from the erythrocytes of the bull shark (Carcharhinus leucas) and the tiger shark (Galeocerdo cuvieri).

**Experimental Procedure**

**Reagents**—All chemicals used in these studies were reagent grade. The metal salts used to prepare metal ion solutions or in the preparation of the Co(II) carbonic anhydrases were spectrographically pure (Johnson Matthey Company, Ltd., London). Metal-free HCl and NaOH solutions were prepared as previously described (29). Dialysis tubing (Arthur H. Thomas Company, Philadelphia, Pennsylvania) was initially prepared by heating to 60° and washing with metal-free water. Dialysis tubing was made metal-free by soaking in 10⁻¹ M, 1,10-phenanthroline. The DEAE-cellulose (Whatman DE-52) was prehydrated in 0.5 M HCl then 0.5 M NaOH prior to use. Hydroxyapatite (Bio Rad ITT) was recycled by suspension in deionized water to lower the phosphate concentration. Sephadex G-150 (Pharmacia) was prepared by swelling the dry gel in deionized water and equilibrated by passing several void volumes of buffer through the column. All resins were extensively “defined” before use and after each recycling.

For the studies on the Co(II)-substituted enzyme, all buffers were freed of contaminating metals by extraction with dithizone in CCl₄. Dithizone was prepared by the method of Kagi and Vallee (30). 2-Hydroxy-5-nitro-α-toluene sulfonic acid sulfate was prepared by the method of Kaiser and Lo (31).

**Enzymatic Activity**—All routine assays of enzymatic activity of shark carbonic anhydrase utilized the esterase activity of these enzymes using 2-hydroxy-5-nitro-α-toluene sulfonic acid as the substrate (11). The assay mixture contained 50 microliters of 4 × 10⁻² M 2-hydroxy-5-nitro-α-toluene sulfonic acid sulfate in acetonitrile, an aliquot of the carbonic anhydrase to be assayed and 0.025 M Tris-sulfate to a final volume of 2.0 ml. The final enzyme concentration for these assays was in the range of 10⁻⁷ M. The change in optical density at 352 nm was recorded with a Cary model 15 recording spectrophotometer. The pH-rate profile at 25° was determined in a series of 0.025 M Tris-phosphate buffers. The pH of these buffers was adjusted with either sulfuric acid or with concentrated solutions of NaOH. The assay mixtures were as described above.

Inhibition assays were run using p-nitrophenyl acetate as the substrate. In all cases the assay solution contained 100 μl of 2 × 10⁻² M p-nitrophenyl acetate in acetonitrile and the final assay volume was 2.0 ml. The buffer was 0.025 M Tris-sulfate pH 7.5 with a final enzyme concentration in the range of 10⁻⁴ M.

Carbon dioxide hydration activity was determined by a stopped flow method as previously described (32, 33). The assays were run at pH 7.5 in 0.025 M sodium phosphate, 25°. The CO₂ concentration in the reaction mixture was either 7.25 mM or 4.35 mM. The enzyme concentration was approximately 3 × 10⁻⁵ M in the reaction mixture and was diluted just prior to use from a 10⁻⁴ M stock solution. p-Nitrophenol was used as the indicator (32, 33).

**Column Assays**—In order to follow the purification of the enzymes, an arbitrary measurement of activity was adopted. One such unit is equal to the number of nanomoles of sulfone hydrolyzed per ml of standard assay solution per min by a 0.25-ml aliquot of protein solution. These units are plotted on the right-hand ordinates of the column figures and can be converted to relative specific activity by dividing by the optical density at 280 nm.

