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Carbonic Anhydrase C in the Human Renal Medulla

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ABSTRACT

Soluble carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1) from the papillary and inner medullary regions of thoroughly perfused human donor kidneys was isolated by affinity chromatography. The purified enzyme was homogenous with respect to sedimentation in the ultracentrifuge and iso-electric focusing. It had an amino-acid composition and behaved chromatographically, kinetically, electrophoretically and immunochemically like the high-activity (with respect to CO_2) erythrocyte carbonic anhydrase HCA-C, and the renal enzyme previously isolated from extracts of the whole kidney. The results suggest that all regions of the human kidney contains one soluble form of carbonic anhydrase similar to and probably identical with HCA-C. Small immunoassayable amounts (1/30 of total enzyme protein) of the low-activity erythrocyte isoenzyme HCA-B were also found, but are considered to be a contaminant. There was no indication for the presence of sulfonamide-resistant isoenzymes.

INTRODUCTION

To be able to evaluate the physiological role of renal carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1) for the acidification of the urine and excretion of electrolytes, it is of obvious importance to know the type and properties of the enzyme in the various regions of the kidney. Several cytoplasmic forms of the enzyme have been isolated from mammalian tissues and found to differ greatly with respect to substrate and inhibitor kinetics. They could have different physiological functions (1). There are conflicting reports in the literature about the distribution and properties of the enzyme both in animal (cf. ref. 2) and human kidneys. In our laboratory we have isolated and characterized the soluble form of carbonic anhydrase from extracts of the whole human kidney. The enzyme was found to be similar and probably identical with the high-activity (with respect to CO_2) C-form of the erythrocytes (3). In another study (4), how-

ever, the human renal cortex was reported to contain only the C-form whereas the medulla, in addition to the C-form, was found to contain relatively large amounts of the erythrocytic B-form. Moreover, the human kidney has been claimed (5) to have a tissue-specific isoenzyme different from the erythrocyte forms.

Since the controversy mainly concerns what type of enzyme is present in the renal medulla, we have chosen in the present work to study the medullary and papillary regions of the human kidney in more detail. We have used an affinity chromatographic technique capable of separating and purifying isoenzymes with different affinities for sulfonamide inhibitors, and a new radioimmunosorbent technique for the assay of B- and C-types of carbonic anhydrase in human tissues. The study was done on fresh donor kidneys which had been thoroughly perfused free from blood.

MATERIALS AND METHODS

Materials

Sepharose 6 B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), carrier empholytes, Ampholine^{R} from LKB-Produkter (Bromma, Sweden), acrylamide and bisacrylamide were from Eastman Kodak (Rochester, Ill., U.S.A.). Sulfanilamide was from Sigma (St. Louis, Mo., U.S.A.) and acetazolamide from Lederle Laboratories (Pearl River, N.Y., U.S.A.). All other chemicals employed were reagent grade. Human erythrocyte carbonic anhydrases HCA-B and HCA-C were prepared by affinity chromatography in our laboratory as reported previously (3).

Preparation of the kidney extracts

Fresh kidneys from two donors were used. There was no history of renal disease. Thiazide or other drugs of importance had not been administered to the donors, as far as it is known. Immediately after removal, the kidneys were perfused with 400-100 ml of cold Ringer-dextran type of solution (Perfadex^{R}, Pharmacia, Uppsala, Sweden), containing 10 million IU benzylpenicillin, 50 ml 2% lidocaine (Xylocaine^{R}, Astra, Södertälje, Sweden) and 5000 IU heparin per 1000 ml solution. This was followed by perfusion with 400-1000 ml of 10% invertose solution (Inverdex^{R}, Pharmacia, Uppsala, Sweden) to which sodium bicarbonate had been added. The perfusion pressure was 150 mm Hg. The warm ischaemic period varied between 30 and 60 min. The kidneys were stored at 4^{o}C and tissue preparation started within 24 h of perfusion. Slices were carefully dissected out from the inner medulla and the papillary region of both kidneys and gently homogenized for 3 min in ice-cold 0.2 M sucrose using a glass homogenizer and a motor-driven Teflon^{R} pestle. An aliquot of the homogenate was taken for assay of the carbonic anhydrase activity and its inhibition by acetazolamide (see below). The homogenate was centrifuged at 100,000 x g for 60 min. The particulate fractions and the fluffy top layer were removed. The superna-

tant was dialyzed against 10 mM HEPES buffer, pH 8.3, containing 200 µM sodium EDTA. It was frozen before the immunoassay of C- and B-types of carbonic anhydrase (see below). Its content of protein was determined by the method of Lowry et al. (6) and of hemoglobin by the cyan-methemoglobin method (7). This latter method probably overestimates the content of hemoglobin by about 50% due to the presence in the kidney of other iron porfyrin containing enzymes (4). The supernatant was charged to the affinity chromatographic column as described in the legend of Fig. 1-2.

