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PARTICULATE CARBONIC ANHYDRASE IN HOMOGENATES OF HUMAN KIDNEY

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Summary

About 2% of human kidney carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) has been found in particulate fractions. Its distribution in the particulate fractions obtained by differential centrifugation suggests that it may be concentrated in the brush border. The particulate enzyme is like red cell carbonic anhydrace C in its susceptibility to inhibition by anions.

Particulate carbonic anhydrase is firmly bound to the membrane and is not released by incubation at pH 10.6 and 37°C or by addition of Triton X-100 or deoxycholate. In 10% Triton X-100 at pH 11.3 and 37°C, the particulate enzyme is inactivated with a half time of about 20 min, and this is at least an order of magnitude slower than the inactivation of soluble enzymes in the presence or absence of membranes. The soluble enzymes are inactivated within a few minutes at 25°C in 3—4% sodium dodecyl sulfate, but the particulate enzyme is relatively stable under those conditions, and its half-time of inactivation at 14°C with a detergent-protein ratio of 25 was about 24 h. Gel filtration with Ultragel AcA-44 in sodium dodecyl sulfate indicates that the membrane carbonic anhydrase has a molecular weight of less than 66 000, so its stability is not due to association with large membrane fragments or vesicles. These results suggest that the membrane enzyme may be a different isozyme than the soluble carbonic anhydrases. Although present in relatively small amounts, its localization on the membrane could give it functional significance.

Introduction

The distribution, function and characterization of mammalian carbonic anhydrase (EC 4.2.1.1) has been the subject of many studies [1—3]. Most of

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the work has been done on soluble carbonic anhydrases from red cell enzyme, and the human red cell enzymes have been studied in greatest detail. The three-dimensional structures of human red cell carbonic anhydrases B and C have been determined from X-ray diffraction studies [4,5] and the primary sequences [6,7]. These well-characterized enzymes provide good models to which other carbonic anhydrase isozymes can be compared.

Studies on carbonic anhydrases from other mammalian tissues indicate that they are similar to or identical with the red cell enzymes [8—15]. Some common characteristics include a molecular weight of about 30 000, one zinc per molecule, CO_2-hydrating and esterase activities, and inhibition by sulfonamides. A notable exception is carbonic anhydrase from male rat liver which is similar to the red cell enzymes in many respects but is relatively insensitive to inhibition by sulfonamides [16]. Although most of the carbonic anhydrases are soluble, there are reports of particulate carbonic anhydrase in some tissues [8—13]. However, many of these studies were carried out at low salt concentrations where nonspecific adsorption is likely to occur.

The soluble carbonic anhydrase in human kidney was purified, and its amino acid content and chromatographic, electrophoretic, antigenic and kinetic properties were found to be the same as for red cell enzyme C [10]. Studies with dog, rat and human kidney homogenates indicated that about 3—5% of the carbonic anhydrase was in the microsomal fraction [9,10]. The finding of particulate carbonic anhydrase was especially interesting since it has been proposed that bicarbonate reabsorption in the proximal tubules is accelerated by carbonic anhydrase bound to the luminal side of the tubule membrane [17]. This membrane surface is densely populated with microvilli of the brush border, so we attempted to isolate a particulate fraction which is enriched in brush border to see if carbonic anhydrase is associated with it.

Methods

Preparation of kidney membranes

Normal human kidneys were obtained from autopsy at Jackson Memorial Hospital, Miami, Fla. with the cooperation of Dr. A. Morales, Chairman of Pathology at the University of Miami. Most of them were frozen for storage. Approximately 500 g of kidney was processed at a time. After thawing the frozen kidneys, ureters, pelves, and large arteries and veins were stripped out and discarded. The remaining tissue was cut up and homogenized in four volumes of cold standard assay buffer (100 mM triethanolamine sulfate/83 mM Na_2SO_4/ 1 mM EDTA, pH 8.2) by a Waring blendor at high speed for six minutes with intermittent periods for cooling on ice.

The homogenate was fractionated by differential sedimentation. Four fractions were sequentially sedimented at the following speeds and durations: fraction I, 16 000 $\times g$ for 15 min; fraction II, 27 000 $\times g$ for 2 h; fraction III, 27 000 $\times g$ for 6 h; fraction IV, 100 000 $\times g$ for 1 h. Each particulate fraction was washed three times by resuspension in greater than four times their volume in standard assay buffer and centrifuged again. The first wash supernatant was added to the next higher fraction. The second centrifugation was done at higher speed to obtain a clear supernatant.