**Preparation of Metal-free Shark Carbonic Anhydrase**—All glassware used in the preparation of the metal-free shark carbonic anhydrases was either soaked in 1:1 concentrated H₂SO₄:HNO₃ or was soaked in 10⁻² M 1,10-phenanthroline in 10⁻² M sodium acetate, pH 5.0, and was then thoroughly rinsed in deionized water made metal-free by passage over a mixed bed resin column. The Zn(II) was removed from the native enzyme by dialysis of the native enzyme against 10⁻³ M 1,10-phenanthroline, 10⁻³ M sodium acetate at either pH 5.0 or pH 5.0, 4°. At pH 5.0 the tiger shark enzyme precipitates. After three changes of the 1,10-phenanthroline solution, each lasting 48 hours, the enzyme was dialyzed against three changes of metal-free 0.05 M Tris-chloride, 0.1 M NaCl. The tiger shark enzyme nearly completely redissolves and is found to contain approximately 4% of the native Zn(II) concentration. The Zn(II) of the native bull shark enzyme is more tightly bound and is only about 60% removed by dialysis with a Cary model 15 recording spectrophotometer.

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packed cells in an equal volume of cold distilled water. 

Ultracentrifugation—The ultracentrifuge studies on the shark carbonic anhydrases were run on a Beckman model E ultracentrifuge equipped with ultraviolet scanner optics. The velocity run using tiger shark carbonic anhydrase was performed on a sample containing 0.4 mg of protein per ml with a rotor speed of 56,300 rpm, 23.4°. The observed S value was corrected to S20,0 using correction factors given by Swedberg and Pederson (34). Molecular weight determinations by the method of Yphantis were performed for both the bull and tiger shark carbonic anhydrases as previously described (39). The rotor speed was 26,100 rpm. The partial specific volume used to calculate the molecular weight was calculated from the amino acid composition to be 0.736 ml g⁻¹.

Acrylamide Gel Electrophoresis—Regular analytical gels were 7.5% acrylamide polymerized with ammonium persulfate. The procedure has been previously described (36). Protein bands were visualized by staining with Coomassie brilliant blue.

Zinc Determination—All Zn(II) determinations were carried out by atomic absorption analysis using a Jorrell Ash (Waltham, Massachusetts) spectrometer equipped with a recorder. Samples in the range of 1 to 10 µmol Zn(II) were analyzed by the “spike-height” method (37).

Circular Dichroism—This was measured with a Cary 60 spectropolarimeter equipped with a circular dichroism attachment. Circular dichroism is expressed as molecular ellipticity, (θ) = 2.303 (4500/π) (ε220 − ε208). Molecular ellipticity has been expressed throughout on the basis of the moles of protein present.

RESULTS AND DISCUSSION

Specimen Collection—The sharks were caught on lines 2 to 5 miles out in the Gulf of Mexico off the southwest coast of Florida. Sharks were from 6 to 12 feet in length and weighed between 200, and 700 pounds. Blood was obtained from freshly caught sharks by dissection and direct removal of blood from the sinus venosus. Several physiological characteristics differentiate the environment of the shark erythrocyte from that of the mammalian enzyme is chromatography on Sephadex G-75 (12). Mammalian carbonic anhydrase elutes after hemoglobin. Chromatography on Sephadex G75 of bull shark hemolysate indicated that the carbonic anhydrase-active material eluted slightly before the hemoglobin, so separation on Sephadex G-100 was attempted. The results are shown in Fig. 1. The fractions showing carbonic anhydrase activity have an apparent molecular weight greater than that of hemoglobin. Thus the shark enzyme either aggregates under these conditions or is associated with other high molecular weight components of the hemolysate (see below). The observed specific activity of the enzyme purified by this method was quite low, so the method was not employed further.

Purification—Several methods were initially investigated in an attempt to purify the shark carbonic anhydrase from the crude hemolysate. One mild method which has been employed for the mammalian enzyme is chromatography on Sephadex G-75 (12). Mammalian carbonic anhydrase elutes after hemoglobin. Chromatography on Sephadex G75 of bull shark hemolysate indicated that the carbonic anhydrase-active material eluted slightly before the hemoglobin, so separation on Sephadex G-100 was attempted. The results are shown in Fig. 1. The fractions showing carbonic anhydrase activity have an apparent molecular weight greater than that of hemoglobin. Thus the shark enzyme either aggregates under these conditions or is associated with other high molecular weight components of the hemolysate (see below). The observed specific activity of the enzyme purified by this method was quite low, so the method was not employed further. Initial purification by ammonium sulfate fractionation also proved to be unsuccessful since other components of the mixture, including the hemoglobin, precipitated at the same ammonium sulfate concentration as the carbonic anhydrase and little purification was obtained.

