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Renal Cortical Basolateral Na^+/HCO_3^- Cotransporter: I. Partial Purification and Reconstitution

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Abstract. The renal basolateral Na^+/HCO_3^- cotransporter is the main system responsible for HCO_3^- transport from proximal tubule cells into the blood. The present study was aimed at purifying and functionally reconstituting the Na^+/HCO_3^- cotransporter protein from rabbit renal cortex. Highly purified rabbit renal cortical basolateral membrane vesicles (hereafter designated as original basolateral membrane), enriched 12-fold in Na-K-ATPase, were solubilized in 2% octylglucoside, and then reconstituted in L-α-phosphatidylcholine (proteoliposomes). Na^+/HCO_3^- cotransporter activity was assessed as the difference in ^{22}Na uptake in the presence of HCO_3^- and gluconate. The activity of the Na^+/HCO_3^- cotransporter was enhanced 18-fold in the solubilized protein reconstituted into proteoliposomes compared to the original basolateral membranes. The reconstituted solubilized purified protein exhibited kinetic properties similar to the cotransporter from original basolateral membranes. In addition, it was like the original cotransporter, inhibited by disulfonic stilbene SITS, and was electrogenic. The catalytic subunit of protein kinase A significantly inhibited Na^+/HCO_3^- cotransporter activity in proteoliposomes. The octylglucoside-solubilized protein was further purified by hydroxylapatite column chromatography, and this resulted in an additional enhancement of Na^+/HCO_3^- cotransporter activity of 80-fold over the original basolateral membranes. The fractions containing the highest activity were further processed by glycerol gradient centrifugation, resulting in a 124- to 300-fold increase in Na^+/HCO_3^- cotransporter activity compared to the original basolateral membranes. SDS-PAGE analysis showed an enhancement of a protein doublet of 56 kD MW in the glycerol gradient fraction. Our results demonstrate that we have partially purified and reconstituted the renal Na^+/HCO_3^- cotransporter and suggest that the 56 kD doublet protein may represent the Na^+/HCO_3^- cotransporter.

Key words: Na^+/HCO_3^- cotransporter — Purification— Reconstitution — Proteoliposomes — Basolateral membranes — Cellular transport

Introduction

To completely understand the mechanisms responsible for acidification in the proximal tubule, it is important to purify the proteins involved in the acid-base transport. The Na^+/HCO_3^- cotransporter has been recognized as the main system responsible for HCO_3^- transport out of the proximal tubule cells into the blood and plays an important role in HCO_3^- transport under physiologic and pathophysiologic conditions [1]. The ionic mechanism of the Na^+/HCO_3^- cotransporter has been characterized, and it has been well established that the system functions via 1:1:1 cotransport of $CO_3^=$, HCO_3^- and Na^+ on different sites [18]. This system functions in an integrated fashion with the brush border Na^+-H^+ antiporter and the activities of these two systems vary in parallel [2]. The activity of the renal Na^+/HCO_3^- cotransporter is stimulated by acidosis and inhibited by alkalosis [15]. In addition, cyclic AMP and calmodulin inhibit while

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protein kinase C stimulates the Na^+-H^+ antiporter and the Na^+/HCO_3^- cotransporter in a symmetric fashion [16]. Despite the importance of the Na^+/HCO_3^- cotransporter in physiologic and pathophysiologic conditions, the protein has not been purified. We used a systematic approach to purify and reconstitute the Na^+/HCO_3^- cotransporter from renal cortical basolateral membranes. Our results showed a 124- to 300-fold purification of protein fractions exhibiting Na^+/HCO_3^- cotransporter activity.

Material and Methods

Materials

^{22}Na was purchased from Amersham. L-α-phosphatidylcholine, octylglucoside, molecular weight standards, Coomasie R-250, SDS, acrylamide, bisacrylamide and catalytic subunit of protein kinase A were bought from Sigma. Hydroxylapatite (Biogel HT™), was purchased from Biorad. General laboratory chemicals were of analytical grade and were obtained from Sigma or Fisher.

Basolateral Membrane Preparation

Renal cortical basolateral membrane vesicles were prepared from New Zealand White rabbits by means of differential and gradient centrifugation with ionic precipitation as described previously [20]. This procedure results in highly purified basolateral membranes enriched on the average of 12- to 14-fold in Na-K-ATPase activity compared with homogenates, and with less than 5% cross contamination with brush border membranes.

