Membrane-associated Carbonic Anhydrase Purified from Bovine Lung*

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Philip L. Whitney† and Thomas V. Briggle
From the Pulmonary Division, University of Miami School of Medicine, Miami, Florida 33101

We found carbonic anhydrase activity associated with particulate fractions of homogenates of rat, rabbit, human, and bovine lungs. These membrane-associated carbonic anhydrases were remarkably stable in solutions containing sodium dodecyl sulfate (SDS). The bovine enzyme was dissolved with SDS and purified by affinity chromatography and gel filtration. The purified enzyme contains cystine, an amino acid that is absent for this conclusion. The apparent function of the enzyme in hydrating activity was about half that of the erythrocyte enzyme. Acetazolamide inhibited the enzyme in SDS, but did not inhibit the membrane-associated enzyme. Other investigators have accumulated a good deal of evidence for carbonic anhydrase on the luminal surface of pulmonary capillaries. The enzyme described here appears to be a new isozyme whose properties are consistent with such a localization.

Studies on the rate of CO$_2$ hydration and HCO$_3^-$ dehydrogenase in lung capillaries have pointed to the presence of carbonic anhydrase on the luminal surface of the pulmonary endothelial cells (1-3). Localization of carbonic anhydrase in cultured endothelial cells by activity and by light and electron microscopic techniques (4, 5) have provided further support for this conclusion. The apparent function of the enzyme in this location is to catalyze the dehydration of serum bicarbonate. The apparent molecular weight of SDS-polyacrylamide gel electrophoresis (52,000) may be higher than the actual molecular weight due to the presence of carbohydrate. The enzyme contains cystine, an amino acid that is absent in bovine erythrocyte carbonic anhydrase. Dithiothreitol greatly accelerated the rate of inactivation of the membrane-associated enzyme in SDS, so disulfide bonds appear to stabilize this enzyme. The specific CO$_2$-hydrating activity was about half that of the erythrocyte enzyme. Acetazolamide inhibits the membrane-associated enzyme ($K_i = 10$ ns) nearly as well as the erythrocyte enzyme ($K_i = 3$ ns). Antibody to bovine erythrocyte carbonic anhydrase did not inhibit the membrane-associated enzyme. Other investigators have accumulated a good deal of evidence for carbonic anhydrase on the luminal surface of pulmonary capillaries. The enzyme described here appears to be a new isozyme whose properties are consistent with such a localization.

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† To whom correspondence should be addressed, at Pulmonary Division R120, University of Miami School of Medicine, P. O. Box 016960, Miami, FL 33101.

DISCUSSION

Many properties of the membrane-associated enzyme from bovine lung are very similar to those of the erythrocyte enzyme. The molecular weights of the polypeptides are probably similar. The specific activity of the membrane-associated enzyme is about half that of the erythrocyte enzyme and its affinity for acetazolamide is about a third of the erythrocyte enzyme. Acetazolamide bound to both enzymes at the same rate. It is known that the erythrocyte carbonic anhydrase must have Zn$^{2+}$ in the active site to promote strong binding of acetazolamide (10). Since the membrane-associated enzyme binds acetazolamide strongly, it is likely that it also contains Zn$^{2+}$. If it does contain Zn$^{2+}$, it is not removed by EDTA at pH 8; the erythrocyte enzyme also does not transfer Zn$^{2+}$ to EDTA at this pH.

On the other hand, there are differences between these enzymes. Antiserum to the erythrocyte enzyme completely inhibited the activity of the erythrocyte enzyme but did not inhibit the membrane-associated enzymes. However, this does not prove that there are no similar antigenic sites on the two proteins. Perhaps the antibody did not bind to the membrane-bound enzyme due to steric hindrance by neighboring membrane components. Furthermore, it is probable that some antibody molecules could bind to the membrane-associated enzyme without inhibiting its activity, especially with a small substrate like CO$_2$. Thus, the lack of inhibition does not rule out the formation of enzyme-antibody complexes.

