Membrane-associated Carbonic Anhydrase from Rat Lung

PURIFICATION, CHARACTERIZATION, TISSUE DISTRIBUTION, AND COMPARISON WITH CARBONIC ANHYDRASE IVs OF OTHER MAMMALS*

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Carbonic anhydrase (CA) IV was purified to homogeneity from rat lung microsomal and plasma membranes. The single N-terminal amino acid sequence showed 55% similarity to that reported for human CA IV. A monospecific antibody to the 39-kDa rat enzyme that cross-reacts on Western blots with CA IVs from other mammalian species was produced in rabbits. Digestion of rat lung enzyme with endoglycosidase (peptide-N-glycosidase F) reduced the $M_r$ to 36,000, suggesting that rat CA contains one N-linked oligosaccharide chain. All of eight additional mammalian CA IVs that were examined also contained oligosaccharide chains, as evidenced by reduction in $M_r$, from 52,000 (cow, sheep, and rabbit), 42,000 (pig, guinea pig, and dog), and 39,000 (mouse and hamster) to 36,000 after treatment of the respective lung microsomal membranes with peptide-N-glycosidase F. The 36-kDa human enzyme showed no change in molecular mass with this treatment. Thus, the human CA IV is the exceptional one in lacking carbohydrate.

Rat lung CA IV was found to be relatively resistant to sodium dodecyl sulfate and to be anchored to membranes by a phosphatidylinositol-glycan linkage; both properties were found to be shared by other mammalian CA IVs. Western blot analysis indicated distribution of CA IV in rat tissues other than kidney and lung, where it was previously known to be present. CA IV was particularly abundant in rat brain, muscle, heart, and liver, all locations where the CA IV enzyme was not known to be present previously. None was detected in rat skin or spleen.

The carbonic anhydrase isozymes are zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide ($CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$). They vary in their subcellular localization, with cytoplasmic (CA I, CA II, CA III, and CA VII) (1, 2), cell surface membrane (CA IV) (3-5), mitochondiral (CA V) (6), and secretory (CA VI) (7-9) forms having been described. The cytoplasmic isozymes have been studied in greatest detail. Until recently, poor yields of the membrane-bound enzyme (CA IV) from cumbersome purification procedures have prevented extensive studies on the structure/function relationships of the membrane-bound isozyme.

The first membrane-associated CA purified to homogeneity was obtained from bovine lung (3). It was characterized as a disulfide bond-containing glycoprotein with an apparent molecular weight of 52 kDa and designated CA IV to distinguish it from the then known cytoplasmic isozymes CA I, CA II, and CA III (3). Bovine CA IV had the unique property of being stable for up to several hours in 1-5% SDS solution, which facilitated its isolation by affinity chromatography. However, the enzyme was unstable in SDS after 24 h and could not be characterized extensively. Several years later, a different type of purification of a membrane-bound carbonic anhydrase from human kidney membranes was reported (4, 10). The apparent molecular weight was initially reported to be 68,000 (10), but more recent purifications by this method yielded an inactive polypeptide with an $M_r$ of 34,400 on SDS-PAGE (4). The N terminus of the homogeneous protein was reported to be blocked on amino acid sequencing. Paradoxically, antisemur raised against SDS-treated 34.4-kDa enzyme reacted only with a polypeptide of 55 kDa, which was found in many tissues and was attributed to CA IV (11), despite the difference in molecular weight from the antigen to which the antibody was raised.

Recently, Zhu and Sly (5) reported purification of CA IV to homogeneity from human lung and kidney. Their biochemical and immunological results differed substantially from those reported by Wistad and Knuttila (4) and Carter et al. (11). First, the 35-kDa homogeneous CA IV from human lung and kidney was catalytically active. Second, it had unblocked N termini, and amino acids could be sequenced by Edman degradation for up to 17 cycles without interruption. Third, the monospecific antisemur raised to human lung CA IV reacted only with polypeptides of appropriate $M_r$ (35,000) in membrane homogenates of lung, kidney, and other CA IV-containing tissues. The 35-kDa human CA IV was found to contain no carbohydrate, but it did contain disulfide bonds that appeared to account for its relative stability in sodium dodecyl sulfate. The human CA IV was shown to be anchored to lung and kidney membranes through phosphatidylinositol-glycan linkages.