Solubilization of Renal Cortical Basolateral Membrane and Purification by Hydroxylapatite Chromatography and Glycerol Gradient Centrifugation

One part purified basolateral membrane vesicles (5 mg/ml) was incubated with 1.25 parts 2% *n*-octyl β-D-glucopyranoside (octylglucoside) in 50 mM HEPES and 250 mM mannitol (buffer A), pH 7.2, on ice for 15 min. The concentration of the detergent octylglucoside was 1.1% and the protein concentration was 2.2 mg/ml. The protein-detergent mixture was spun for 30 min at 110,000 × *g*. The supernatant which was recovered was dialyzed extensively for 18 hr in buffer A using a membrane with molecular weight cut off of 6–8 kD. Twenty milligrams of solubilized protein was loaded onto a hydroxylapatite column (20 cm × 5 cm) previously prepared and washed sequentially with 50 ml each of 0.5 M Tris Cl pH 7.2, 50 ml distilled water and 50 ml of buffer HA containing 10 mM Tris Cl, pH 7.2, 0.2% OG, 0.5 mM EDTA, 10% glycerol and 10 mM DTT. The protein was eluted with a continuous gradient of Na_2HPO_4 (0–0.4 M, 100 ml total), pH 7.20 prepared in buffer HA. The fractions which contained Na^+/HCO_3^- cotransporter activity were treated with $(NH_4)_2SO_4$ to a final concentration of 45% and then centrifuged at 100,000 × *g* for 35 min. The pellet resuspended in buffer HA was loaded on a 15–35% (w/w) continuous glycerol gradient and then centrifuged at 170,000 × *g* in a Beckman SW 41 rotor for 22 hr at 4°C. In each purification step, the protein fractions were collected concentrated, the protein measured, and stored at −70°C. The protein fractions from purification steps were subjected to 10–12% SDS-PAGE. Likewise, each purified protein fraction was reconstituted into liposomes and Na^+/HCO_3^- cotransporter activity measured.

Reconstitution of Solubilized and Purified Protein Fraction into Liposomes

The protein (2.5 mg/ml) was reconstituted into liposomes by mixing 1.6 part of protein (v/v) with one part of L-α-phosphatidylcholine (35 mg/ml) which was sonicated for 10 min. The combination of proteins and lipids was dialyzed for 18 hr in buffer A using a membrane restricting the passage of 6–8 kD molecules. In all uptake assays, the amount of reconstituted solubilized protein was kept constant at 5 µg in each tube. ^{22}Na uptake was performed in the presence of $NaHCO_3$ or Na gluconate in the original basolateral membrane vesicles (BLMV), in liposomes and in the proteoliposomes, as will be described below.

Measurement of Na^+/HCO_3^- Cotransporter Activity

HCO_3^--dependent ^{22}Na uptake (a measurement of Na^+/HCO_3^- cotransporter activity) was measured at 3 sec by the rapid-filtration technique as previously described [15]. In brief, basolateral membrane vesicles or proteoliposomes were preincubated for 1–2 hr at room temperature in a solution containing 200 mM sucrose, 50 mM HEPES, pH 7.50 with Tris and 1 mM Mg gluconate. The suspension was spun at 30,000 revolutions/min with a Beckman Ti 50.2 rotor for 30 min at 4°C, and the resulting pellet was resuspended in the same solution. The assay was started by addition of reconstituted proteins to uptake medium containing (in mM) 40 Na gluconate, 60 K gluconate, 1 Mg gluconate, and 50 HEPES, pH 7.50 with KOH, as well as 1 µCi $^{22}NaCl$ in the presence of 25 mM HCO_3 or gluconate. After 3 sec incubation at room temperature, the reaction was stopped by adding 4 ml ice-cold stop solution containing 200 mM sucrose and 50 mM HEPES, pH 7.50 with Tris, and subsequently poured on a 0.45-µm pore size prewetted Millipore filter. Filters were washed three times more, and radioactivity was measured by scintillation spectroscopy. Na^+/HCO_3^- cotransporter activity (HCO_3-dependent ^{22}Na uptake) was taken as the difference in ^{22}Na uptake in the presence or absence of an inwardly directed HCO_3^- gradient (HCO_3^- was replaced by gluconate).

Regulation of the Reconstituted Na^+/HCO_3^- Cotransporter by cAMP-PKA

Solubilized proteins (2.5 mg/ml) were incubated in phosphorylating solution containing 100 µM of Mg, 50 µM of ATP and catalytic subunit (CSU) of cAMP-dependent protein kinase A (40 mU/ml). The reaction was carried out at 30°C for 15 min, and terminated by the addition of cold asolectin. The phosphorylated solubilized proteins were reconstituted into proteoliposomes, and HCO_3^--dependent ^{22}Na uptake was measured as described above.

SDS-Polyacrylamide Gel Electrophoresis Analysis

SDS-PAGE was carried out on discontinuous systems as described by Laemmli [11]. Low molecular weight standards were used (from 14 to 92 kD). Electrophoresis was done on slab gels of 10–12% acrylamide. Coomassie Brilliant Blue or silver stains were used as indicated.

Data Analysis

Results are presented as mean ± SEM. The *t*-test for paired or unpaired data was used to analyze the data wherever appropriate.

Results

Solubilization of Basolateral Na^+/HCO_3^- Cotransporter with Octylglucoside

The experimental design to achieve purification of the rabbit renal basolateral Na^+/HCO_3^- cotransporter included trials with different detergents to determine the best detergent which solubilizes this protein and yet maintains the activity of the Na^+/HCO_3^- cotransporter [9]. In particular, it was important to find the optimal detergent to protein ratio which is a critical step in achieving successful solubilization.