The purification method which proved most useful was an initial precipitation of nonecarboxic anhydrase components by addition of cold chloroform and ethanol (43). Carbonic anhydrase from this treatment could be dialyzed and lyophilized. Best results were obtained if the enzymes were not dialyzed for extended periods of time. In at least one species of shark, however, the hammerhead, we found this procedure to result in complete inactivation of the enzyme.

The lyophilized shark carbonic anhydrase was then chromatographed on a DEAE-cellulose column using a linear 0 to 0.7 M NaCl gradient in 0.05 M Tris-chloride, pH 8.8 (Fig. 2A). Fractions containing carbonic anhydrase activity were then dialyzed and introduced on to a hydroxypapitate column equilibrated in 0.004 M potassium phosphate, pH 7.0. The enzyme was eluted with a linear 0.004 to 0.4 M potassium phosphate gradient (Fig. 2B). The material from this column was dialyzed and lyophilized. The final purification step was chromatography on Sephadex G-150 (Fig. 2C). The latter step results in a nearly symmetrical peak containing carbonic anhydrase of constant
specific activity across the peak. As can be seen in all of the purification steps, large amounts of noncarbonic anhydrase material which absorbs at 280 nm are present. Since shark erythrocytes contain nuclei there is a full complement of cellular proteins which complicates the purification procedure considerably. The purification procedure employed for the carbonic anhydrases studied in this work is summarized in Table 1.

Gel Electrophoresis—The progress of the purification was monitored by gel electrophoresis on the active fractions after each procedure. Representative results on both the bull and tiger shark enzyme are pictured by the densitometer tracings of the gels in Fig. 3. The gels from intermediate column steps demonstrate the diversity of other proteins in addition to carbonic anhydrase and hemoglobin present in the shark erythrocyte. The final step yields nearly homogeneous enzyme, with one or two very minor components in addition to a large symmetrical band. We have found no evidence for electrophoretically distinct isozymes. The very small amount of protein in the secondary peaks may represent denatured enzyme. This interpretation is supported by the gel electrophoresis of the enzymes following Sephadex G-150 chromatography (Figure 3, E and H) which always showed more of these minor peaks than the enzyme from the hydroxylapatite column (Fig. 3D), the immediately preceding step. The final gels are representative of the preparations upon which the physicochemical studies described here were performed.

Electrostatic Focusing—During the initial purification attempts the bull shark enzyme was observed to adsorb so tightly to DEAE-Sephadex A-50 that it was almost impossible to elute at alkaline pH even with high salt. This behavior suggested that the protein was highly negatively charged, which was confirmed by the electrophoretic focusing of the chloroform-ethanol supernatant of bull shark hemolysate. The bull shark enzyme migrates to a position corresponding to pH 4.5 (Fig. 4A). The large number of other proteins present in the shark erythrocyte are evident in the electrostatic focusing of the impure material.

Highly purified bull shark enzyme also focuses between pH 4.2 and 4.5 under the same electrostatic focusing procedure (Fig. 4B). Direct assay of fractions across the peak showed a specific activity of ~2500 moles of sultone hydrolyzed per min per mole of enzyme at pH 7.5. This is about half of the normal specific activity of the enzyme (Table 1), and undoubtedly reflects the extremely low pH of the fractions before assay. Dialysis of the fractions against pH 7.5 buffer containing 10−6 M zinc before assay, however, restored the specific activity to 90% of the original enzyme. Thus irreversible denaturation had not occurred. The apparent isoelectric point near pH 4.5 of the shark enzyme is lower than any reported for mammalian carbonic anhydrases and is compatible with the relatively high contents of aspartic and glutamic acid found in the shark enzymes (see below) if it is assumed that a large number of these residues are in the acid rather than the amide form.