In order to demonstrate the efficacy of the washing procedure in removing

soluble protein, a small preparation was monitored for the release of ^3H-labeled human carbonic anhydrase B which was added as a marker for soluble protein. The labeled protein was prepared by reacting enzyme B with iodo-[^3H]acetate under conditions reported for reaction with bromoacetate [18]. Kidney material (20 g) was fractionated as before after labeled enzyme was added ($5 \cdot 10^6$ cpm) before the Waring blendor treatment. After washing, the fractions were assayed for carbonic anhydrase activity and for radioactivity. Tritium was measured by liquid scintillation using a medium containing 15 g PPO, 0.3 g POPOP in 2 liters of toluene plus 1 liter of Triton X-100 [19]. Samples were first dissolved in 1 M NaOH; 0.05 ml aliquots were counted. Quenching was assessed by recounting vials after addition of a known amount of pure labeled enzyme containing a relatively large amount of tritium.

Assays

The CO_2-hydrating activity of carbonic anhydrase was measured from the rate of change of pH after addition of CO_2 into the assay buffer containing enzyme at 2°C [20]. The reaction was initiated by adding 1 ml of 90 mM Na_2SO_4 saturated with CO_2 at atmospheric pressure to 4 ml of enzyme in assay buffer (100 mM triethanolamine/83 mM Na_2SO_4/1 mM EDTA, pH 8.2) in a water jacketed cell and stirred with a magnet. The decrease in pH was recorded from a Corning model 10 pH meter. The time required to produce a given pen deflection (corresponding to about 0.03 pH units) was determined, and an activity unit was defined as $t_b/t_e - 1$ where t_b is the time for the blank and t_e is the time with enzyme. The blank was determined in the absence of added enzyme or in the presence of a carbonic anhydrase inhibitor, acetazolamide.

Alkaline phosphatase activity was measured using kit number 109 from Sigma Chemical Co., St. Louis. This employs *p*-nitrophenyl phosphate as substrate in a glycine buffer at pH 10.5. Since alkaline phosphatase requires Zn^{2+} and the membranes were prepared in buffer containing 1 mM EDTA, the membrane aliquot was preincubated for 30 min at 37°C in assay media with 1 mM $ZnCl_2$ added.

Succinate-cytochrome *c* reductase was assayed as described by Tisdale [21]. The reduction of cytochrome *c* was followed in a Cary 14 spectrophotometer equipped with a scattered transmission accessory to minimize loss of energy by scattered light. Latent activity due to intact mitochondria was assessed after treatment with Lubrol PX [22].

Protein concentration was determined from the absorbance values at 260 nm and 280 nm [23]. Since the fractions of interest were not soluble, they were first dissolved by warming in 10% dodecyl sulfate. Absorbance by the detergent was moderate and was subtracted from the readings with protein. A standard curve of the absorbance parameter vs concentration of added bovine serum albumin was linear up to mg per ml. For a mixture of membrane fractions III and IV, the protein concentration determined by absorbance was standardized against the total protein determination from amino acid analysis and found to be high be a factor of 1.30.

Other proteins and reagents

Bis (2-hydroxyethyl) imino-tris (hydroxymethyl) methane (Bis-Tris), do-

decyl sulfate, Triton X-100, Lubrol PX, sodium deoxycholate, bovine heart cytochrome *c*, bovine serum albumin and assay kits for alkaline phosphatase were purchased from Sigma Chemical Co. Acetazolamide was obtained from Aldrich, and iodo[2-^{3}H]acetic acid was from New England Nuclear. Human erythrocyte carbonic anhydrases were prepared as described [18].

Results

Cell fractionation

Four particulate fractions were obtained by differential sedimentation and washed three times in standard assay buffer (pH 8.2) to remove soluble proteins. Since most of the carbonic anhydrases in the kidney are soluble kidney and red blood cell enzymes, the washing must be thorough. In initial experiments, 0.25 M sucrose was used for homogenization and washing, but only about 10% of the carbonic anhydrase activity was washed off fraction III membranes in the first wash with sucrose. Over 80% of the activity was removed by one wash with assay buffer.