The interesting similarities and differences between bovine lung CA IV (3) and CA IV from human lung and kidney (5) led us to purify the membrane-associated CA IV from rat lung microsomal and plasma membranes. We hoped to assess the versatility of the new purification procedure for other mammalian CA IVs, to provide antigen for immunological studies of rat CA IV, and to study the physicochemical properties of other mammalian CA IVs to define their common properties.

In this paper, we provide evidence that rat lung CA IV is a...
glycoprotein and is also anchored to membranes via a phosphatidylinositol-glycan linkage. We also report immunologic studies with antibody raised to the purified rat lung CA IV that show that these two properties are also shared by CA IVs from nine different species. These studies show that the large molecular weight differences between the human CA IV and the CA IVs in various other mammalian species are almost entirely explained by differences in carbohydrate content. Finally, we present the first evidence for the tissue distribution of CA IV in rat tissues other than kidney.

MATERIALS, EXPERIMENTAL PROCEDURES, AND RESULTS

The successful application of the purification procedure we reported for CA IV from human lung to CA IV from rat lungs demonstrates the versatility of this procedure for producing relatively large amounts of homogeneous enzyme in a form that is active and stable to storage. The same purification has been applied successfully to CA IV from bovine lungs, porcine lungs, and rabbit lungs. The success of this procedure relies on one property that all the CA IVs appear to have in common, namely relative stability in 1–5% sodium dodecyl sulfate. This property allows them to be selectively adsorbed to an affinity column under conditions where nearly no other proteins (or other carbohydrate anchors) are adsorbed. The resistance to denaturation in sodium dodecyl sulfate probably reflects the stabilizing effect of one or more disulfide bonds (19), since the same level of sodium dodecyl sulfate completely inactivates CA IVs in the presence of reducing agents (3). Once the enzyme is adsorbed to the column, replacing the sodium dodecyl sulfate with a nonionic detergent before elution allows the hydrophobic CA IVs to be recovered under conditions where they are active and stable to storage (5).

A second property that mammalian CA IVs appear to have in common is a hydrophobic anchor that is sensitive to phosphatidylinositol-specific phospholipase C. The phosphatidylinositol-glycan anchor provides a means to target CA IV to the extracellular surface of the plasma membrane. In polarized cells, the phosphatidylinositol-glycan anchor has been suggested as a means of targeting protein specifically to the apical surface (22). However, in rat kidney, CA IV was identified on both the apical and basolateral surfaces of certain portions of the proximal convoluted tubules and the thick ascending limb (23). Whether it is anchored differently on the apical and basolateral membranes in the rat nephron has not yet been determined.

The presence of N-linked oligosaccharide chains was found in 9 of 10 mammalian CA IVs examined. Only human CA IV lacked N-linked oligosaccharides. The studies reported here demonstrated a wide variation in the carbohydrate content among the nonhuman CA IVs with no obvious biochemical differences between those with less and those with more carbohydrate. This observation, and the fact that the glycosylation is not conserved evolutionarily, suggests that the oligosaccharide chains do not have an important role in the respiratory and renal functions of CA IV.

The availability of the antibody described here provided the opportunity to study the tissue distribution of CA IV. One prior study on the distribution of CA IV in human tissues has been reported. However, it is not possible to interpret the results of that study with confidence. The presumptive antigen used to immunize the rabbit was a 35-kDa protein purified from human kidney that was thought to be CA IV. However, the only peptide detected with the antibody in human tissues was a 55-kDa polypeptide that was present in every tissue examined. It seems probable that the antigen surveyed in this study was a contaminant protein, rather than the 35-kDa human CA IV.

The tissue distribution of rat CA IV seen in the present work is quite extensive. Yet its absence in skin and spleen indicates that CA IV is not ubiquitous. Immunoreactive protein at 39 kDa was found in homogenates of brain, kidney, lung, liver, skeletal muscle, stomach, large intestine, and small intestine. Faint bands of larger molecular weight (50,000) cross-reacting species were also found in brain, stomach, and large intestine. Whether these bands of cross-reacting proteins have carbonic anhydrase activity is not yet clear. Nor is it clear which cell types account for the immunoreactivity found in the tissue homogenates that contained CA IV. Detailed immunohistochemical studies recently defined the cellular distribution of CA IV in rat kidney, where the CA IV is present on the apical and basolateral surfaces of specific segments of the nephron (23), and in the human eye, where the CA IV is found in a specific capillary bed, the choriocapillaris (24). Similar studies using the antibody described here can help clarify which cells account for the immunoreactivity in the additional rat tissues we found to contain CA IV in this study.