Affinity chromatography

Sulfanilamide coupled to agarose via oxirane groups is a stable, effective and selective adsorbant for both low- and high-activity forms of carbonic anhydrase (8). The unspecific adsorption is low and the enzymes eluted are highly purified (2,3). The activated matrix is formed by reaction of Sepharose 6 B with 1,4-bis(2,3-epoxypropoxy-)butane (EGA Chemie, Steinheim, GFR). Sulfanilamide is coupled to the long hydrophilic spacer by an alkylamine bond. The amount of coupled sulfanilamide was 200 µmol per ml gel, by sulphur and nitrogen analysis. After addition of the supernatant to the column, the proteins which were non-specifically retained by the gel were first desorbed by increasing the ionic strength of the buffer. The low-activity B-forms of carbonic anhydrase, bound to the gel, were then eluted by adding chloride, an anionic inhibitor which inhibits the B-form stronger than the C-form of the enzyme. The latter forms were eluted by raising the chloride concentration and lowering the pH of the buffer. Finally sulfanilamide was added to desorb all retained enzyme proteins.

The same technique as described previously (3) was used except for the application of a gradient elution system (Ultrograd^{R} gradient mixer, LKB 11300, LKB-Produkter, Bromma, Sweden). The chromatographic procedure is described in detail in the legend to Fig. 1.

Enzyme assays

The enzyme activities in the tissue homogenate, in the supernatant and in the chromatographic fractions were measured by a pH-changing method of Philpot and Philpot (9). Briefly, aliquots of samples were added to a phenol red solution containing Na_2CO_3 and saturated with CO_2. After the addition of bicarbonate buffer the time required for the indicator color to shift from red to yellow is recorded. One enzyme unit, U, is the amount of enzyme that halves this time. The sensitivity of the enzyme activity towards inhibition was expressed as I_{50}, which is the molar concentration in the final assay system to reduce 50% of the enzyme activity. Immunoassayable levels of the isoenzymes

CA-C and CA-B were determined in the same tissue fractions by a radioimmunosorbent technique (10). The sensitivity of this method is about 0.2 ng/ml for both isoenzymes and the precision is about 5% in duplicate determinations. The antibodies used were monospecific against CA-C and CA-B and preparaed as described previously (11). The presence in a tissue of up to 20 times higher concentrations of one isoenzyme will not interfere with the assay of the other. The titration curves obtained with the renal C and B types of enzymes were parallel with those of the purified erythrocyte enzyme HCA-C and HCA-B (not shown). Concentrations of the pure enzyme was also measured from the absorbance at 280 nm using a value, $A_{280} \cdot cm^{-1}$, of 18.7 (3).

Isoelectric focusing

This was done in tubes according to a procedure similar to that of Convay-Jacobs and Lewin(12). A pH gradient between 3.5 and 9.5 was obtained by mixing carrier ampholytes (Ampholine^{R}) in polyacrylamide gels. Focusing was performed by a step-wise increase of the voltage to give a power per tube not exceeding 200 mW. Equilibrium was achieved after 4 h. The gels were stained by Coomassie brilliant blue (Svenska ICI AB, Gothenburg, Sweden) and destained according to the procedure given by LKB-Produkter (Bromma, Sweden).

Amino acid analysis

This was performed by ion exchange chromatography according to Moore and Stein (13) on a Beckman 121 M automatic amino acid analyzer. Protein samples were hydrolyzed in 6 M HCl at 110^{o}C for 24 h.

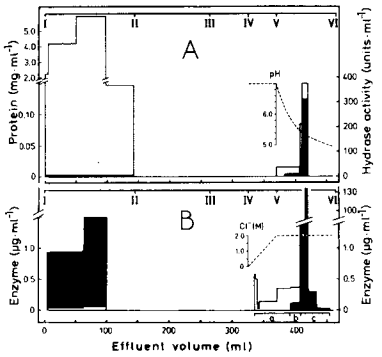
Analytical ultracentrifugation

Sedimentation velocity experiments were performed in a Spinco Model E analytical ultracentrifuge, equipped with a UV-scanning optical system. The rotor speed was 40,000 rev./min for sedimentation runs. Exposures were taken every 4 min for 3 h. The sedimentation constant was calculated and corrected to give values in water at 20^{o}C, $s_{20,w}$, according to Svedberg and Pedersen (14).