Molecular Weight and Zinc Content—The molecular weight and equivalence weight for both the tiger and bull shark enzymes have been investigated by several methods. The decrease in enzymatic activity upon titration of the enzyme with ethoxzolamide was first used by Marek, Parcell, and Malik (44) to determine the equivalence weight of enzyme present. The low dissociation constant for this inhibitor leads to a very sharp end point. Using the hydrolysis of p-nitrophenyl acetate as the assay, ethoxzolamide titration gave an equivalence weight of 42,500 for the bull shark enzyme and 41,200 for the tiger shark enzyme, as-
TABLE I

Summary of purification of shark carbonic anhydrase

| Purification step | Total protein Bull | Volume Bull | Total activity Bull | Yield* Bull | Specific activity Bull |
|-------------------|-------------------|------------|---------------------|-------------|----------------------|
| Hemolysate        | ~57,000           | 1,000      | 30,000              | 42          | 20                   |
| CHCl3-C2H5OH precipitation (determined on dissolved protein after dialysis and lyophilization) | 500 | 85 | 12,500 | 88 | 2,500 |
| DEAE-cellulose chromatography (determined on pooled fractions) | 171 | 510 | 11,000 | 88 | 2,500 |
| Hydroxyapatite chromatography (determined on pooled fractions) | 144 | 400 | 10,800 | 96 | 2,700 |
| Sephadex G-150 chromatography (determined on pooled fractions) | 62 | 225 | 7,900 | 73 | 4,600 |

* Yield values refer to the individual purification step and not to the over-all yield which was 26% in the case of the bull shark enzyme.

Substrate was 5-nitro-2-hydroxy-α-toluene sulfonic acid sultone in 0.025 M Tris-sulfate, 4% acetonitrile, pH 7.5, 25°C. The amount of enzyme present was calculated using the extinction coefficient for the purified enzyme.

Fig. 3. Densitometer traces of stained acrylamide gels of shark carbonic anhydrase after different stages of purification. A, crude bull shark hemolysate; B, after chloroform-ethanol treatment of bull shark hemolysate; C, after DEAE-cellulose chromatography of the chloroform-ethanol-treated bull shark hemolysate; D, after hydroxyapatite chromatography of bull shark carbonic anhydrase from Step C; E, after Sephadex G-150 chromatography of the carbonic anhydrase from Step D; F, after chloroform-ethanol treatment of tiger shark hemolysate; G, after DEAE-cellulose chromatography of tiger shark carbonic anhydrase from

37,700 for the tiger shark enzyme and 35,900 for the bull shark enzyme. The weight average molecular weights calculated by averaging all the Yphantis determinations are 39,500 for the tiger shark enzyme and 36,000 for the bull shark enzyme.

The zinc content as measured by atomic absorption shows 1 g atom of zinc per 39,500 g of protein and 1 g atom per 34,500 g of protein for the tiger and bull shark enzymes, respectively.

Although the various means of determining equivalence weight are based on assumptions of stoichiometry and show at least ±15% variation, these values together with the determination of molecular weights by equilibrium centrifugation indicate a significantly larger polypeptide chain than in the case of the mammalian enzymes. This conclusion is also supported by sedimentation velocity centrifugation of the tiger shark enzyme. At a protein concentration of 0.4 mg per ml, s20,w value was 3.34 compared with values of 2.8 to 2.9 for the mammalian enzymes at comparable protein concentrations.