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Materials

Triton X-100 was purchased from Boehringer. Goat anti-rabbit IgG-peroxidase and alkaline phosphatase conjugates and isoelectric focusing marker proteins were obtained from Sigma. Molecular weight standards were from Bio-Rad. Peptide-glycosidase F was from Boehringer. Immobilon-P, PVDF-membrane was purchased from Millipore. Goat anti-rabbit IgG-peroxidase and alkaline phosphatase conjugates and isoelectric focusing marker proteins were obtained from Sigma. Molecular weight standards were from Bio-Rad. Peptide-glycosidase F was from Boehringer. Immobilon-P, PVDF-membrane was purchased from Millipore.

All other chemicals used were of analytical grade.

Experimental procedures

Purification of rat lung CA IV. Homogeneous rat CA IV was obtained from 250 g of rat lung. Purification and purification of the enzyme was performed as described by Zhu and Sly (5). In this procedure, the SDS used for extraction of the enzyme was replaced with the nonionic detergent, Igepal. During purification of the enzyme on the inhibitor-affinity column. The homogenous enzyme obtained was stable for several months at 4°C. Carcinoembryonic antigen activity was measured using the procedure of Marx (12) as described (13). The protein concentration of the enzyme preparation was determined by the Bio-Rad assay (14). Homogeneity of the enzyme on SDS-gel electrophoresis (SDS-PAGE) (15).

Immunoblotting. The polypeptides from SDS-PAGE were electrophoretically transferred to Immunoblot membrane as described (16) followed by immunostaining of the polypeptides using 11000 diluted rabbit-anti-rat lung CA IV antibody and 11000 diluted goat antiserum. Peroxidase activity was stained using 4-chloro-1-naphthol and hydrogen peroxide.

N-terminal amino acid sequencing. Amino acid sequencing from N-terminal of the rat lung CA IV was carried out using an Applied Biosystems automated A77 protein sequencer (17).

Phase separation in Triton X100 detergent. Microsomal membranes equivalent to 50 μg protein of homogenous rat lung CA IV. 50 μg were solubilized in 100 μl Triton X-100 at 4°C and flashin of proteins to detergent phase was accomplished as described (18).

Endoglycosidase digestion. Homogenous enzymes (500 μg) or lung microsomal membranes equivalent to 50 μg protein were denatured and treated with 200 μg of endoglycosidase (PNGase F) in 10 μl reaction mixture (15, 17) except the denaturation buffer used was 0.1 M sodium phosphate pH 8.4 containing 1% NP-40, 0.2% SDS and 1% N-serpentine. Before addition of PNGase F, 1 μM of N-acetylglucosamine (PNGase F) and 5 μM of N-acetylglucosamine (PNGase F) were added to the reaction mixture as substrate inhibitors. The deamidolysis treatment was analyzed by SDS-PAGE.

Treatment of lung microsomal membranes with phospholipid-based specific phospholipase C (PAS-P) from Bacillus Caldotenax was performed as described (19).

Preparation of rat lung CA IV from bovine lung and CA VI from human saliva. CA IV from bovine lung was purified as described (1) and CA VI from human saliva was prepared according to Karaman and Sly (4).

Preparation of lung microsomes. Frozen lung tissue from different mammals was obtained from Pel-Freeze. Tissue were cut into small pieces and homogenized in 5.5 ml volume of 100 mM Tris-HCl pH 7.5 containing 7.5 μl buffer containing 1 mg peritectic, 0.5 mM benzamidine, 0.2 mM EDTA and 5 mM of iodide. Microsomes were obtained and washed as described (5). Micropore pellets were dispersed in 100 mM Tris-HCl pH 7.5 buffer and frozen at -70°C.

Preparation of CA IV from bovine lung and CA VI from human saliva. Purified bovine lung CA IV was purified as described (1) and CA VI from human saliva was prepared according to Karaman and Sly (4).