Initial studies included attempts at solubilizing renal cortical basolateral membranes with different detergents (Triton X, SDS and octylglucoside). Of these detergents, only octylglucoside, resulted in solubilization of the basolateral membranes while maintaining Na^+/HCO_3^- cotransporter activity. In initial experiments we found that ^{22}Na uptake (measured at 3 sec with 40 mM Na) was always higher in the presence of HCO_3 than in the presence of gluconate (11.9 ± 1.6 *vs.* 6.1 ± 1.5 nmol/mg protein/3 sec, $P < 0.001$). The difference was taken as HCO_3-dependent ^{22}Na uptake, a measure of Na/HCO_3 activity.

Figure 1 shows the time course of HCO_3^--dependent ^{22}Na uptake in proteoliposomes. It is clear that ^{22}Na uptake increased with time and reached a plateau at 15 sec and thus all experiments were performed at 3 sec, the linear phase of uptake. Figure 2 shows a summary of Na^+/HCO_3^- cotransporter activity (expressed as HCO_3^--dependent ^{22}Na uptake at 3 sec) in basolateral membranes and in octylglucoside-solubilized membranes reconstituted in L-α-phosphatidylcholine (proteoliposomes). Na^+/HCO_3^- cotransporter activity was increased, on the average 18-fold (range 3–40), in proteoliposomes compared to the original basolateral membranes (17.8 ± 5.4 *vs.* 1.0 ± 0.2 nmol/mg protein/3 sec, $P < 0.01$). It is clear from the Table that on the average 89% of the total activity of the Na^+/HCO_3 cotransporter was recovered in the solubilization step. When the extravesicular osmolality was raised by 200 mOsm, HCO_3-dependent ^{22}Na uptake decreased by 40%, suggesting that the uptake was occurring into vesicular space. To exclude the possibility of ^{22}Na binding to the protein, ^{22}Na uptake was done in proteoliposomes after incubation with 8% octylglucoside to completely dissolve the lipids. Under these conditions, ^{22}Na uptake decreased to background levels, suggesting no detectable binding to the protein.

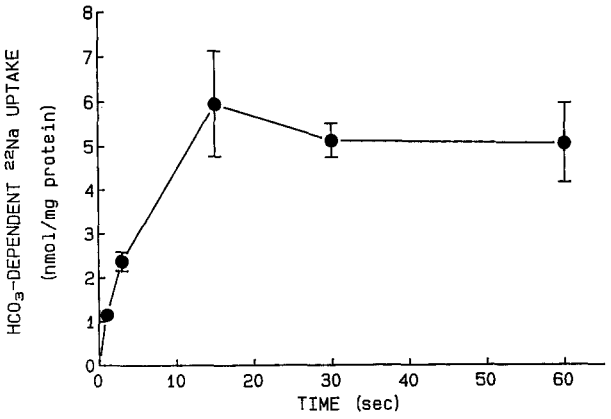

Fig. 1. Time course of HCO_3^--dependent ^{22}Na uptake by octylglucoside-solubilized protein reconstituted into proteoliposomes. HCO_3^--dependent ^{22}Na uptake was measured in the proteoliposomes at different time points. HCO_3^--dependent ^{22}Na uptake assay was done by rapid filtration technique as described in Materials and Methods. The figure depicts the average of five different experiments measured in triplicates in different proteoliposome preparations.

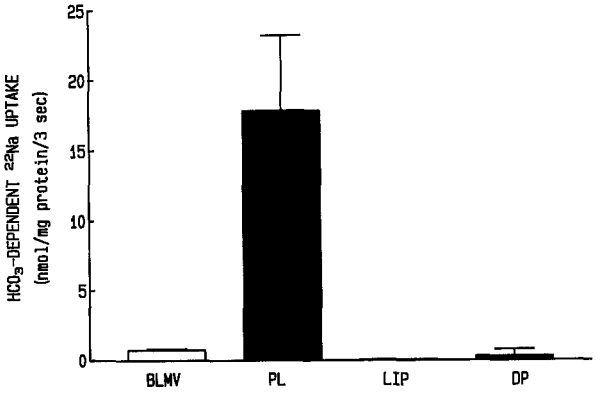

Fig. 2. HCO_3^--dependent ^{22}Na uptake in proteoliposomes. HCO_3^--dependent ^{22}Na uptake in original basolateral membranes (*BLMV*), octylglucoside-solubilized protein reconstituted into proteoliposomes (*PL*), liposomes without protein (*LIP*) and denatured solubilized protein (*DP*) reconstituted into liposomes. Basolateral membranes were solubilized with 2% octylglucoside as described in Materials and Methods. The solubilized proteins were either directly reconstituted into or boiled for 15 min and then later reconstituted into proteoliposomes. L-α-phosphatidylcholine alone (LIP) or blank liposome contained no protein. The results shown represent the average of at least four different experiments done in triplicates in various proteoliposome preparations.

