Purification of a carbonic anhydrase from the inner ear of the guinea pig
(cochlea/spiral ligament/stria vascularis/acetazolamide/endolymph)

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ABSTRACT A soluble carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) has been purified to homogeneity from the membranous lateral wall (stria vascularis, spiral ligament, spiral prominence, and outer sude) of the guinea-pig inner ear. About 1% of the protein of the membranous lateral wall is carbonic anhydrase. The specific activity of the enzyme in homogenates of the lateral wall is 1.6-1.5 times that of whole blood; for homogenates of the components, stria vascularis and the fraction containing the spiral ligament, the specific activities are 0.9 and 2.6 times the specific activity of whole blood, respectively. No other cochlear fraction contains appreciable carbonic anhydrase. The purified enzyme has a molecular weight of about 30,000, a specific activity 60-80% that of carbonic anhydrase C from blood, and an electrophoretic mobility similar to that of the blood enzyme. Cochlear carbonic anhydrase is half-maximally inhibited by \(4 \times 10^{-9} \) M acetazolamide, is completely inhibited above \(10^{-5} \) M acetazolamide, and forms a fluorescent complex with 5-dimethylaminonaphthalene-1-sulfonamide, by which it can be distinguished on polyacrylamide gels. This report describes both another isolation of a carbonic anhydrase from a source other than blood and the isolation of an inner-ear enzyme.

Carbonic anhydrases (carbonate hydro-lyases, EC 4.2.1.1) catalyze the hydration of carbon dioxide to form carbonic acid, which dissociates to bicarbonate and hydrogen ions. Carbonic anhydrases in erythrocytes speed the hydration of CO\(_2\) from respiring tissues and catalyze the dehydration of H\(_2\)CO\(_3\) in the lungs where CO\(_2\) is exhaled (1). The enzymes also furnish H\(^+\) or HCO\(_3^-\) in ion-transporting systems, such as mammalian kidney (2), bird oviduct (3), and other ion-transporting organs (4). Erulkar and Maren first reported carbonic anhydrase activity in the cochlea of the cat (5). Because cochlear endolymph contains high K\(^+\) and low Na\(^+\) concentrations (6), an ion-transporting system has been implicated in the cochlear membranous lateral wall involving adenosine triphosphatase (7) and carbonic anhydrase (5, 8). Although the physiological significance of the high concentration of endolymphatic K\(^+\) is still unclear (9), the high K\(^+\) is likely to be necessary for normal functioning of the mammalian inner ear. As a first step in investigations of the molecular properties and possible role of carbonic anhydrase in the cochlea, I have studied the distribution of the activity of this enzyme in cochlear fractions of the guinea pig and purified a major soluble form of carbonic anhydrase from cochlear membranous lateral wall.

MATeRIALS AND METHODS

All chemicals used in these studies were of reagent grade or better. 5-Dimethylaminonaphthalene-1-sulfonamide (DNSA) was from K & K Fine Chemicals, Plainview, N.Y., and the chromatographic resins were from Bio-Rad, Richmond, Calif.

The animals were normal, colored guinea pigs of both sexes weighing 160-320 g. Animals were decapitated and their blood was collected in containers on ice. Blood from each animal was diluted \(1/500 \) or \(3/500\) in deionized water at 4°C. Temporal bones were dissected with surgical scissors and placed on ice within 1 min of death. Intact cochleas were then removed and immersed in a dissecting medium of 10 mM Tris-sulfate, 100 mM Na\(_2\)SO\(_4\), pH 7.4 at 1-2°C. Dissections were performed in the medium using a dissecting microscope. A sample of bony shell that covered the fourth turn was chipped away with a forceps and the remaining cochlear bone was removed, exposing the intact spiral of the membranous lateral wall, which was unwound (10). The basilar and tectorial membranes, the remaining cochlear epithelium, and a small amount of bone, comprising the "organ-of-Corti fraction," were scraped from the bony spiral with a finely pointed forceps. The auditory nerve was dissected from the modiolus to the internal auditory meatus. Separate experiments, the medulla oblongata was exposed and the left temporal bone was removed after the cranial nerves entering it were cut. Then the whole cochlear nucleus was dissected with a small spatula. All dissected tissues were rinsed in cold medium, placed in ground-glass homogenizers (0.2-ml nominal capacity; Kontes Glass Co., Vineland, N.J.), homogenized in 50 \(\mu\)l of deionized water for 15-30 min on ice, and diluted with water to the following volumes: 30 \(\mu\)l/sample of cochlear bone containing 16 \(\mu\)g of protein, 2 \(\mu\)l/cochlear nucleus, 300 \(\mu\)l/auditory nerve, 70 \(\mu\)l/organ-of-Corti fraction from one cochlea, and 220 \(\mu\)l/membranous lateral wall from one cochlea. Each fraction, except cochlear nucleus and whole blood, contained tissue samples from more than one animal. Each experiment (Table 1) consisted of the analysis of one fraction. The average number of animals used per fraction can be determined from Table 1.