Amino Acid Composition—The amino acid compositions of the two shark enzymes are given in Table II. For purposes of comparison, the amino acid compositions of the bovine enzyme, a typical mammalian carbonic anhydrase, and parsley carbonic anhydrase are also given in Table II. The molecular weight used to calculate the number of residues was 36,000 for the bull shark enzyme and 39,500 for the tiger shark enzyme. The most striking difference between the shark and mammalian carbonic anhydrases is the high content of cysteine. The parsley enzyme, on the other hand, does have 7 half-cystine residues per molecule. In the shark enzymes these cysteine residues all appear to be present in disulfide linkage since the enzyme in both the native and denatured state does not react with sulfhydryl reagents. 5,5'-Dithiobis(2-nitrobenzoic acid) gives an increased absorption at 412 nm when it reacts with cysteine sulfhydryl groups (48). This reagent gave no increased absorption when added to either the bull or tiger shark enzyme in the presence or absence of 6 M guanidine hydrochloride. p-Chloromercuribenzoate titration of the chloroform-ethanol supernatant; H, Sephadex G-150 chromatography of purified tiger shark carbonic anhydrase previously purified by chloroform-ethanol precipitation, DEAE-cellulose chromatography, and hydroxyapatite chromatography.
Fig. 4. A, electrostatic focusing of the chloroform-ethanol supernatant from the bull shark hemolysate. The shaded area represents carbonic anhydrase activity as described under "Experimental Procedure"; (-) absorbance at 280 nm. An LKB column using Ampholite pH gradient buffers was employed. B, electrostatic focusing of homogeneous bull shark enzyme. Conditions as in A. ---, pH; ---, absorbance at 280 nm; shaded peak height equals micromoles of sultone hydrolyzed per min by the 2-ml fractions.

the shark enzymes in the presence or absence of guanidine hydrochloride also failed to show any increase in absorption at 250 nm (49). Native shark carbonic anhydrase does not label with 3H-iodoacetate. Following treatment with mercaptoethanol, however, both shark enzymes reacted with 3H-iodoacetate and the number of residues reacted was approximately equal to their cysteine content. Although the native enzymes do not react with iodoacetate, prior reduction with mercaptoethanol, followed by alkylation with iodoacetate results in the appearance of large amounts of carboxymethylcysteine upon amino acid analysis. These findings suggest that these primitive animal carbonic anhydrases have a number of disulfide cross-links as part of their structure, a feature that has been lost as the enzyme evolved in higher animals.

Absorption Spectra and Optical Activity—Both the bull and tiger shark enzymes have near ultraviolet absorption spectra typical of proteins. For tiger shark carbonic anhydrase $E_{280}^m = 16.0$ ($E_{280} = 6.3 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ for a molecular weight of 39,500). Calculation of the molar extinction coefficient of the tiger shark enzyme at 280 nm from the observed contents of tyrosine and tryptophan is in good agreement with the observed value, a moderate hyperchromia being observed in the native state. For bull shark carbonic anhydrase $E_{280}^m = 20.9$ ($E_{280} = 7.5 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$ for a molecular weight of 36,000). The bull shark enzyme, however, apparently shows an unusually large hyperchromia of its tyrosine and tryptophan chromophores, since the calculated extinction coefficient ($4.2 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$) falls considerably short of the value ($7.5 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$) observed in the native enzyme.

Circular dichroic spectra of the bull and tiger shark enzymes show multiple large near ultraviolet ellipticity bands between 295 and 240 nm (Fig. 6). The general contours of the CD spectra in this region, a large minimum near 270 nm with small bands superimposed on the long wavelength side, are similar to the near ultraviolet CD spectra of mammalian carbonic anhydrases. Thus the particular environment of the aromatic residues which induces these large Cotton effects must be a particularly ancient feature of the structure of carbonic anhydrase. Direct evidence relating a particular aromatic structure or structures to the function of carbonic anhydrase is not available yet, but appearance of tryptophan phosphorescence in bovine carbonic anhydrase when a bound sulfonamide chromophore is excited by light of an energy too low to excite tryptophan phosphorescence from the protein alone suggests the presence of a tryptophanyl residue near the active site cavity (50). Clusters of aromatic residues have also been observed in the structure of human carbonic...
The mass of the extinct mammalian carbonic anhydrases, relative hypochromia of the tiger shark protein in the region around 200 nm allowed the recording of the circular dichroism down to 190 nm revealing a positive ellipticity band located at 195 nm. Although the presence of a negative band near 220 nm suggests the presence of some a helix, the shift in the positive band from 190 nm to 195 nm and the relatively low magnitude of this band in comparison to the 220 nm trough suggest a relatively high content of b structure (17). The x-ray structure of human carbonic anhydrase C at 2 A resolution shows b-pleated sheet to be the predominant feature of the secondary structure in the primate enzyme, a feature that may also be present in the elasmobranch enzyme.