Octylglucoside solubilization resulted in significant loss of peripheral membrane proteins (Table). In liposomes containing no protein or in liposomes reconstituted with denatured protein, no Na^+/HCO_3^- cotransporter activity was detected (Fig. 2). When the amount of protein in the liposome was varied to increase the protein/lipid ratio, a linear relationship between the activity of the Na^+/HCO_3^- cotransporter and lipid protein ratio was observed with a correlation coefficient of 0.98 (Fig. 3).

Table. Purification of Na^+-HCO_3^- cotransporter from renal basolateral membrane

Purification Step	Protein (mg)	Recovery (%)	Na^+-HCO_3^- activity (nmol/mgPr/3 sec)	Enrichment of activity	Recovery of total activity (%)
BLMV	400	100	1.0 ± 0.18	1	100
OG	20	5	17.8 ± 5.4	18	89
HAC	4	1	80 ± 4.4	80	80
GG Fraction 1	1	0.2	300	300	75
Fraction 5	0.5	0.1	124	124	15

Highly purified renal cortical basolateral membrane vesicles were solubilized in 2% octylglucoside and subsequently processed through hydroxylapatite column chromatography and glycerol gradient centrifugation. Basolateral membrane vesicle proteins (BLMV), octylglucoside-solubilized proteins (OG), fractions from the hydroxylapatite column chromatography (HAC) and glycerol gradient centrifugation (GG) were reconstituted into proteoliposomes and assayed for HCO_3^--dependent ^{22}Na uptake by rapid filtration technique at 3 sec.

We performed additional experiments to show that the reconstituted protein has kinetic characteristics and inhibitor sensitivity similar to the original basolateral Na^+/HCO_3^- cotransporter. Figure 4 shows that the Na^+/HCO_3^- cotransporter activity increases as a function of Na^+ concentration. It is clear that Na^+ uptake increases with increasing Na^+ concentration reaching a maximum at 15 mM with an apparent K_m of 10.2 mM, a value similar to the original basolateral membrane cotransporter [3]. The original Na^+/HCO_3^- cotransporter is inhibited by disulfonic stilbene to a variable degree [16] and Fig. 5 (right panel) shows that SITS (10^{-4}M) inhibited Na^+/HCO_3^- cotransporter activity in proteoliposomes by 75% (from 8.5 ± .91 to 2.1 ± .20 nmol/mg protein/3 sec, $P < 0.05$).

In renal cortical basolateral membranes, the cotransporter is electrogenic and functions via 1:1:1 cotransporter of $CO_3^=$, HCO_3^- and Na^+ and thus carries a negative charge. We investigated whether the reconstituted cotransporter was electrogenic by performing experiments in the presence of a positive intravesicular potential generated by an inwardly directed K^+ gradient (outside K 85 mM, inside K 1 mM) and 7 μM valinomycin. Figure 5 (left panel) shows the Na^+/HCO_3^- cotransporter reconstituted in liposomes is electrogenic, as evidenced by an increase in the Na^+/HCO_3^- cotransporter activity from 8.5 ± 0.9 to 34.3 ± 6.7 nmol/mg protein/3 sec, $P < 0.01$). Taken together, the above results show that the octylglucoside resulted in solubilization and partial purification of the Na^+/HCO_3^- cotransporter (Table). In addition, the reconstituted Na^+/HCO_3^- cotransporter is electrogenic and has kinetic properties and inhibitor sensitivity very similar to that of the original basolateral membranes.

The Na^+/HCO_3^- cotransporter of basolateral membranes is inhibited by the cAMP/PKA system. To demonstrate that the reconstituted Na^+/HCO_3^- cotransporter is also regulated by PKA, we measured HCO_3-dependent ^{22}Na uptake in the absence and in the presence of catalytic subunit of PKA. The proteoliposomes

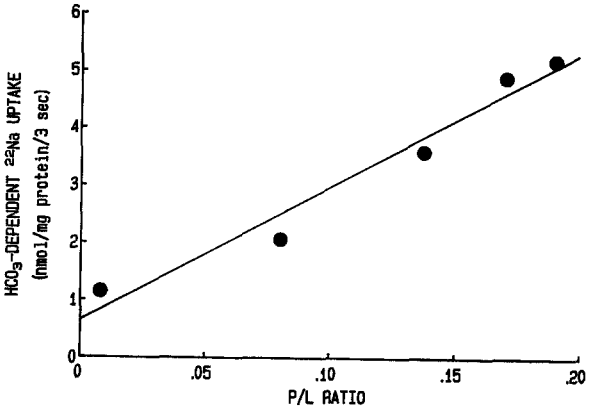

Fig. 3. HCO_3-dependent ^{22}Na uptake in proteoliposomes as a function of the amount of the reconstituted protein. Dependence of HCO_3^--dependent ^{22}Na uptake on the protein concentration reconstituted in proteoliposomes. Purified basolateral membrane vesicles were solubilized by 2% octylglucoside. Varying amounts of octylglucoside-solubilized proteins were reconstituted with fixed amounts of L-α-phosphatidylcholine into proteoliposomes. HCO_3^--dependent ^{22}Na uptake assay at 3 sec was done in each sample by rapid filtration technique. The values shown represent the average of three separate experiments measured in triplicates in different proteoliposome preparations.

were loaded with 50 mM ATP and 40 mU/ml of catalytic subunit of protein kinase A by the method of Weinman et al. (23) as previously described [16]. In five separate experiments, the CSU of PKA decreased the Na^+/HCO_3^- cotransporter activity from 54.9 ± 4.5 to 19.7 ± 6.0 nmol/mg protein 3 sec ($P < 0.01$). Thus, the reconstituted cotransporter protein, like the original basolateral membranes, is inhibited by CSU of PKA.