Lyophilized tissue was used in experiments where the stria vascularis was separated from the spiral ligament fraction, because the use of fresh tissue caused loss of carbonic anhydrase into the medium when the fractions were separated. Fresh cochleas were dissected free of most of the cochlear shell, the soft tissues, and all of the modiolus, leaving a 6-mm strip of membranous lateral wall that adhered to the bony shell of parts of turns 1 and 2 (11). This section was located on the inner side of the cochlear wall about 6 mm through 12 mm from the round window, measured along the length of the membranous wall. These samples were rinsed once in distilled water, frozen on dry ice, and lyophilized for 24 hr. After lyophilization, for some samples the intact strip of membranous lateral wall was separated from the bone with a jeweler's forceps. For other samples, the pigmented stria vascularis was separated from the spiral ligament fraction before the latter was scraped away from the bone. The fractions were homogenized as described previously. For each 6-mm strip of tissue, volumes were: 90 \(\mu\)l/membra-
Biochemistry: Drescher

Table 1. Carbonic anhydrase activity of inner-ear fractions and other tissues

<table>
<thead>
<tr>
<th>Fresh tissue</th>
<th>Membranous tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cochlear bone</td>
</tr>
<tr>
<td>Total no. of animals</td>
<td>31</td>
</tr>
<tr>
<td>No. of experiments</td>
<td>6</td>
</tr>
<tr>
<td>Enzyme activity*</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>(27)</td>
<td>(100)</td>
</tr>
<tr>
<td>Total protein†</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>(30)</td>
<td>(100)</td>
</tr>
<tr>
<td>Specific activity (units/mg of protein)</td>
<td>0</td>
</tr>
<tr>
<td>Relative specific activity‡</td>
<td>0</td>
</tr>
</tbody>
</table>

Procedures are detailed in Materials and Methods. Each assay value is reported as mean ±SEM (n determinations).

* For cochlear bone, enzyme units/sample of apical bony wall containing 16 µg of protein; for cochlear nucleus, units/cochlear nucleus; for whole blood, units/µL; for other fresh tissue fractions, units/cochlea; for lyophilized tissue fractions, units/6-mm strip from cochlear turns 1–2 (see text).

† Expressed as µg of protein/each sample described in previous footnote.

‡ Relative to whole blood.

ous lateral wall, 60 µl/spiral ligament fraction, and 5 µl/stria vascularis.

Carbonic anhydrase activity was measured at 4°C by the method of Wilbur and Anderson (12) as adapted by Rickli et al. (13), scaled down for the present studies. One unit of activity is the amount of enzyme that increases the reaction rate 10% above that of the uncatalyzed reaction (13). Linear response of the assay was always established for each fraction. For inhibition studies, a solution of 0.1 M sodium acetazolamide, pH 9.2, was diluted serially in assay buffer to yield the desired concentrations of inhibitor. Uncatalyzed reaction rates varied little with inhibitor concentration. Protein in crude fractions was estimated by the method of Lowry et al. (14). Protein in purified fractions was determined by absorbance at 280 nm, with specific extinction coefficient, E280 cm⁻¹% = 17. Hemoglobin was estimated by absorbance at 410 nm.