Enzymatic Activity of Shark Carbonic Anhydrases—Like mammalian carbonic anhydrases, the shark enzymes catalyze both the reversible hydration of CO₂ and the hydrolysis of certain aromatic esters. This is in contrast to the plant enzymes which do not appear to hydrolyze esters (47). Turnover numbers for the hydration of CO₂ as measured by a stopped flow method (7) at two CO₂ concentrations are given in Table III. The magnitude of the specific activity for both shark enzymes is similar to that observed for human carbonic anhydrase C and other high activity forms of the mammalian enzyme. These numbers compare favorably in order of magnitude with the values for the specific activity of the dogfish enzyme reported by Maren and Wiley (25). From the limited amount of data available from the stopped flow measurements, only a rough estimate of Kₘ and Vₘₐₓ can be made. Kₘ for CO₂ is approximately 100 μm, while Vₘₐₓ is in the range of 10⁶ moles of CO₂ per mole of enzyme per sec.

**Table II**

| Amino acid residuе | Tiger shark | Bull shark | Bovine B (40)* | Parsley (41)* |
|-------------------|-------------|------------|----------------|---------------|
| Lysine            | 29          | 26         | 19             | 21            |
| Histidine         | 14          | 9          | 11             | 5             |
| Arginine          | 13          | 10         | 9              | 5             |
| Aspartic acid     | 40          | 33         | 32             | 24            |
| Threonine         | 13          | 11         | 15             | 10            |
| Serine            | 31          | 29         | 10             | 18            |
| Glutamic acid     | 40          | 41         | 24             | 21            |
| Proline           | 21          | 24         | 20             | 22            |
| Glycine           | 32          | 30         | 20             | 22            |
| Alanine           | 23          | 19         | 17             | 17            |
| Half-cystine      | 18 (11)     | 25 (18)    | 0              | 7             |
| Valine            | 13          | 10         | 20             | 24            |
| Methionine        | 3           | 2          | 3              | 4             |
| Isoleucine        | 16          | 12         | 5              | 8             |
| Leucine           | 34          | 32         | 26             | 21            |
| Tyrosine          | 9           | 8          | 8              | 8             |
| Phenylalanine     | 18          | 12         | 11             | 17            |
| Tryptophan        | 7           | 5          | 7              | 3             |
| Zinc              | 1           | 1          | 1              | 1             |
| Total residues    | 374         | 338        | 264            | 257           |

* Number in parenthesis is reference number.

† Number given is the value from performic acid oxidation.

The value in parentheses is the average cysteine value obtained from the standard amino acid analysis on the oxidized protein.

* Value given was determined by the Edelhoch (41) spectrophotometric method. The same value was observed for tyrosine determined spectrophotometrically and by standard amino acid analysis.

## Fig. 6. Ultraviolet circular dichroic spectra for native shark carbonic anhydrases in 0.025 M Tris-sulfate, pH 7.5: ----, tiger shark; ----, bull shark.
Table III

Enzymatic activity of shark carbonic anhydrases

| Enzyme     | CO₂ hydration² | Esterase activity |
|------------|----------------|-------------------|
|            | [CO₂]          | \(v_{\text{act}}/E\) | p-Nitrophenylacetate² | Sulfone² |
|            | mM             | sec⁻¹              | moles substrate/mole enzyme/min |
| Tiger shark| 7.25           | 1.4 x 10⁴          | 70                         | 9.8 x 10³ |
|            | 4.35           | 0.89 x 10⁴         | 70                         | 5 x 10³   |
| Bull shark | 7.25           | 1.14 x 10⁴         | 70                         | 5 x 10³   |
|            | 4.35           | 0.45 x 10⁴         | 70                         | 5 x 10³   |

² Values were determined by the stopped flow assay described under "Experimental Procedure."

The conditions were 10⁻⁴ M p-nitrophenylacetate, 0.025 M Tris-SO₄, 2.5% acetonitrile, pH 7.5, 25°.