After the fractions were washed, they were assayed for carbonic anhydrase and marker enzymes for mitochondria and brush border (Table I). On the basis of estimates of hemoglobin absorption at 420 nm, about 50% of the soluble carbonic anhydrase activity was due to red cells. Fraction IV contained 17 units of carbonic anhydrase activity per gram of kidney. The distribution of particulate carbonic anhydrase is similar to that of the brush border marker, alkaline phosphatase. The nine-fold enrichment of alkaline phosphatase in fraction IV is about the same as that achieved in other brush border preparations [24].

The distribution of the mitochondrial marker enzyme, succinic-cytochrome *c* reductase, is different from that of the particulate carbonic anhydrase. The maximum activity of the mitochondrial marker can be assessed only if the

TABLE I

DISTRIBUTION OF ENZYMES IN FRACTIONS FROM DIFFERENTIAL SEDIMENTATION

The homogenate of 500 g of kidney was fractionated by differential centrifugation: I, 16 000 × *g* for 15 min; II, 27 000 × *g* for 120 min; III, 27 000 × *g* for 360 min; IV, 100 000 × *g* for 60 min. The fractions were washed with assay buffer four times. The numbers in parentheses were calculated after subtracting the activities of the soluble enzymes from the total activity in the homogenate.

A. Results as per cent of the total volume or activity in the homogenate.

B. Activity per mg protein normalized to 1.00 for the homogenate.

	Fraction	Volume	Carbonic anhydrase	Alkaline phosphatase	Succinate-cytochrome c reductase
A.	I	5.9 (62)	1.1 (18)	6.8 (8)	57 (65)
	II	1.2 (12)	0.9 (14)	5.8 (7)	13 (15)
	III	1.5 (16)	1.6 (28)	16 (20)	18 (20)
	IV	1.0 (10)	2.5 (42)	54 (65)	0.4 (1)
	Super	92	95	18	12
B.	I		0.032 (0.6)	0.42 (0.5)	3.5 (4.0)
	II		00.075 (1.3)	0.67 (0.8)	1.4 (1.6)
	III		0.24 (4.2)	3.00 (3.6)	0.3 (0.4)
	IV		0.32 (5.7)	8.80 (10.6)	0.1 (0.1)

mitochondria are damaged or broken open so that the large substrate, cytochrome *c*, can get inside. Further treatment of these mitochondria by sonication or Lubrol PX did not produce any increase in activity. Therefore, the mitochondria were not intact and the results do not bear upon the unresolved issue of the existence of soluble carbonic anhydrase in mitochondria.

A separate experiment was carried out with a soluble, ^{3}H-labeled protein which was added before homogenization to serve as a marker for cytoplasmic enzymes. The labeled protein was chemically modified carbonic anhydrase B from human erythrocytes. Enzyme B was chosen to assess nonspecific binding since the particulate enzyme was C-type and might be the soluble enzyme in a specific membrane site. Also, the activity of the labeled protein was too low to interfere with measurements of carbonic anhydrase activity in any of the fractions. Washed fractions III and IV contained 3.6% and 1.3% of the carbonic anhydrase activity and 0.4% and 0.3% of the ^{3}H, respectively. The amount of tritium in these particulate fractions is only a small percentage of the total, and the degree of contamination could be overestimated in this experiment if a trace of labeled protein was denatured and sedimented with the particulate fractions. These results indicate that most of the carbonic anhydrase in the particulate fractions cannot be attributed to nonspecific contamination by soluble enzymes.

Some experiments were done using homogenates of kidney which had not been frozen. Compared with homogenates of frozen kidneys, this preparation contained about the same amount of particulate carbonic anhydrase but about twice as much total carbonic anhydrase activity.

Inhibition of activity

At pH 8.2 and 2°C, the inhibition by 60 mM I^- was 93% with red cell enzyme B and 48% with enzyme C or with the membrane enzyme. Assays with 50 mM Cl^- gave 85% inhibition of enzyme B, but no detectable inhibition of enzyme C or membrane enzyme. Deoxycholate (1%) caused 50% inhibition of red cell enzyme C and particulate enzyme. Triton X-100 did not inhibit any of the enzymes. The levels of these detergents were 5—10 times their critical micelle concentrations, so the concentrations of free detergent were maximal. The particulate enzyme was quite stable at 37°C with large concentrations of either detergent. All the enzymes are inhibited completely by low concentrations of acetazolamide.