Purification of Na^+/HCO_3^- Cotransporter by Hydroxylapatite Chromatography and Glycerol Gradient Centrifugation

The basolateral membrane fraction solubilized by octylglucoside and enriched in the Na^+/HCO_3^- cotransporter

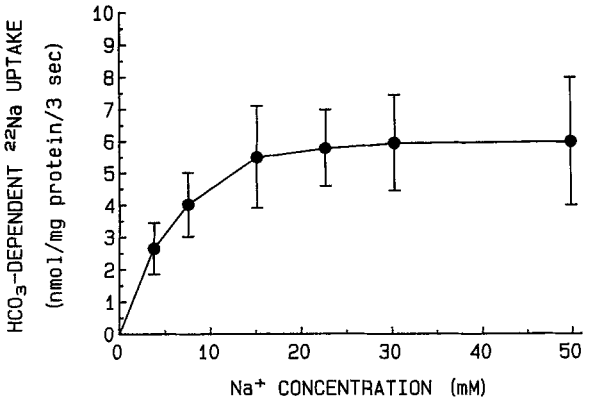

Fig. 4. HCO_3-dependent ^{22}Na uptake in proteoliposomes as a function of Na concentration. HCO_3^--dependent ^{22}Na uptake measured at 3 sec by rapid filtration techniques as described in Materials and Methods. Na^+ concentration was varied by replacing K gluconate with equimolar amounts of Na gluconate, thus maintaining the osmolality constant. The results shown represent the average of six separate experiments done in triplicates on different proteoliposome preparations.

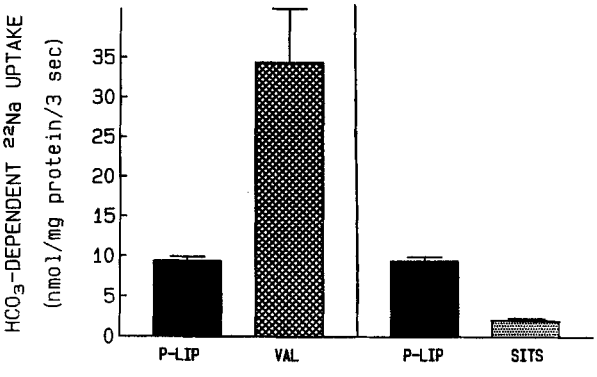

Fig. 5. HCO_3^--dependent ^{22}Na uptake in proteoliposomes in the presence of valinomycin and SITS. The effect of valinomycin (left panel) and SITS (right panel) on HCO_3^--dependent ^{22}Na uptake in solubilized protein reconstituted in liposomes. Reconstituted proteoliposomes were preincubated in the loading medium containing (in mM) 200 sucrose, 1 Mg gluconate, 50 HEPES pH 7.50 with Tris and 7 μM valinomycin for 2 hr at 25°C. Preloaded proteoliposomes were added in uptake medium consisting of (in mM) 50 HEPES, pH 7.5 with KOH, 1 Mg gluconate, 40 Na gluconate, 60 potassium gluconate, and 25 mM of either potassium bicarbonate or potassium gluconate and 7 μM valinomycin. In experiments involving SITS, proteoliposomes were preloaded as described above in Materials and Methods. SITS (0.5 mM) was added to the uptake medium alone when indicated. Each bar represents five different experiments measured in triplicates on various proteoliposome preparations.

was further purified by hydroxylapatite chromatography. Twelve fractions were collected (Fig. 6) and Na^+/HCO_3^- cotransporter activity was present in the first five fractions with the highest activity in fraction 1 (F_1). The Table shows that F_1 exhibits an 80-fold enhancement of activity over the basolateral membrane and a 4-fold enhancement over the octylglucoside pu-