There are other notable differences between the two enzymes. The membrane-associated enzyme binds strongly to membranes and is remarkably stable in SDS solutions, properties that the erythrocyte enzyme lacks. The membrane-associated enzyme also has carbohydrate and cysteine residues, components that the erythrocyte enzyme lacks. The disulfide bonds in the membrane-associated enzyme are important in protecting the enzyme from denaturation with

1 Portions of this paper (including "Experimental Procedures," "Results," Figs. 1 and 2, Tables I-III, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 8M-875, cite the authors and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviation used is: SDS, sodium dodecyl sulfate.

Even when the membrane-associated carbonic anhydrase is dissolved in SDS, we continue to designate it as the membrane-associated enzyme to distinguish it from soluble carbonic anhydrase. It is very likely that the membrane associated enzyme is a new isozyme which could be designated as CA IV.
SDS, since activity is rapidly lost if dithiothreitol is added with the SDS. The carbohydrate probably has little influence on the stability of the enzyme in SDS but may be important in directing it to the appropriate membrane site.

Since our analyses were not very extensive (because we had so little purified enzyme), the exact nature of the carbohydrate is not yet known. It contains mannose and glucosamine but not galactosamine, so carbohydrate chains would probably be linked to the protein via N-glycosidic linkage from N-acetylglucosamine to asparagine residues. Amino acid analysis showed about 6 residues of glucosamine/100 amino acids, a value which is somewhat low because of partial destruction during acid hydrolysis. If the protein is the same size as the erythrocyte enzyme (with about 270 amino acid residues/molecule) and if the carbohydrate chains contain an average of 4 residues of glucosamine, then there would be 4–6 carbohydrate chains/molecule. The presence of glucose in the sample is not easily explained, because most known mammalian glycoproteins do not have glucose incorporated into the carbohydrate chains. It is possible that glucose-containing glycolipids are associated with the enzyme and remain attached during purification.

These results permit some speculation about the location of the membrane-associated carbonic anhydrase in lung. The enzyme is firmly bound to membrane and is most concentrated in the pellet (84,000 × g), results consistent with earlier ideas that it is located on the luminal surface of endothelial cells (1–3). Such localization is supported by studies of acetazolamide binding during perfusion through rabbit lung (11) and by rates of bicarbonate diffusion across the alveolar-capillary barrier (12). It is also supported by histochemical studies of cultured bovine pulmonary endothelial cells (4, 5) and of human, monkey, and rat lungs (13, 14). In human and monkey lung, the activity was limited to the capillary walls. In rat lung, carbonic anhydrase activity was found only on that portion of the capillary wall that was in close contact with alveoli. This suggests that not all endothelial cells in rat lung possess significant amounts of the membrane-associated enzyme.

The localization of carbonic anhydrase to capillary walls in close contact with alveoli might explain earlier findings of the activity of the membrane-associated carbonic anhydrase in developing rat lung (15). The activity was low in newborns, rose slowly for the first 2 weeks, and then rose rapidly to adult levels during the third week. It is during the third week that the capillary-alveolar barrier thins (16–17) to form the structure where carbonic anhydrase was found histochemically.

The level of vascular carbonic anhydrase activity varies markedly from tissue to tissue. It was high in lung and low in liver and hindlimb (18). The distribution of membrane-associated carbonic anhydrase in kidney is different from that found in lung. Histochemical studies of rat kidney showed no activity in the glomeruli and Bowman’s capsule, but the brush-border and basolateral membranes of the convoluted proximal tubules showed intense activity (19). These results agree with biochemical studies that found carbonic anhydrase activity in purified brush-border and basolateral membranes (9). The membrane-associated carbonic anhydrase in human kidney is unusually stable in sodium dodecyl sulfate and is about the same size as the enzyme from bovine lung (6). We think that the membrane-associated carbonic anhydrase of lung is the same as that in the brush-border of the kidney proximal tubules even though the enzyme is apparently associated with endothelial cells in a lung and epithelial cells in kidney. The distribution of the membrane-associated carbonic anhydrase may be similar to that of angiotensin-converting enzyme, an enzyme usually found only on the capillary but which is also found in the brush-border of kidney proximal tubules (20–22). Future studies of membrane-associated carbonic anhydrase may provide important information on the structure and function of membrane proteins and on factors which control their synthesis and distribution.

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Additional references are found on p. 12069.
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Supplemental Material

Membrane-associated Carbonic Anhydrase

Justified from bottom long.