Treatment of membranes at alkaline pH

Fraction III membranes were suspended in 150 mM $NaHCO_3$ at pH 8.2 and titrated to about pH 10.5 with dropwise addition of 150 mM NaOH. The membranes were then incubated at 37°C, cooled in ice water, and centrifuged at 3°C at 57 000 × *g* for 2 h. Only 19% of the protein and 19% of the carbonic anhydrase was released into the supernatant by incubation at pH 10.75 at 4°C for 1 h, but about 60% of the protein and 50% of the carbonic anhydrase was released in 15 min after increasing the temperature to 37°C. The release of protein or carbonic anhydrase at 37°C and pH 10.5 was not affected by doubling the incubation time or changing the protein concentration. If the particulate suspension is incubated at pH 10.5 and 37°C for 15 min and then titrated back

to pH 7.4 with 150 mM bis (2-hydroxylethyl) imino-tris (hydroxymethyl) methane (Bis-Tris) sulfate buffer (pH 5.0) and reincubated for 15 min at 37°C, only about 10% of the activity remained in the supernatant after centrifugation at 57 000 × *g* for 2 h. Pure human erythrocyte carbonic anhydrase C added to the membrane suspension remains soluble during these treatments.

The enzyme which was released at alkaline pH was characterized by gel filtration on a column of Sephadex G-100. In some cases the material was first sonicated to assure passage through the column. The sonication caused membrane suspensions to become nearly clear. Blue dextran and nitrophenol were used to indicate the positions for very high and very low molecular weight material. The G-100 column was calibrated with cytochrome *c*, carbonic anhydrase C, transferrin and Blue dextran.

The results of chromatography of membranes which had been subjected to the disaggregation procedure at pH 10.5 are shown in Fig. 1. Recovery of activity was 75%. The first peak of activity appeared in the void volume in fractions with high turbidity, indicating a molecular weight in excess of 150 000. The second peak of activity eluted in a position expected for a globular protein with a molecular weight of 30 000. The second peak represents only 10% of the total activity and is probably due to contaminating cytoplasmic enzyme.

Material in the first peak was considered to be free of contamination by soluble enzymes. In order to check this, the three peak tubes were pooled, incubated at 35°C for 30 min at pH 10.6 and a portion rechromatographed at pH 10.5 shortly thereafter. Essentially all of the activity eluted in the void volume. This experiment indicates that the particulate enzyme aggregates or has not been detached from large particles.

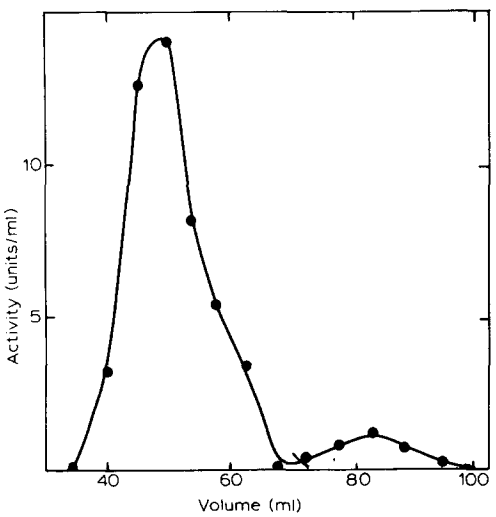

Fig. 1. Gel filtration of fraction IV after alkaline treatment. A sample of washed fraction IV (14 ml) was incubated at 37°C for 15 min in 100 mM sodium carbonate buffer, pH 10.5. It was then cooled, sonicated, and run on a 1.5 × 70 cm column of Sephadex G-100 at 6°C. The elution buffer was 100 mM sodium carbonate, pH 10.0.

Treatment of membranes with detergents

Deoxycholate and Triton X-100 were not effective in releasing the particulate carbonic anhydrase. After 11 h at 37°C in 10% Triton X-100 at pH 8.2, only 16% of the activity remained in the supernatant after centrifugation at 5°C (2 h at 57 000 × *g*) and the supernatant was slightly cloudy. Addition of 4% sodium deoxycholate to the Triton-containing medium resulted in the release of an additional 16% of the activity into the ultrasupernatant.

In order to find out if the carbonic anhydrase in the ultrasupernatant after treatment with Triton X-100 was really solubilized, one sample was chromatographed on Sephadex G-100. The sample was first treated at high pH to remove any contaminating soluble enzymes and then suspended in 4% Triton X-100 to give a detergent-protein ratio of 8. It was chromatographed in 1% Triton, and all the activity was recovered in the breakthrough peak. In a control experiment, human carbonic anhydrase C was chromatographed under these conditions. The position at which the C enzyme eluted was not affected by Triton.