The conditions were 10⁻⁴ M sulfone, 0.025 M Tris-SO₄, 2.5% acetonitrile, pH 7.5, 25°.

Activity data for the hydrolysis of two aromatic esters, p-nitrophenylacetate and 2-hydroxy-5-nitro-α-toluenesulfonic acid sulfone are given in Table III at a fixed substrate concentration and pH value. The specific activities for the esterase reaction are similar to those reported for mammalian carbonic anhydrases (11, 45, 94). Complete pH-rate profiles for the two shark enzymes catalyzing the hydrolysis of the sulfone are shown in Fig. 7 and are compared with a detailed pH rate profile for human carbonic anhydrase B catalyzing the same reaction. All three pH rate profiles can be adequately fit by a simple sigmoid curve. While the maximum specific activity observed is similar to that of the primates enzyme, the pKₐ for the two shark enzymes, pH 6.6, is lower than the pKₐ values reported thus far for any mammalian carbonic anhydrase catalyzing ester hydrolysis. The pH of elasmobranch plasma is reported to average 7.6, similar to the pH of human plasma (55). While it is not certain that this represents the pH of the immediate environment of the enzyme, a lower pKₐ for carbonic anhydrase would favor the hydration reaction over the dehydration reaction, since the pH rate profiles for the forward and reverse reactions are inverted, hydration being favored at pH values above the pKₐ. A physiological advantage of the low pKₐ is not immediately apparent, but one striking feature of the CO₂ = HCO₃⁻ equilibrium in elasmobranchs suggests a possible advantage. Aquatic gas exchange results in a \(p_{\text{CO₂}}\) of ~4.6 mm Hg in elasmobranch venous blood or about 10% of that of mammalian venous blood (55). HCO₃⁻ concentration is ~5 meq per liter or about 20% of that of man (55). Thus the relatively lower \(p_{\text{CO₂}}\) favors the dehydration reaction over hydration, other factors being equal. The lower pKₐ for the pH rate profile of the elasmobranch enzyme would tend to compensate for this by favoring the catalysis of hydration relative to the mammalian enzyme at pH 7.6. Thus an organism operating in an environment where the \(p_{\text{CO₂}}\) favors free CO₂ loss might gain some advantage in rapid \(\Pi^+\) adjustment with an enzyme favoring hydration. Calculations for the many components of this equilibrium, however, are difficult and precise physiological conclusions are hard to draw (56).

Removal of Zn(II) from the shark enzymes results in loss of enzymatic activity. The best preparations of the apoenzymes showed between 3 and 7% of the original zinc and had a specific esterase activity corresponding to the residual zinc. A complete titration of the bull shark apoenzyme with Zn(II) showed restoration of esterase activity in direct proportion to the amount of Zn(II) added. Although the end points for these titrations were somewhat variable, full activity was always restored after the addition of 1 to 1.5 g atoms of Zn(II) per mole of protein.

Like other animal carbonic anhydrases, the shark enzymes are inhibited by sulfonamides and metal-binding anions. \(K_i\) values of \(5 \times 10^{-7}\) M for acetazolamide, \(10^{-4}\) M for cyanide, and 0.4 M for acetate were calculated from inhibition data on both the bull and tiger shark enzymes. Acetazolamide appears to be a somewhat weaker inhibitor of the shark carbonic anhydrases than for bovine carbonic anhydrase where a \(K_i\) value of \(1.3 \times 10^{-1}\) M has been reported (57).