Sedimentation equilibrium ultracentrifugation was done using the long-column meniscus-depletion technique of Chervenka (15). The enzyme was dissolved in 10 mM HEPES 300 µM EDTA buffer, pH 8.3; its concentration had an absorbance value A_{280} (1.2 cm^{-1}) of 0.7. A 12-mm 4^{o} capillary-type synthetic boundary double-sector cell was used at 20^{o}C. The centrifuge was run at 28,000 rev./min for about 20 h. The molecular weight was calculated from the slope of ln C versus r^{2}.

Enzyme purification

The elution of the proteins from the first affinity chromatographic run is seen in Fig. 1 A-B. More than 99.8% of the applied protein was not retained by the column, steps I-II. Small amounts of CA-B (3.5 µg), CA-C (111 µg) and hydrase activity were found among this protein (Fig. 1 A-B). These fractions were therefore rechromatographed on a fresh column and this time all material with hydrase activity and antigenic properties like CA-C and CA-B became specifically bound to the gel (Fig. 2 A-B).



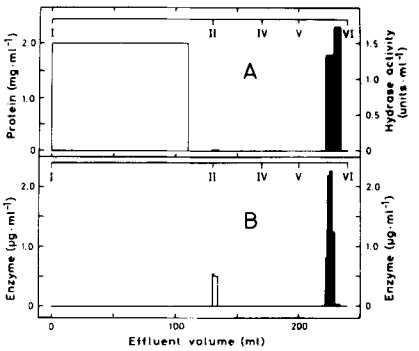


Fig.1 (left). Bioaffinity chromatography on sulfanilamide agarose of carbonic anhydrase from the medullary and papillary regions of the kidney.
First chromatography
A. Desorption as followed by protein determinations (open columns) and hydrase activities (filled columns).
B. Desorption as followed by radio-immunological determination of the B (open columns) and C (filled columns) types of enzyme.
The column was equilibrated with 10 mM HEPES, pH 8.3 and loaded with 0.43 g renal soluble-protein mixture in 97 ml of the same buffer, step I. Nonspecifically bound proteins were displaced by increasing the ionic strength, 50 mM HEPES-1 M Na_2SO_3, pH 8.3 (step II) followed by washing with 50 mM HEPES, pH 8.3 (step III). The carbonic anhydrases bound to sulfanilamide were liberated by first increasing the concentration of the anionic inhibitor chloride (step IV) in the buffer eluate by mixing 50 mM HEPES, pH 8.3, with 250 mM HEPES, -2M NaCl pH 7.0, and then lowering the pH by mixing 250 mM HEPES, -2M NaCl pH 7.0 with 1.25 M HEPES, pH 5.0 (step V). Finally the column was washed extensively with 0.1 M sulfanilamide in 10 mM HEPES buffer, pH 8.3, to desorb all retained enzyme. Fractions were usually of 3.3 ml volume. They were pooled, concentrated and dialyzed against 10 mM HEPES, pH 8.3, 200 µM EDTA. The flow was 10 ml/h, the temperature was 4°C.

Fig.2 (right). Rechromatography of the non-specifically bound material from the first run.
A. Desorption as followed by hydrase activity (filled column) and protein determination (open column).
B. Desorption as followed by radio-immunological determination of the B (open column) and C (filled columns) types of enzyme. The procedure was the same as described in legend of Fig. 1. The elution pattern shows that the non-adsorbed and non-specifically bound material with hydrase activity of the first chromatographic run (Fig. 1 A-B) became specifically bound when rechromatographed on a fresh column.

When the specific desorbtion started in the first chromatography by applying the chloride gradient, step IV, a small amount of protein was eluted, immunochemically identified as CA-B (a, Fig. 1 B). It was hardly detectable catalytically or by the protein assay. When applying the pH gradient (Fig. 1 A) while maintaining the high chloride concentration, step V, more of the B-type and some C-type of enzymes were eluted (b, Fig. 1 B), still hardly detectable as hydrase activity. However, between pH 5.5 and 5.0 (Fig. 1 A) a large sharp protein peak (c, Fig. 1 B) appeared which reacted immunochemically like a C-type of enzyme. It had a catalytic activity of 1570 U/mg protein similar to that of HCA-C (Table III). Since the supernatant contained 8.2 U/mg protein (Table I) the purification was 192-fold (Table I. The addition of 0.1 M sulfanilamide, step VI, did not displace further amounts of protein. The elution pattern of the rechromatographic run (Fig. 2 A-B) exhibited the same sharp peak of hydrase activity and CA-C type of protein. Like in the previous study (3) where affinity chromatography was used to isolate the renal enzyme from extracts of the whole kidney, the recovery of enzyme activity was about 80% (Table I). It was somewhat lower in the immunochemical determinations, particularly for HCA-B. Very little blood was contaminating this kidney as judged from the values of hemoglobin in the supernatant charged to the column. The contribution of HCA-C and HCA-B from erythrocytes was therefore small as calculated in the legend of Table I.