Under more drastic conditions of Triton X-100 at higher pH the particulate enzyme was not completely stable. However, these conditions were useful for comparing its stability with those of the soluble enzymes. In these experiments, the particulate carbonic anhydrase was prepared from kidneys which had not been frozen.

The rate of inactivation of fraction IV enzyme is an order of magnitude slower than the rates for the red cell enzymes (Fig. 2). Inactivation of a mixture of red cell enzyme C and fraction IV gives a biphasic curve (Fig. 3a) and the activity associated with the slower inactivation is quantitatively the same as the amount of membrane enzyme activity added. It is clear that the membranes do not confer special stability to the red cell enzyme.

A similar biphasic curve is obtained when whole kidney homogenate is treated this way (Fig. 3b). The fraction which is inactivated slowly accounts for about 1% of the total activity in the homogenate. This corresponds to about 15 units of activity per gram of kidney.

The particulate enzyme also displays unusual stability in the presence of dodecyl sulfate. Again, kidneys were used which had not been frozen. The effect of 4% dodecyl sulfate on carbonic anhydrase in kidney homogenate containing 2.3% protein is shown in Fig. 4. Most of the activity is lost immediately, but about 1% is resistant. The stable carbonic anhydrase again corresponds to about 15 units of activity per gram of kidney. In contrast, the carbonic anhydrase activity in the ultrasupernatant (obtained by centrifuging the homogenate at 57 000 × *g* for 2 h) is completely lost upon addition of the detergent. Almost no carbonic anhydrase activity is lost within 1 h after addition of dodecyl sulfate to fraction IV membranes at 4°C or 25°C, and more than half the activity remains after 24 h at 14°C with a weight ratio of dodecyl sulfate to protein of 25.

This stability of particulate carbonic anhydrase made it possible to characterize the enzyme by gel filtration in the presence of dodecyl sulfate. A sample of fraction III + IV membranes containing 2.5 mg protein per ml and 63 mg dodecyl sulfate per ml was chromatographed on Ultragel AcA-44 (Fig. 5). A small amount of activity eluted in the void volume, but 85% eluted later. Al-