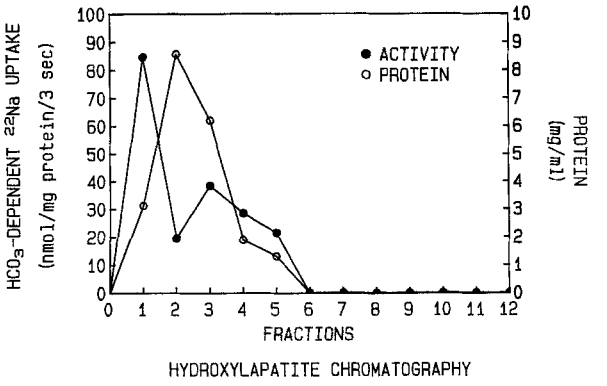

Fig. 6. HCO_3^--dependent ^{22}Na uptake in proteoliposomes prepared from the hydroxylapatite column chromatography purification. Purified basolateral membrane vesicles were solubilized by 2% octylglucoside in the presence of HEPES/mannitol buffer pH (7.2). Solubilized proteins were loaded onto hydroxylapatite column (1 ml hydroxylapatite/5 mg protein) and eluted by continuous gradient of Na_2 HPO_4 (0–0.4 M). Each eluted fraction was reconstituted into proteoliposomes as described in Materials and Methods and HCO_3^--dependent ^{22}Na uptake was measured in triplicates by rapid filtration technique. The elution profile shown is representative of four different hydroxylapatite chromatography experiments.

rification step. Hydroxylapatite chromatography resulted in only 1% recovery of protein (Fig. 6 and the Table). HCO_3-dependent ^{22}Na uptake in the F_1 fraction was inhibited 80% by SITS. The F_1 fraction of the hydroxylapatite chromatography was further purified by a glycerol gradient centrifugation step. Figure 7 shows that the highest Na^+/HCO_3^- cotransporter was present in F_1 and F_5 which showed an enhancement of 300- and 125-fold, respectively, over the original basolateral membranes. The other fractions showed less enrichment in Na^+/HCO_3^- cotransporter activity. There was substantial loss of protein with this purification step, with recovery of about 0.3% of the original protein in fractions F_1 and F_5. Figure 8 shows the SDS-PAGE analysis of the basolateral membranes and of fractions of each purification step. It can be seen that the glycerol gradient fraction shows an enhancement of a protein doublet of 56 kD molecular weight.

Discussion

This study was aimed at isolating the basolateral renal Na^+/HCO_3^- cotransporter, the main protein responsible for HCO_3 transport from proximal tubule cells into the blood. Despite its importance in HCO_3 transport, this protein has not been purified and functionally reconstituted. The kinetic characteristics and ionic requirements for this protein have been well described [1, 3, 8]. We undertook a systematic approach to solubilize and purify this protein from rabbit renal cortex. After trials with different detergents, we found that octylgluco-

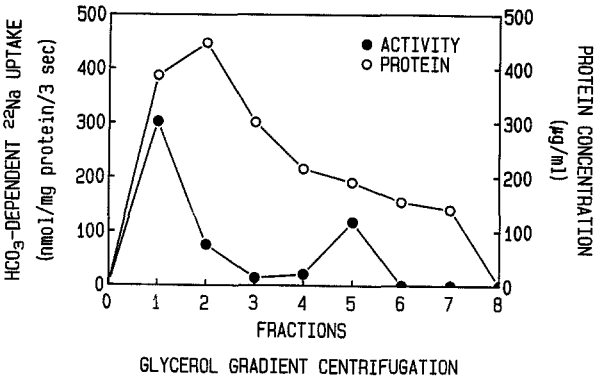

Fig. 7. HCO_3^--dependent ^{22}Na uptake in proteoliposomes prepared from the glycerol gradient centrifugation. Glycerol gradient centrifugation purification fractions containing Na^+-HCO_3^- cotransporter activity purified from hydroxylapatite chromatography were precipitated with $(NH_4)_2\ SO_4$ and resuspended in buffer HA, loaded on a 15–35% (w/w) continuous glycerol gradient and centrifuged at 170,000 × *g* for 22 hr. One ml fractions were collected and proteins measured. Each fraction was reconstituted into proteoliposomes and HCO_3^--dependent ^{22}Na uptake was assayed at 3 sec by rapid filtration technique. The graph is representative of three different glycerol gradient centrifugation experiments.

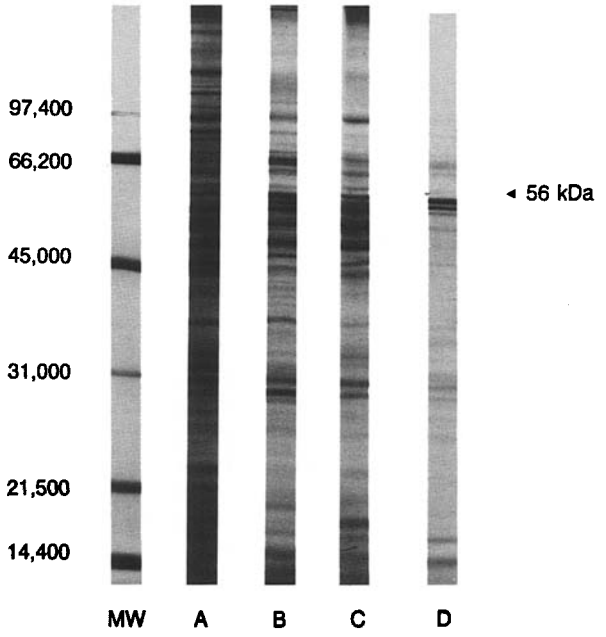

Fig. 8. SDS-PAGE analysis of the basolateral membrane fractions and each fraction from each purification step. (Lane *MW*) molecular weight markers. (Lane *A*) Basolateral membrane vesicles (30 μg). (Lane *B*) Octylglucoside basolateral membrane solubilized proteins (10 μg). (Lane *C*) Hydroxylapatite chromatography fraction (5 μg). (Lane *D*) Glycerol gradient centrifugation fraction (5 μg). Glycerol gradient fraction shows enhancement of a protein doublet of 56 kD molecular weight.