Table I. Purification of carbonic anhydrase from human renal medulla. The amount of starting material was 15.6 g of renal tissue. CO_2 hydration activity was measured by the indicator method of Philpot & Philpot (9). The B- and C-forms or the enzyme was assayed by a radioimmunosorbent technique (10). Concentrations of the pure enzyme was determined by absorbance measurements at 280 nm or by the immunoassay. The supernatant was chromatographed two times on the affinity column (Fig. 1,2). Values are the total recoveries from these runs.

Purification step	Total enzyme activity (units)	Total immunoassayable enzyme (µg)		Specific activity (units/mg extractable protein)
		B	C	
Homogenate	4064	40	1290	3.5
Particulate fraction	289	-	-	1.6
Supernatant*	3526	44.6	1300	8.2
Affinity chromatography	2895	25.8	907	1570

*The supernatant contained 0.64 mg of hemoglobin. Contaminating blood would therefore contribute 7.7 µg HCA-B and 1.0 µg HCA-C, since blood contains 12.1 µg HCA-B and 1.5 µg HCA-C per mg Hb (10).

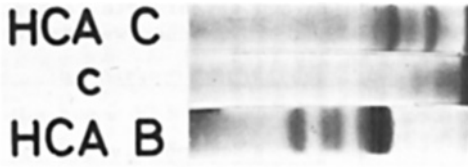


Fig. 3. Isoelectric focusing pattern of carbonic anhydrase isoenzymes separated by affinity chromatography. The renal fraction (c, Fig. 1 B) with hydrase activity and immunologically similar to HCA-C, is compared with HCA-B and HCA-C from hemolysate. About 20 µg of the proteins were applied. The concentrations of ampholytes in the gels was 2.1% (w/v) and acrylamide T=5.8% (w/v) and C=4.8% (w/v)(28).

Criteria of purity

The purity of the renal enzyme was tested by isoelectric focusing where it exhibited a pattern similar to that of HCA-C (cf. c and HCA-C of Fig. 3). The major bands of this enzyme and of HCA-C were found to focus at identical positions, pH 7.3, different from that of HCA-B. Minor enzyme forms, more acidic in nature than the major bands, were also found, particularly for the erythrocyte forms. They correspond to those reported previously (4). The enzyme was homogenous with respect to sedimentation in the ultracentrifuge. In the sedimentation velocity experiment the enzyme sedimented as a single symmetrical distribution curve throughout the run of 3 h. The molecular weight distribution also indicated a mono-disperse system.

Comparative physical and chemical properties of the renal and erythrocyte enzymes

The analyses of the amino-acid composition of the renal medullary enzyme and of the erythrocyte enzyme HCA-C and HCA-B were run simultaneously. From data of Table II is seen that the renal enzyme and HCA-C are identical within the limits of the method and different from HCA-B. The values for the number of amino acids are from one 24-h hydrolysis only, and differ therefore slightly from those previously reported (16) where more extensive hydrolysis was done. Determinations of the molecular weight and the Svedberg units of the enzyme from the centrifugation data gave values slightly lower than those previously obtained for HCA-C (Table III). The reason for this difference is not apparent.

Kinetic properties

The homogenate contained enzyme activity corresponding to 260 U/g wet tissue (Table I). Its activity was inhibited by acetazolamide giving an I_{50} of 34 nM. The I_{50} for the particulate fraction and the supernatant was 56 and 18 nM respectively, values rather similar to that of the pure renal enzyme (Table III). The specific activity of the renal enzyme was similar to that of HCA-C and different from that of HCA-B (Table III).

Table II. Amino-acid composition of human renal medulla and erythrocyte carbonic anhydrases. Values of one 24-h hydrolysis. The number of residues was calculated assuming the total number of amino acids to be 259 (27).