Homogenate CA IV in lung microsomes from different mammals was performed as described (4). Microsome pellets were dispersed in 100 mM Tris-HCl pH 7.5 buffer and frozen at -70°C.

Expression of CA IV in lung microsomes. Purified bovine lung CA IV (100 μg) was subjected to SDS-PAGE by immunoblotting. Figure 1 shows the apparent molecular weight of the polypeptides was determined. CA IV in lung microsomes from different mammals was performed as described (4). Microsome pellets were dispersed in 100 mM Tris-HCl pH 7.5 buffer and frozen at -70°C.

Figure 1. SDS-polyacrylamide gel electrophoretic analysis of rat lung CA IV. Rat lung microsomal membranes (lane 1), homogenous rat lung CA IV (lane 3) and molecular weight markers (lane 2) were subjected to electrophoretic transfer of the polypeptides to PVDF membrane. Lane 1 was subjected to immunoblotting and lanes 3 and 2 were stained for proteins with anodeblack stain.
In order to explore the generality of phosphatidylinositol-glycan anchoring of mammalian lung CA IV, lung microsomes of several additional mammals were obtained and microsomal membranes were treated with PI-PLC. Microsomal membranes equivalent to 50 μg protein from different mammals were treated with buffer alone (−) and with endoglycosidase (+). Deglycosylation was analyzed by SDS-PAGE followed with immunoblotting. Open arrow head shows proteolytically clipped polypeptides. +HCA human, rat, mouse, hamster, pig, guinea pig, and dog membranes; B. bovine, sheep, and rabbit membranes.

Figure 3 (A and B) Effect of endoglycosidase digestion on CA IV from lung microsomal membranes. Microsomal membranes equivalent to 50 μg protein from different mammals were treated with buffer alone (−) and with endoglycosidase (+). Deglycosylation was analyzed by SDS-PAGE followed with immunoblotting. Open arrow head shows proteolytically clipped polypeptides. +HCA human, rat, mouse, hamster, pig, guinea pig, and dog membranes; B. bovine, sheep, and rabbit membranes.

Phosphatidylinositol-glycan anchoring is anchored to the membrane through a phosphatidylinositol-glycan linkage. The hydrophobic nature of the human enzyme can be demonstrated by showing phase separation into the detergent phase following Triton X114 extraction (16). The purified rat lung CA IV also partitioned to the Triton X114 phase (results not shown) suggesting that the homogeneous rat CA IV also contains a hydrophobic domain. When rat lung microsomal membranes were treated with PI-PLC, membrane bound carbonic anhydrase activity was released into the supernatant. The results in Table II show that about 4% of the CA IV enzyme activity was released from the membrane into the supernatant. The purified rat lung CA IV, which is phosphatidylinositol-glycan anchored, was studied under identical conditions, 50% of the 1′-nucleotidase was solubilized (see Table II).

**Table II**

| Treatment  | Enzymes                      | Carbonic anhydrase | 1′-nucleotidase |
|------------|------------------------------|-------------------|----------------|
|            | EU/ml (μg protein) | EU/ml (μg protein) |                |
| Membrane alone | 4.4 (90) | 9.1 (91) | 1.4 (81) |
| +PI-PLC    | 5.8 (91) | 4.0 (10) | 6.0 (40) |

Numbers in parentheses are percent of total enzyme units in membrane and soluble fractions.

**Figure 4** Solubilization of CA IV from lung microsomal membranes by PI-PLC. Microsomal membranes equivalent to 50 μg protein from rat (1), mouse (2), hamster (3), pig (4), guinea pig (5), dog (6), bovine (7), sheep (8), and rabbit (9) were treated with buffer alone (−) and with PI-PLC (+). A. soluble enzyme and B. membrane associated enzymes were analyzed by SDS-PAGE followed by immunoblotting. All results were treated with buffer only (+) or with PI-PLC, results with buffer only are shown only for rat (1).

**Figure 5** Immunoblotting of lung microsomal membranes by PI-PLC. Membrane-bound CA IV was solubilized by PI-PLC and subjected to SDS-PAGE followed by immunoblotting. The polypeptides corresponding to CA IV and CA II were marked. Filled arrow shows crossreacting protein of 50 kDa.