Disc gel electrophoresis was performed by the procedure of Davis (15). Gels were stained as described by Crambach et al. (16), and photographed. Stained gels were equilibrated with 7% acetic acid, 40% ethylene glycol, and stored at -20°C. For electrophoresis of carbonic anhydrase–DNSA complex, 0.2 ml of homogenate of cochlear membranous lateral wall containing 1.3 mg of protein was mixed with 0.2 ml of 0.1 mM DNSA, 9 mM Tris-sulfate, pH 8.9, and incubated for 30 min at 25°C. A sample of 0.4-ml volume was applied to each gel and electrophoresed. Fluorescent bands were visualized during electrophoresis and after removal of gels using an ultraviolet lamp producing at least 160 µW/cm² radiation centered at 366 nm. The gels were not stained, but were equilibrated with saturated (NH₄)₂SO₄ and stored at 4°C, which preserved their fluorescence. Electrophoresis of protein standards and DNSA indicated that the DNSA combined specifically with carbonic anhydrases.

RESULTS

The membranous lateral wall of the inner ear is a rich source of carbonic anhydrase, with an average of 61 enzyme units/cochlea (Table 1). The organ-of-Corti fraction, auditory nerve, and cochlear bone are poor sources of the enzyme. As shown for lyophilized tissues, the large amount of enzyme in membranous lateral wall is due largely to the spiral ligament fraction, which has about 20 times as much carbonic anhydrase activity and about 10 times as much protein as the stria vascularis. The enzyme activity and protein of the membranous lateral wall are, within limits of error, the sums of the activity and protein, respectively, of the spiral ligament fraction and the stria vascularis. Lyophilization lowers slightly the specific carbonic anhydrase activity of the fresh membranous lateral wall (see Table 1), and has the same effect on other cochlear fractions. Such differences are on the order of 10%. The specific activities and specific activities relative to whole blood are also shown for each fraction (last two rows of Table 1). The specific activity of the membranous lateral wall is 1.6–1.8 times that of whole blood. The components of the wall, the spiral ligament fraction and the stria vascularis, are 2.0 and 0.9 times, respectively, as active per mg of protein as is blood. The organ-of-Corti fraction, auditory nerve, and cochlear nucleus all have specific activities considerably lower than that of blood, and bone has negligible relative specific activity.

The elution diagram for the initial purification of cochlear carbonic anhydrase is shown in Fig. 1. The majority of the enzyme activity was recovered at 1.6 void volumes (large dashed peak), an elution position corresponding to a molecular weight of about 30,000, as determined by gel filtration of the blood carbonic anhydrases with molecular weights near 30,000 (17). About 3% of the total carbonic anhydrase activity was also eluted in a small peak at 40 ml. The majority of inactive protein (54–60 ml, solid curve) was eluted before the large peak of carbonic anhydrase, resulting in a 7-fold purification of the enzyme at this step. Two peaks of absorbance at 410 nm (dotted curve) corresponded to yellowish turbidity at the front and to a small amount of hemoglobin at 1.3 void volumes. Very little additional protein or activity was eluted from 80 to 220 ml. Fig. 2 shows elution diagrams for the further purification of cochlear carbonic anhydrase by anion exchange and gel filtration. Chromatography of crude enzyme from the initial gel filtration (Fig. 1) on DEAE-agarose (Fig. 2, bottom) yielded a peak of activity at 26 ml, eluted by 10 mM Tris-sulfate, pH 8.9. Protein of this active peak was incompletely separated from protein of
Tissues were of membranous Na₂SO₄, pH and applied anhydrase (CA, stripped buffer sulfate eluted anhydrase carbonic hydrase from zyme in dilute solutions in Amicon stirred cell 10 mM Instrument Corp., Lexington, Mass.). was Cochlear material from ml/(cm² by shown rate, 10⁻¹² peak, 10⁻⁹ M. FIG. 1. Isolation of crude carbonic anhydrase from a homogenate of membranous lateral wall (60 cochleas) by gel filtration on Bio-Gel P-100 (100–200 mesh). All isolation steps were performed at 2–4°C. Tissues were homogenized, and the homogenate was brought to 1 ml and applied to the column. Bed dimensions were 2.6 x 32 cm and flow rate was 2.3 ml/(cm² h). Eluting buffer was 10 mM Tris-sulfate, 100 mM Na₂SO₄, pH 7.4. Enzyme units are defined in the text. The positions of elution of hemoglobin (Hb, small dotted peak) and carbonic anhydrase (CA, dashed peak) are shown.