Philip L. Whitney and Thomas F. Brink

EXPERIMENTAL PROCEDURE

Preparation of membrane-associated carbonic anhydrase. Following removal of large airways, 100 ml of blood (heparinized by Dr. S.L. Cook) was added and homogenized in a Burrell Denver after adding 5 ml assay buffer (0.10 M NaCl, 0.05 M Tris, pH 7.4, 0.10 M NaHCO3) was added to the homogenate and centrifuged at 3000g for 30 min at 4°C to remove intact cells, nuclei, and mitochondrial. The supernatant fraction was filtered through glass wool and centrifuged at 10,000 rpm for 60 min (at 28°C) to separate plasma membranes. The membrane fraction was washed by resuspending in 100 ml assay buffer and then reincubating at 3000 rpm for 30 min. Each step was repeated 2 times.

The pellet (120g) was resuspended in 80 ml buffer with the bicarbonate.

Membrane-associated carbonic anhydrase was dissolved by adding 500 ml of 800 mM NaCl. The solution was then centrifuged at 8000 rpm for the presence of 800. All subsequent procedures were done quickly. The solution was centrifuged at 10,000 rpm for 15 min at 37°C and the pellet (matt color) was dissolved.

The primary purification step was affinity chromatography using monomer-nitrocellulose coupled to CM-Sephadex A-15. Affinity gel (100ml) was added to the dissolved microsomal fractions (100ml), mixed for 30 min and allowed to settle. The affinity gel was then added to a column (10-cones) and washed with 200 ml of sodium acetate (pH 5.6) and 100 ml NaCl, 0.05 M NaHCO3. The enzyme was then eluted with 10 ml of 0.1 M sodium acetate (pH 5.6) and 0.05 M NaHCO3.

The active fraction was then collected. The column was pre-washed and stored at 0°C overnight. The inactive 800 was removed by centrifugation. The supernatant fraction was then centrifuged at 10,000 rpm for 60 min. The pellet was washed with 200 ml of 0.1 M sodium acetate (pH 5.6) and 0.05 M NaHCO3. The enzyme was then eluted with 10 ml of 0.1 M sodium acetate (pH 5.6) and 0.05 M NaHCO3. The enzyme was then eluted with 10 ml of 0.1 M sodium acetate (pH 5.6) and 0.05 M NaHCO3.

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Membrane-associated Carbonic Anhydrase

Membrane-associated Carbonic Anhydrase was detected in the plasma membrane fraction of rabbit erythrocytes. The enzyme was characterized by its ability to bind to the plasma membrane and its resistance to inhibitors commonly used to inhibit cytosolic carbonic anhydrase. The membrane-associated enzyme was purified by a combination of centrifugation and isoelectric focusing, followed by affinity chromatography on a column of affinity gel.

The enzyme was shown to have a molecular weight of about 100,000, as determined by gel electrophoresis. The active site of the enzyme was sensitive to inhibition by sulfonamides, suggesting a potential role in drug resistance mechanisms.

Table II: Activity of Restriction of Membrane-associated Carbonic Anhydrase by 28-29°C

| Source     | Fraction | Temp. | %ECA Concentration | DPT Concentration |
|------------|----------|-------|--------------------|-------------------|
| Novela pellet | 25°C     | 0.57  | 0.15               | 0.15              |
| (O,0000g,135%)|          |       |                    |                   |
| Galena     | 25°C     | 0.45  | 0.10               | 0.10              |
| (0,0000g,135%)|          |       |                    |                   |
| Barium     | 25°C     | 0.30  | 0.05               | 0.05              |
| (0,0000g,135%)|          |       |                    |                   |

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The enzyme was also shown to have a high degree of homology with the cytosolic form of carbonic anhydrase, suggesting a common evolutionary origin for the two forms of the enzyme.

The activity of the membrane-associated enzyme was found to be inhibited by sulfonamides, but not by other common inhibitors of cytosolic carbonic anhydrase.

The membrane-associated enzyme was found to be stable at temperatures up to 28-29°C, suggesting a potential role in maintaining cellular pH homeostasis in the face of rapid changes in external pH.

The membrane-associated enzyme was found to be resistant to inhibitors commonly used to inhibit cytosolic carbonic anhydrase, suggesting a potential role in drug resistance mechanisms.

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