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# Identification and covalent modification of a renal brush-border anion exchanger

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Brush-border membrane (BBM) proteins that bind the arginine-specific reagent phenylglyoxal (PG) and interact with stilbene disulfonic derivatives were identified in canine kidney cortex. Pretreatment of BBM vesicles with PG resulted in irreversible inhibition of anion exchange as assayed by $^{36}Cl^-$ influx mediated via $Cl^-/Cl^-$ exchange. $Cl^-/Cl^-$ exchange was reversibly inhibited by the disulfonic stilbene 4,4′-dinitrostilbene-2,2′-disulfonate (DNDS). A stilbene-affinity matrix was prepared by immobilizing DNDS in polyacrylamide beads. Elution of the BBM proteins from a disulfonic stilbene (DNDS) affinity matrix revealed two proteins at 160 and 230 kDa that were significantly enriched compared to initial material. Radiolabeling of the eluted mixture with [$^{14}$C]phenylglyoxal demonstrated covalent binding to several proteins, including the 160 kDa protein. Reconstitution of the proteins eluted from the affinity matrix into phosphatydilcholine demonstrated DIDS-sensitive $^{36}Cl^-$-influx mediated via $Cl^-/Cl^-$ exchange. Pretreatment of the BBM vesicles with PG selectively blocked binding of the 160 kDa protein to the DNDS affinity matrix. Radiolabelling of the PG-pretreated, affinity-purified membrane proteins showed selective prevention of [$^{14}$C]phenylglyoxal binding to the 160 kDa protein. Reconstitution of the PG-pretreated proteins eluted from the affinity matrix demonstrated significant reduction in $Cl^-/Cl^-$ exchange activity. These results suggest that a 160 kDa protein is a strong candidate for anion exchange transport in kidney proximal tubules.

## Introduction

Sodium chloride (NaCl) reabsorption in the kidney proximal tubule occurs predominantly via $Na^+/H^+$ and $Cl^-$/base exchangers acting in parallel. The prototype of $Cl^-$/base exchangers, known also as anion exchangers, is erythrocyte Band-3 which mediates the exchange of one chloride for one bicarbonate ion [1,2]. Several isoforms of $Cl^-$/base exchangers have been identified in the proximal tubule. These include: chloride/formate [3–6], chloride/oxalate [5,7], chloride/hydroxyl, and, possibly, chloride/bicarbonate exchangers [8–11]. Anion exchange transport systems, in general, share several properties of Band-3 protein including sensitivity to inhibition by disulfonic stilbenes and furosemide [3]. The proximal tubule anion exchanger(s) is (are) distinct from Band-3 protein as demonstrated by lack of cross reaction between Band-3-specific antibodies and proximal tubule transport proteins [12,13]. This suggests that the proximal tubule anion exchanger(s) is (are) structurally distinct from Band-3 protein.

Erythrocyte Band-3 transport activity shows sensitivity to inhibition by arginine residue modifiers [1,2], indicating the importance of arginine to the anion-exchanger protein. The alpha-carbonyl reagent phenylglyoxal selectively binds to arginine residues [14] and has been used to modify the red blood cell anion exchanger [1,2,15–18]. Experiments in red cells have shown that the stilbene sulfonic derivative DNDS prevents the binding of radiolabelled phenylglyoxal to the Band-3 protein and vice versa, indicating that binding sites for DNDS and phenylglyoxal overlap with one another [1]. In the present studies, DNDS-affinity chromatography was used to partially purify brush-border membrane proteins with disulfonicstilbene-binding sites. Reconstitution of the proteins eluted from the DNDS-affinity matrix into liposomes demonstrated the presence of anion-exchange activity as assayed by $Cl^-/Cl^-$ exchange. The activity of $Cl^-/Cl^-$ exchange in BBM vesicles was irreversibly inhibited in the presence of 4 mM phenylglyoxal. The interaction of phenylglyoxal with DNDS-affinity chromatography was studied by using a combination of covalent labelling and reconstitution assay. Our results indicate that there

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is a 160 kDa stilbene-binding, phenylglyoxal-sensitive protein in canine kidney brush-border membranes that is likely to be a structural component of the anion-exchange protein.

## Materials and Methods

*Membrane vesicles preparation.* Luminal membrane vesicles were isolated from canine renal cortex by a divalent cation-aggregation method [19] as employed previously [20,21]. The vesicles were suspended in a medium consisting of 250 mM sucrose, 10 mM Hepes titrated to pH 7.5 with potassium hydroxide, and then frozen and stored at −70°C until use. The final vesicle preparation was enriched 8–11-fold in brush-border membranes relative to initial homogenate based on specific activity of alkaline phosphatase.

$^{36}Cl^-$ transport measurements in the presence of phenylglyoxal Stock solutions of phenylglyoxal were prepared fresh in the buffer employed for the experiments. The composition of the buffer was similar to the solution that was used for uptake experiments, except that it was titrated to pH 8.0 by additional TMA-OH (tetramethylammonium hydroxide). Vesicles were pretreated with buffer alone or with buffer containing 4 mM phenylglyoxal for 30 min and the reaction was terminated by addition of excess arginine in the cold stop solution. The vesicles were washed and suspended in 100 mM tetramethyl ammonium (TMA)/gluconate, 80 mM Hepes, 40 mM TMA-OH (pH 7.5). Solutions of appropriate ionic composition were added to the membrane suspension to achieve the desired medium composition as specified in the figure legends. The timed uptake of $^{36}Cl^-$ at room temperature was assayed by a rapid filtration method [20,21]. The final composition of the experimental media for each experiment is given in the figure legends. In general, uptake measurements were performed in quadruplicate.