The preceding inactive peak. Development with 50 mM Tris-sulfate eluted no detectable activity or protein. The column was stripped of remaining protein, which was inactive, by applying buffer of high ionic strength. Gel filtration of active material from the anion-exchange column resulted in a major peak of carbonic anhydrase of molecular weight of about 30,000 and a minor peak of active material of lower molecular weight, probably breakdown product (Fig. 2, inset).

The purification steps illustrated in Figs. 1 and 2 are outlined in Table 2. Specific activity increased regularly during the isolation, resulting in a 150-fold purification and 0.6% yield of carbonic anhydrase at the last step. Causes of enzyme loss during purification included inactivation of carbonic anhydrase in dilute solutions (18) and adsorption of the enzyme by the membranes during concentration. Larger amounts of the enzyme were obtained by again purifying cochlear carbonic anhydrase from homogenates by these procedures; by omitting monitoring steps of Table 2, 5–7 μg could be obtained. This quantity of enzyme was sufficient clearly to demonstrate homogeneity on polyacrylamide gels. The specific activity of purified cochlear enzyme ranged from 41,000 to 57,000 units/mg of protein. Therefore, about 1% of the protein of the membranous lateral wall is carbonic anhydrase (see Table 1).

Blood carbonic anhydrases C and B had specific activities of 70,000 and 11,000 units/mg, respectively. Stained polyacrylamide gels of blood carbonic anhydrases C and B, and of the purified cochlear enzyme, are shown in Fig. 3. The cochlear carbonic anhydrase had the same mobility as carbonic hydrase C from blood.

The interaction of the cochlear enzyme with each of two aromatic sulfonamides is shown in Fig. 4. Cochlear carbonic anhydrase, in crude or purified form, is inhibited by acetazolamide with half-maximal inhibition at 4 x 10⁻⁹ M (Fig. 4A). The enzyme activity approaches complete inhibition near 10⁻⁵ M acetazolamide. Fig. 4B shows a single fluorescent band after electrophoresis of a mixture of a homogenate of membranous lateral wall and DNSA. This band is one of about fifteen that are visualized by staining the gels with Coomassie blue (not illustrated). When blood lysate is electrophoresed with DNSA, two brightly fluorescing bands appear, corresponding to carbonic anhydrases C and B, while hemoglobin is not detectable by fluorescence (not illustrated). The fluorescent band in Fig. 4B, which appears to be a specific complex between the cochlear carbonic anhydrase and DNSA (19), has the same electrophoretic mobility as the fluorescent complex of DNSA and blood enzyme C. Control homogenates, lacking carbonic anhydrase activity, showed no fluorescent bands when electrophoresed with DNSA at comparable protein concentrations.

**DISCUSSION**

The data of this report indicate that there is a true cochlear carbonic anhydrase that is not derived from cochlear blood, that most of the enzyme is present in the spiral ligament fraction, and that the enzyme resembles carbonic anhydrase C from blood. Also, the cochlear carbonic anhydrase is inhibited by acetazolamide and binds the fluorescent sulfonamide, DNSA.

There are two lines of evidence that indicate the existence of a true cochlear carbonic anhydrase. One is that the specific carbonic anhydrase activities of the membranous lateral wall and the spiral ligament fraction are considerably higher than the specific activity of whole blood (Table 1). The specific activities of these cochlear fractions are also higher than the specific activity of erythrocytes, which contain the blood carbonic anhydrases (1). Red blood cells represent about 2% of the protein of whole blood, and therefore, by multiplying the values

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**Table 2. Purification of carbonic anhydrase from the cochlear membranous lateral wall**

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Enzyme activity (units)</th>
<th>Protein (μg)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>3978</td>
<td>7123</td>
<td>558</td>
</tr>
<tr>
<td>P-100 peak</td>
<td>1920</td>
<td>495</td>
<td>3,879</td>
</tr>
<tr>
<td>DEAE-agarose peak (Fig. 2)</td>
<td>672</td>
<td>25</td>
<td>26,880</td>
</tr>
<tr>
<td>P-60 peak (Fig. 2)</td>
<td>24</td>
<td>0.59</td>
<td>40,677</td>
</tr>
</tbody>
</table>

* See text and previous illustrations for details.
in the last row of Table 1 by %, the specific activities relative to that of erythrocytes can be obtained. Whole blood was used as a reference in Table 1 because the cochlea contains whole blood as a compartment. A second line of evidence for a true cochlear enzyme is that the carbonic anhydrases due to blood account for only a small part of the total activity of the membranous lateral wall, shown as follows: The ratio of carbonic anhydrase protein to hemoglobin protein is about 1/100 in the erythrocyte (13), and the small amount of hemoglobin present in the cochlear preparation can be estimated from its A 410 nm and its elution position (Fig. 1). Knowing the specific activities of the pure erythrocyte enzymes (17), the contribution of blood carbonic anhydrases to the total activity can be calculated. It is found to be about 4%. Although one could argue that a potent activator might amplify the small amount of carbonic anhydrase due to cochlear blood, this possibility is unlikely, and it does not explain enzyme activity in fractions containing no blood.