Amino acids	Number of residues		
	Renal medulla	HCA-C	HCA-B
Lysine	23.4	24.1	18.0
Histidine	11.7	12.1	11.2
Arginine	7.5	7.5	7.4
Aspartic acid	27.6	29.2	30.4
Threonine	12.2	11.9	14.0
Serine	16.4	17.7	27.6
Glutamic acid	25.0	25.7	20.0
Proline	16.9	16.3	17.4
Glycine	21.3	22.3	15.6
Alanine	13.8	13.6	18.1
Valine	15.3	13.7	16.6
Methionine	1.1	1.1	2.1
Isoleucine	8.1	7.0	7.3
Leucine	26.0	24.7	20.8
Tyrosine	8.1	7.7	7.7
Phenylalanine	12.7	11.5	10.2
Cysteine	0.8	0.5	0.3

Table III. Comparative properties of the carbonic anhydrases from human renal medulla and erythrocytes

Property	Isoenzyme		
	Renal medulla	HCA-C	HCA-B
Molecular weight (from centrifugation)	27400	28000	25000
Partial specific volume	0.734	0.734 (16)	0.729 (16)
$S^{o}_{20,w}$ (Svedberg units)	3.0	3.3 (16)	3.2 (16)
Isoelectric point (pH) (Fig. 3)	7.3	7.3	6.6
Amino acid composition, Table II	Same as HCA-C	Same as kidney	Different from kidney and HCA-C
Specific activity, U/mg	1570	1500	83
Inhibition by acetazolamide, I_{50} (nM)	18	20	560
Immunochemical reactivity			
Against anti-HCA-C	+	+	-
Against angi-HCA-B	-	-	+

DISCUSSION

The carbonic anhydrase isolated from the medullary and papillary regions of the human kidney is similar to and probably identical with the high-activity erythrocyte from HCA-C (Table III) and the renal enzyme, previously (3) isolated from extracts of the whole kidney. We propose that this enzyme is the only cytoplasmic form present in the kidney, and believe that the small amount of the B-form detected immunochemically (Fig. 1,2; Table I) originated from red cells contaminating the kidney tissue. Our main reason for this is that the amount of CA-C isolated from different kidneys has been found to be rather constant, whereas that of CA-B varies and apparently so with the blood content. In the first preparation of the renal enzyme in this laboratory CA-B was about 30% of the total enzyme protein but of a magnitude which was similar to that which could be calculated to originate from contaminating blood. In the present preparation, where strong precautions were taken to avoid blood contamination, the amount of CA-B was only 2.7% of the total amount of extracted enzyme (Table I). It is true that the measured amount of CA-B was higher than could be calculated to originate from the blood content (Table I) but it is possible that the simultaneous presence of high amounts (about 40 times higher) of CA-C in the sample interferes in the immunoassay of CA-B so that the values of this enzyme become overestimated. The same cross-reaction between high concentrations of the C-enzyme and the antibodies against CA-B used in the immunoassay of the B-enzyme, probably explains why the cortex of well perfused monkey (Macaca nemestrina) kidneys was found to contain 29 times more C- than B-enzyme (17). But it does not explain the finding (4) that the medulla contains rather similar amounts, 1.73 and 0.99 mg/g protein, of immunoassayable CA-C and CA-B. However, in this latter study an autopsy kidney was used which contained much more hemoglobin than did our perfused fresh donor kidneys, which should make the correction for blood contamination more difficult.

The specific activity of the purified enzyme was 1570 enzyme units/mg protein whereas the renal tissue contained 2710 units/mg (3526 units/1.3 mg CA-C; Table I). The relatively high activity in the tissue could depend on the presence of other isoenzymes, immunochemically different from CA-C and CA-B. However, this is not likely, since all material with catalytic activity was bound to the affinity column when rechromatographed and was eluted as a single peak which contained only pure CA-C (Fig. 1 and 2). The activity of the whole homogenate and supernatant was inhibited by acetazolamide like that of the pure renal enzyme (Results section) which speaks against the presence of sulfonamide-resistant carbonic anhydrases in the human kidney. Such enzymes have been shown to exist in liver (18) and skeletal muscle (19), and have been claimed (20) to be present also in the rat kidney. Taken together, the present data, however,

suggest that CA-C is the only cytoplasmic carbonic anhydrase in the human kidney. Most animal studies, in dog (21,22), rat (2,22-24) and ox (25), where perfused kidneys were used, have also indicated the presence of only one cytoplasmic enzyme, kinetically similar to the erythrocyte C-form. The function of this enzyme is probably to ensure the rapid hydration of CO_2 which will maintain near CO_2-equilibrium and maximal buffering capacity within the tubular cell during the secretion of acid. There is, however, carbonic anhydrase activity (about 10-15% of total) also in the particulate fraction (Table I) which probably originates from a membrane-bound enzyme different from the cytoplasmic form (26). The function of this membrane-bound enzyme remains an outstanding problem.

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