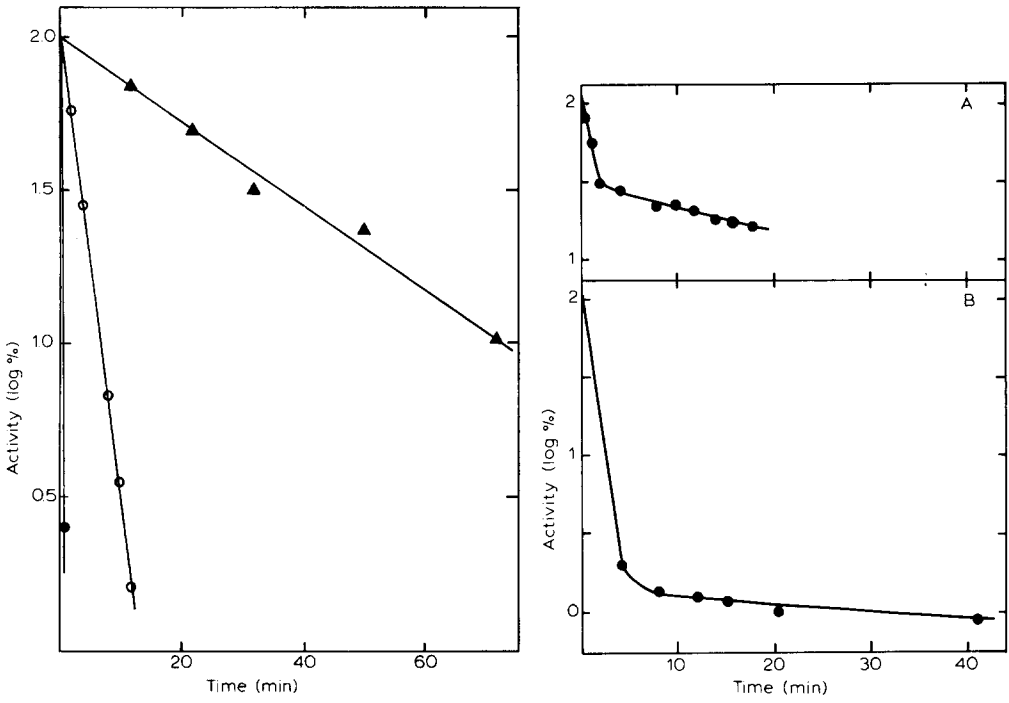

Fig. 2. Inactivation of carbonic anhydrases in alkaline Triton X-100. Triton X-100 was added to enzymes in assay buffer to give a final concentration of 10% detergent by weight. This was titrated with 0.5 M NaOH to pH 12.3 at 2°C. At zero time, the solution was placed in a water bath at 37° C. At that temperature, the pH was 11.3 as measured with a sodium-insensitive glass electrode. Aliquots were then diluted into assay buffer at 2°C for measurement of CO_2-hydrating activity. The enzyme solutions contained 0.012 mg per ml red cell carbonic anhydrase C (●), 0.34 mg per ml red cell enzyme B (○), or fraction IV (▲) containing 8.5 mg protein per ml.

Fig. 3. Inactivation of mixtures of carbonic anhydrases in alkaline Triton X-100. These experiments were carried out as in Fig. 2, except as noted. (A) Red cell carbonic anhydrase C, 0.007 mg per ml, 60 units activity per ml; fraction IV, 15 mg protein per ml, 30 units activity per ml. (B) Homogenate of whole kidney, 24 mg protein per ml, 1250 units activity per ml. The homogenate with detergent was titrated to pH 12.85 at 0°C and warmed to 37°C, where the pH reading was 11.8.

though the concentration of detergent in the eluting buffer was lower than that in the sample, the detergent-protein ratio at the major peak of carbonic anhydrase activity was about 30. A calibration mixture containing Blue dextran, bovine serum albumin, ovalbumin, red cell carbonic anhydrase, and myoglobin was then chromatographed in the same buffer, but without the detergent. If the calibration obtained in the absence of detergent is applied to the results in Fig. 4, the position of the major peak intersects the calibration curve at a point corresponding to a molecular weight of 66 000. These results cannot be used to determine the molecular weight of the particulate enzyme. However, by assuming that dodecyl sulfate does not increase the porosity of the gel, the molecular weight is not likely to be greater than 66 000 and it could be close to the molecular weights of the soluble enzymes.

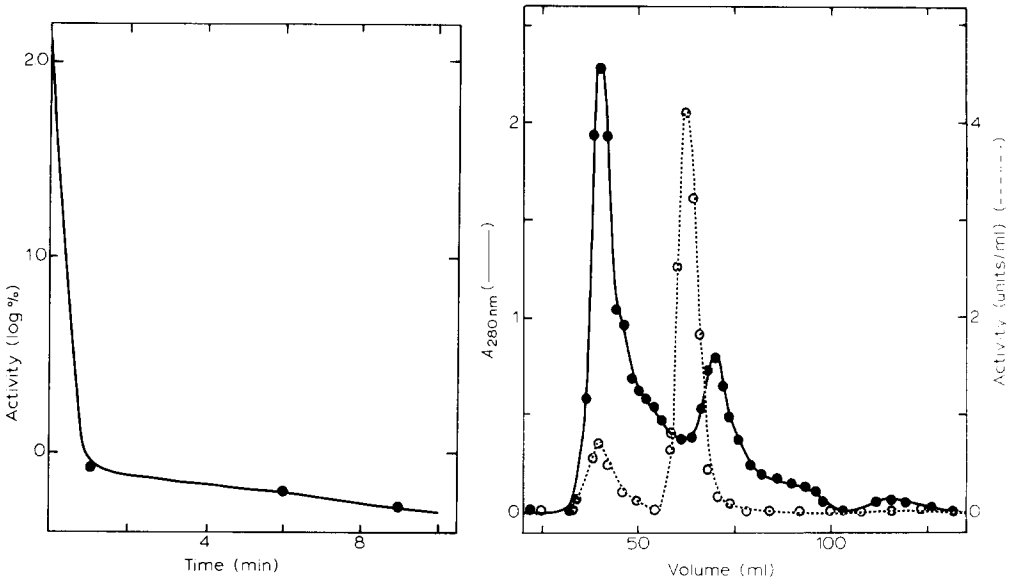

Fig. 4. Inactivation of carbonic anhydrase in kidney homogenate by dodecyl sulfate. Detergent was added to the homogenate in assay buffer (pH 8.2) at 0°C. Final concentrations were 32 mg protein per ml and 40 mg dodecyl sulfate per ml. At zero time, it was put in a 25°C water bath. Aliquots were assayed for CO_2-hydrating activity at 2°C.