An interesting finding is the high level of carbonic anhydrase in the spiral ligament fraction (Table 1). This fraction contains no hemoglobin detectable by A 410 nm. Because the specific activity of the spiral ligament fraction is appreciably higher than that of whole blood, it is likely that the enzyme located here is of cochlear origin. The same cannot be said for carbonic anhydrase of the stria vascularis because its specific activity is comparable to that of blood; however, indigenous strial enzyme is not ruled out. The possible function of carbonic anhydrase in the spiral ligament fraction is not clear, but the existence of high levels of the enzyme indicates that "ligament" may be a misnomer for this cochlear tissue.

Carbonic anhydrases have been purified to homogeneity from several tissues other than blood, namely, kidney (20), liver (21), gastrointestinal mucosa (17), uterus (22), and prostate gland (23). The present purification of carbonic anhydrase from the guinea pig cochlea has revealed a major soluble enzyme that is similar, but not necessarily identical, to the high-specific-activity isoenzyme from blood, carbonic anhydrase C. Points of similarity include electrophoretic mobility, elution position on anion-exchange columns, and specific activity. Although proof of identity awaits detailed chemical analyses, the cochlear enzyme clearly has more of the characteristics of a "C-type" than a "B-type" carbonic anhydrase (24). The evidence for little of the low-specific-activity or B enzyme in the cochlea is 2-fold; first, none was detected by anion-exchange chromatography (Fig. 2), and second, no fluorescent complex other than DNSA-enzyme C was seen after electrophoresis of cochlear homogenates with fluorescent probe (Fig. 4). Larger amounts of starting material may be necessary to detect enzyme B in the cochlea. Enzyme B may also be less stable than enzyme C. Besides the soluble carbonic anhydrase activity, there may also be membrane-bound enzyme in the cochlear membranous lateral wall. Three percent of the activity near the chromatographic front in Fig. 1 may represent carbonic anhydrase bound to material of high molecular weight because nonspecific binding is minimized with the relatively high salt concentrations used for elution (25). Carbonic anhydrase in the cochlea thus may be present in soluble and particulate forms similar to those in the kidney (25).

It is clear from the present studies that soluble cochlear carbonic anhydrase interacts with at least two aromatic sulfonamides. Acetazolamide half-maximally inhibits the enzyme at 10⁻⁵ to 10⁻⁹ M, in which range it inhibits many (4) but not all (21) carbonic anhydrases. DNSA, which combines with bovine carbonic anhydrase (19), forms a brightly fluorescent complex with the cochlear enzyme and does not appear to form such complexes with other soluble proteins of the membranous lat-
eral wall. The electrophoresis of DNA-protein mixtures thus constitutes a simple method for detecting isoenzyme forms of carbonic anhydrase in crude homogenates.

What physiological function might carbonic anhydrase have in the inner ear? Apart from its role in CO₂ removal (1, 4), carbonic anhydrase is thought to function in secretory tissues that transport or accumulate H⁺ or HC₀₃⁻ (4). It has been suggested that ATPase-catalyzed K⁺ transport may be linked in some way to carbonic-anhydrase-catalyzed HC₀₃⁻ production in the formation of cochlear endolymph (26). Support for this contention comes from one report that acetazolamide decreases endolymphatic K⁺ (5), although these results have not been confirmed (27). The effects of carbonic anhydrase inhibitors in the cochlea clearly need more investigation. So too does the macromolecular structure of the membranous lateral wall, which presumably maintains the ionic and electrical gradients of the endolymphatic space. The present study is an initial step in that direction.

I thank Jörgen Fex and Ichiji Tasaki for helping to make this work possible, and Anne Mustafa for technical assistance.