side resulted in solubilization of the basolateral membrane fraction with enhancement of Na^+/HCO_3^- cotransporter activity. Octylglucoside solubilization resulted in significant protein loss and an enrichment in Na^+/HCO_3^- activity on the average of 18, ranging from 3 to 40. Other investigators have also used octylglucoside to solubilize the renal brush Na^+-H^+ antiporter [23] and the bile acid transport protein from hepatocyte sinusoidal plasma membrane [22]. These investigators have reported an average enhancement of 5- and 22-fold for the Na^+/H^+ antiporter and the bile acid transporter, respectively. In our study, we successfully reconstituted the Na^+/HCO_3^- cotransporter in L-α-phosphatidylcholine and then determined that the reconstituted protein had characteristics similar to that of the original basolateral membranes. Evidence for successful reconstitution of this cotransporter was the finding that the activity of the cotransporter varied in a linear fashion with the amount of reconstituted protein and furthermore denatured protein reconstituted into liposomes was not associated with any detectable activity of the cotransporter.

It was important to demonstrate that the solubilized protein when reconstituted into proteoliposomes exhibited kinetic properties and inhibitor sensitivity similar to that of the original basolateral membrane. Like the original cotransporter in the basolateral membrane, the reconstituted protein was electrogenic and was inhibited by the disulfonic stilbene SITS. It is of interest to note that Na^+/HCO_3^- cotransporter activity was inhibited by the disulfonic stilbene SITS to a greater extent than that originally observed in original basolateral membranes 75% *vs.* 30% [15]. Likewise, the catalytic subunit of protein kinase A significantly inhibited Na^+/HCO_3^- cotransporter activity in proteoliposomes like the original basolateral membrane proteins [16]. These data strongly suggest that we have successfully solubilized, partially purified and reconstituted the renal basolateral Na^+/HCO_3^- cotransporter.

We have further purified the octylglucoside-solubilized protein by hydroxylapatite chromatography and glycerol gradient centrifugation. This approach has resulted in successful purification of the chloride transporter from the clathrin-coated vesicles [24]. Hydroxylapatite chromatography resulted in recovery of several fractions containing Na^+/HCO_3^- cotransporter activity (Fig. 6) with the highest activity present in fraction 1, which showed an average purification of 80-fold over the original basolateral membrane. This fraction was then further purified by glycerol gradient centrifugation, which resulted in greatest enrichment in fractions 1 and 5 and lesser enhancement in other fractions. Na^+/HCO_3^- cotransporter activity was enriched 300- and 124-fold in fractions 1 and 5, respectively. Although this step resulted in purification of greater magnitude to that obtained for the chloride transporter, this purification procedure resulted in only 0.2% recovery of the protein in fraction 1. This low recovery is in agreement with that reported for the purification of chloride transporter in clathrin vesicles [24]. This low protein recovery obvi-

ously limited the number of studies that could be performed utilizing fraction 1 and therefore other studies were done using fraction 5. SDS-PAGE analysis of fraction 5 enriched 124-fold in Na^+/HCO_3^- cotransporter activity showed enhancement of a protein doublet at 56 kD.

Several detergents and different techniques have been used to solubilize and partially purify a variety of membrane protein from different tissues [4–7, 10–14, 17, 19, 21–23]. These different techniques have yielded different degrees of purification with variable protein yield. The method utilized in the present study resulted in one of the best enrichment of protein purification by physicochemical process reported in the literature. Another advantage of this method is that the protein was functionally active when reconstituted. However, the major disadvantage of the technique was the low yield of protein.

Although this enrichment of the 56 kD protein in fraction 5 does not prove that this band is a component of the Na^+/HCO_3^- cotransporter, the significant enrichment of the Na^+/HCO_3^- cotransporter activity in this fraction and the enhancement of this band suggest that the 56 kD protein represents the Na^+/HCO_3^- cotransporter or active component thereof. Of course, additional studies using production of antibodies against this protein with subsequent immunopurification will be needed to ascertain that the 56 kD protein is the cotransporter.

In summary, we have solubilized, partially purified and functionally reconstituted the renal basolateral Na^+/HCO_3^- cotransporter, and this should provide the opportunity to further characterize this important protein in acid base regulation.