Fig. 5. Gel filtration of kidney membrane carbonic anhydrase in dodecyl sulfate. A sample of fraction III + IV (6 ml) containing 2.5 mg protein per ml and 63 mg dodecyl sulfate per ml in assay buffer (pH 8.2) was left at 25°C for 1 h and then chromatographed on a 1.5 × 87 cm column of Ultragel AcA-44 at 14°C. The eluting buffer was assay buffer with 10 mg dodecyl sulfate per ml.

Discussion

The objectives of these studies were to verify the existence of a particulate carbonic anhydrase in the human kidney and to begin a characterization of the enzyme and the nature of its binding to membrane. Human kidney was chosen so the kidney enzyme could be compared with the well-characterized human red cell enzymes.

Since the particulate enzyme accounts for only a small fraction of the total carbonic anhydrase activity in the kidney, the particulate fractions had to be thoroughly washed to remove adsorbed soluble enzymes. Maren and Ellison washed the particulate fractions from dog and rat kidney homogenates with 0.25 M sucrose, and this was effective in washing off endogenous soluble enzymes as well as added soluble enzymes [9]. With homogenates prepared from frozen human kidneys, however, we found that sucrose was not effective. A buffer of relatively high pH and ionic strength proved to be suitable and our washed microsomal fraction contained about 17 units of carbonic anhydrase activity per gram of kidney. We also found that about 15 units of activity per gram of kidney was resistant to denaturation by detergents and that this activity was due to the particulate enzyme. These results agree with those of Maren and Ellison who found 12 units/g of dog or rat kidney [9]. This amounts to 3% of the total activity in homogenates of fresh, perfused dog

kidney. In another study, about 5% of the carbonic anhydrase was found in particulate fractions of homogenates of perfused human kidney [10].

Although only 2—3% of the kidney carbonic anhydrase is particulate, two lines of reasoning suggest that it may be physiologically significant. First, 15 units of carbonic anhydrase activity in our assay would produce about a 6-fold enhancement in the rate of hydration in 1 cm^3 at 37°C. If the particulate enzyme is concentrated in certain regions of the kidney, then the effective local catalytic enhancement would be higher. Secondly, normal kidney function is not impaired until over 99.9% of the kidney carbonic anhydrase is inhibited [25], so 2% of the activity is potentially quite significant. Furthermore, the importance of the membrane enzyme would be underestimated in the inhibition experiments if it is more resistant to inhibition than soluble carbonic anhydrase.

The localization of the particulate enzyme in the kidney is not known. The existence of a luminal carbonic anhydrase in the proximal tubule has been questioned on experimental and theoretical grounds [26,27], and our results cannot be used as evidence for or against it. Even if the enzyme is located on the brush border, these results do not indicate whether it is on the luminal or cytoplasmic side of the membrane. Nevertheless, bicarbonate reabsorption is a membrane function, so the localization of some carbonic anhydrase at the site of action would be advantageous.

This particulate carbonic anhydrase is more difficult to solubilize than many other enzymes associated with membranes. Alkaline extraction has been effective in solubilizing protein from several membrane preparations [28], and some brush border enzymes have been solubilized with deoxycholate or Triton X-100 [29], but these treatments did not solubilize this carbonic anhydrase. The solubilization achieved with dodecyl sulfate takes advantage of the exceptional stability of the particulate enzyme in this solvent. Although enzyme stability in dodecyl sulfate has been observed [30], most soluble enzymes (including soluble carbonic anhydrases) are rapidly denatured by this detergent at high detergent-protein ratios. The results of gel filtration of the particulate carbonic anhydrase in 1% dodecyl sulfate suggest that it has a molecular weight of 66 000 or less, so its unusual stability is not due to association with large membrane fragments or vesicles. Its stability is more likely to be due to specific enzyme-detergent interactions which mimic enzyme-membrane interactions.

The reasons for the strong association of particulate enzyme with membrane and its stability in dodecyl sulfate are not known. The particulate enzyme could be soluble kidney enzyme which is bound in a specific membrane site and remains associated with a small membrane component in dodecyl sulfate. However, the soluble kidney enzyme is the same as red cell enzyme C [10], and we did not find any significant binding of enzyme C added to washed or alkaline-treated membranes. A likely alternative is that this isozyme is different than the soluble carbonic anhydrases.

Acknowledgements

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