Visible Absorption and Circular Dichroism of Shark Co(II) Carbonic Anhydrases—One of the most sensitive and highly specific probes of the active center of carbonic anhydrases has been the Co(II) ion. Substitution of Co(II) for the native Zn(II) ion results in an enzyme that is active and possesses moderately intense \(d-d\) transitions in the region 430 to 700 nm. The energies and intensity of the latter are extremely sensitive to the geometry of the active site ligands as well as to the introduction of additional ligands such as the anions or sulfonamides at the active center. Both Co(II) shark enzymes show visible absorption maxima at 510 nm, 555 nm, 618 nm, and 645 nm (Fig. 8). These maxima are within ±2 nm of the band positions reported for the corresponding bands of Co(II) mammalian carbonic anhydrases. Thus the coordination geometry of the metal-binding site in the shark enzymes must be nearly identical with that in higher animals. This suggests that this particular geometry is an invariant feature of carbonic anhydrase and necessary for enzymatic activity. The widely split \(d-d\) transitions suggest that the complex is highly distorted. The observed intensities of the transitions, \(e = 300 \text{ to } 400 \text{ m}^{-1} \text{ cm}^{-1}\) and magnetic susceptibility...
data (21) have led to suggestions that a distorted tetrahedral geometry is present in the native enzyme. Recent electron spin resonance studies of the Co(II) and Cu(II) enzymes show that there appears to be some flexibility in the ligand geometry and that the enzymatically active geometry may be considered as a distortion of a complex that under a number of circumstances assumes axial symmetry (22). The changes in the visible absorption spectrum of the Co(II) shark carbonic anhydrases induced by acetazolamide (Fig. 8) are also almost identical with those observed in mammalian Co(II) carbonic anhydrases (20, 58), implying that the same change in geometry is associated with the probable coordination of the sulfonamide to the metal ion.

It has generally been assumed in studies of small coordination complexes that the signs and the magnitude of the ellipticity associated with the d-d absorption bands is related in a rather direct way to the stereochemistry of the coordination polyhedron, although a completely satisfactory theory is not available and some disagreement exists on details. In the case of Co(II) carbonic anhydrase, an examination of the Co(II) derivatives of a number of species and isozyme variants has shown that both the magnitude and sign of the ellipticity bands associated with the Co(II) d-d transitions vary enormously, even though the energy levels of these d-d transitions are nearly identical in all variants. This suggests that the observed circular dichroism may be more a product of the adjacent asymmetric protein environment than of the absolute geometry or chirality of the central metal complex.

The CD spectra of the Co(II) shark enzymes (Fig. 9) contribute further to the conclusions discussed above, since the bands of the visible CD spectra for both the native Co(II) enzyme and the sulfonamide complexes are different in magnitude and sign from any described previously (58), although the band positions are similar. Maximum Δε values above 400 nm are less than ±1, considerably smaller than shown by the bovine or human C Co(II) enzymes, but larger than those shown by the human B enzyme. On the other hand, the appearance of a striking negative ellipticity band at 600 nm upon combination of these enzymes with a sulfonamide (Fig. 9) is unlike the changes observed in other Co(II) carbonic anhydrase isozymes. The presence of dissymmetrical charged or dipolar protein structure surrounding the Co(II) chromophore may be the prominent structural feature controlling the rotatory strength of the d-d transitions. As has been shown in the mammalian enzymes, the CD spectra reveal that there are absorption bands between 300 and 400 nm associated with the Co(II) complexes.

These physicochemical studies of the shark enzymes reveal a coordination geometry around the active site that is in many ways similar if not identical with the coordination complex at the active site of primate carbonic anhydrases. The likely explanation for this would appear to be that the particular features of protein structure determining the coordination geometry have undergone little change over 400 million years of animal evolution. Although fossil evidence indicates some change in shark morphology since the Devonian period, sharks as a group seem...
to have been subject to relatively few pressures of natural selection in the last 400 million years. In the absence of such pressure it is likely that the carboxy anhydrase molecule has also undergone little evolutionary change in the shark. Hence it seems more likely that the similarities in the elasmobranch and primate enzymes are due to the sharing of a common ancestral gene rather than convergent evolution from rather different molecules. Supporting evidence would have to come from statistical analyses of the primary amino acid sequences (59).

The presence of certain aromatic residues in a highly symmetrical environment inducing large optical activity in their chromophores also appears to be a constant feature of carboxy anhydrase molecules, although the direct relation of this structure to activity is unclear. Other features, such as the length of the peptide chain, the presence of disulfide bridges, and certain features of secondary structure have, however, undergone considerable change in higher animals with shortening of the polypeptide chain and loss of the disulfide crosslinks.

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