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References

1. Alpern, R.J. 1990. Cell mechanisms of proximal tubule acidification. *Physiol. Rev.* **70:**79–114
2. Akiba, T., Rocco, V.K., Warnock, D.G. 1987. Parallel adaptation of the rabbit renal cortical sodium/proton antiporter and sodium/bicarbonate cotransporter in metabolic acidosis and alkalosis. *J. Clin. Invest.* **80:**308–315
3. Akiba, T., Alpern, R.J., Eveloff, J., Calamina, J., Warnock, D.G. 1986. Electrogenic sodium/bicarbonate cotransport in rabbit renal cortical basolateral membrane vesicles. *J. Clin. Invest.* **78:**1472–1478
4. Brose, N., Halpain, S., Suchanek, C., Reinhard, J. 1989. Characterization and partial purification of a chloride- and calcium-dependent glutamate-binding protein from rat brain. *J. Biol. Chem.* **264:**9619–9625
5. Cidon, S., Nelson, N. 1986. Purification of N-ethylmaleimide-sensitive ATPase from chromaffin granule membranes. *J. Biol. Chem.* **261:**9222–9227
6. Corcuera-Lopez, B., Kanner, B.I., Aragon, C. 1989. Reconstitution and partial purification of the sodium and chloride-coupled glycine transporter from rat spinal cord. *Biochim. Biophys. Acta* **983:**247–252
7. Garlid, K.D., Shariat-Madar, Z., Nath, S., Jezek, P. 1991. Reconstitution and partial purification of the Na^+-selective Na^+/H^+ antiporter of beef heart mitochondria. *J. Biol. Chem.* **266:** 6518–6523
8. Grassl, S.M., Aronson, P.S. 1986. Na^+/HCO_3^- co-transport in basolateral membrane vesicles isolated from rabbit renal cortex. *J. Biol. Chem.* **261:**8778–8783
9. Hjelmeland, J.M., Chambrach, A. 1984. Solubilization and functional reconstitution of membrane proteins. *Methods Enzymol.* **104:**305–318
10. Jong, Y-J., Sheldon, A., Zhang, G.H., Kraus-Friedman, N. 1990. Purification of the microsomal Ca^{2+}-ATPase from rat liver. *J. Membrane Biol.* **118:**49–53
11. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227:**680–685
12. Peerce, B.E., Clarke, R.D. 1990. Isolation and reconstitution of the intestinal Na^+/glucose cotransporter. *J. Biol. Chem.* **265:** 1731–1736
13. Philipson, K.D., Longoni, S., Ward, R. 1988. Purification of the cardiac Na^+-Ca^{2+} exchange protein. *Biochim. Biophys. Acta* **945:**298–305
14. Ran, S., Benos, D.J. 1991. Isolation and functional reconstitution of a 38-kD chloride channel protein from bovine tracheal membranes. *J. Biol. Chem.* **266:**4782–4788
15. Ruiz, O.S., Arruda, J.A.L., Talor, Z. 1989. Na-HCO_3 cotransport and Na-H antiporter in chronic respiratory acidosis and alkalosis. *Am. J. Physiol.* **256:**F414–F420
16. Ruiz, O.S., Arruda, J.A.L. 1992. Regulation of the renal Na-HCO_3 cotransporter by cyclic AMP and Ca-dependent protein kinases. *Am. J. Physiol.* **262:**F560–565
17. Soldati, L., Longoni, S., Carafoli, E. 1985. Solubilization and reconstitution of the Na^+/Ca^{2+} exchanger of cardiac sarcolemma. *J. Biol. Chem.* **260:**13321–13327
18. Soleimani, M., Lesoine, G.A., Bergman, J.A., Aronson, P.S. 1991. Cation specificity and modes of the Na:CO_3:HCO_3 cotransporter in renal basolateral membranes vesicles. *J. Biol. Chem.* **266:**8706–8710
19. Talor, Z., Arruda, J.A.L. 1985. Partial purification and reconstitution of the renal Na-Ca exchange system. *J. Biol. Chem.* **260:**15473–15476
20. Talor, Z., Richison, G., Arruda, J.A.L. 1985. High-affinity calcium binding sites in luminal and basolateral renal membranes. *Am. J. Physiol.* **248:**F472–F478
21. Turner, R.J., George, J.M. 1990. Solubilization and partial purification of the rabbit parotid Na/K/Cl-dependent bumetanide binding site. *J. Membrane Biol.* **113:**203–210
22. von Dippe, P., Levy, D. 1990. Reconstitution of the immunopurified 49-kD sodium-dependent bile acid transport protein derived from hepatocyte sinusoidal plasma membranes. *J. Biol. Chem.* **265:**14812–14816
23. Weinman, E.J., Shenolikar, S., Cragoe, E.J., Jr., Dubinsky, W.P. 1988. Solubilization and reconstitution of renal brush border Na^+-H^+ exchanger. *J. Membrane Biol.* **101:**1–9
24. Xie, X.S., Crider, B.P., Stone, D.K. 1989. Isolation and reconstitution of the chloride transporter of clathrin-coated vesicles. *J. Biol. Chem.* **264:**18870–18873
25. Weinman, E.J., Shenolikar, S., Kahn, A.M. 1987. cAMP-associated inhibition of the Na^+-H^+ exchanger in rabbit kidney brush border membranes. *Am. J. Physiol.* **252:**F19–F25