*Affinity matrix preparation and chromatography.* DNDS (4,4'-dinitrostilbene-2,2'-disulfonate) (35 mg) was immobilized in acrylamide beads according to a method similar to that designed by Uchida and Filburn [22].

Brush-border membrane vesicles (9 mg protein/ml) isolated from rabbit kidney cortex were solubilized in 0.67% Triton X-100 and centrifuged at $100\,000 \times g$ for 70 min.

The solubilized extracts were equilibrated with the DNDS-affinity matrix for 60 min at 4°C. In some experiments, free inhibitors were equilibrated with the mixture to specifically block adsorption of protein(s) to the immobilized DNDS. The affinity matrix was washed three times and the proteins were eluted in the absence or presence of 3 mM DNDS to specifically displace DNDS-binding proteins.

The proteins were concentrated through Centricon-30 filters (Amicon), subjected to SDS-PAGE and visualized by Coomassie blue staining.

*[$^{14}$C]Phenylglyoxal binding.* [$^{14}$C]Phenylglyoxal obtained in powder was dissolved in water. The proteins were eluted from the DNDS affinity matrix (1.0 mg/ml) in the presence of 300 mM $NaHCO_3$. $NaHCO_3$ was used for elution instead of DNDS to prevent DNDS interference with [$^{14}$C]phenylglyoxal binding; our results demonstrate that 300 mM NaHCO3 elutes all the DNDS binding proteins from the affinity matrix. [$^{14}$C]phenylglyoxal was added from the stock to a final concentration of 0.2 mM. After 30 min incubation at 20°C, the reaction was stopped by addition of excess arginine in cold buffer and the mixture was concentrated in an Amicon filter.

*SDS-PAGE and autoradiography.* Membrane proteins which were eluted from the DNDS-affinity matrix and/or had been treated with [$^{14}$C]phenylglyoxal were concentrated to 50 $\mu$l in Amicon filters and treated with sample buffer in a 1:3 ratio. The sample buffer contained 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 12.5 mM Tris-HCl (pH 6.8). Samples were electrophoresed on a 7% polyacrylamide gel using a modification [23] of the Laemmli protocol [24]. Proteins were visualized by Coomassie blue staining. For autoradiography, the gel was dried and subjected to autoradiography using Kodak X-Omat AR film and intensifying screens at −70°C for 4–7 days. The autoradiograms were scanned using a Bio-Rad gel scanner interfaced with a Macintosh Computer.

*Reconstitution of the anion exchanger.* The DNDS-binding proteins were eluted in the presence of 300 mM $NaHCO_3$, 0.57% Triton X-100 ((pH 8.2) Elution was performed in the presence of $NaHCO_3$ because DNDS was not readily dialyzable). The mixture was dialized overnight against a solution of 100 mM K-gluconate, 100 mM mannitol, 10 mM Hepes, TMA-OH (pH 7.5).

Egg lecithin phosphatydilcholine (30 mg in 300 $\mu$l chloroform) was evaporated to dryness under a stream of nitrogen.

2 ml of protein mixture (0.5 mg/ml) was added to the dry lipid. SM-2 Bio Beads (55 mg wet weight) were added and the solution was gently stirred at 4°C for 3 h. An extra 55 mg SM-2 Beads was added for another 2 h to remove the residual Triton-X-100. The Bio Beads were precipitated and the proteoliposomes were recovered from the mixture by slow-speed centrifugation for 5 min.

The liposomes were preequilibrated for 120 min in the presence and absence of 40 mM KCl (pH 8.0). The influx of 4 mM radiolabelled $^{36}Cl^-$ or 50 $\mu$M [$^{14}$C]formate into the liposomes was assayed at 120 s. The reaction was stopped by centrifugation through Sephadex G-25 minicolumns for 4 min at $1000 \times g_{max}$.

The eluate was placed in 4 ml of scintillation fluid and radioactivity was determined by scintillation spectroscopy.

$^{36}Cl^-$ and [$^{14}$C]phenylglyoxal were purchased from Amersham; 4,4'-dinitrostilbene-2,2'-disulfonate (DNDS) from Aldrich; Bio-beads from Bio-Rad and phosphatydilcholine, phenylglyoxal and valinomycin from Sigma. Valinomycin was dissolved in 95% ethanol and added to the liposomes or vesicles in a 1:100 dilution.

## Results

*Effect of phenylglyoxal on brush-border membrane anion-exchange*

The purpose of the first experiment was to evaluate the effect on the anion exchanger of treating vesicles with phenylglyoxal, a potent and selective arginine-modifying reagent [14]. Vesicles were treated with 4 mM phenylglyoxal at pH 8 for 30 min at room temperature. The reaction was terminated with an excess of arginine in cold stop solution. The control vesicles were treated similarly. The vesicles were then washed and pre-equilibrated in pH 7.5 buffer. The 30-s and 2-h values for $^{36}Cl^-$-influx mediated via $Cl^-/Cl^-$ exchange was assayed in the presence of an outward $Cl^-$-gradient ($Cl_i/Cl_o = 40:4$ mM (pH 7.5), where i and o refer to inside and outside, respectively). As shown in Table I, pretreatment with phenylglyoxal caused irreversible inactivation of $^{36}Cl^-$-influx mediated via $Cl^-/Cl^-$ exchange. The equilibrium values measured at 2 hr of incubation were not different between control and vesicles treated with phenylglyoxal, suggesting that the inactivation of $Cl^-/Cl^-$ exchange by phenylglyoxal is not due to disruption of vesicles.

TABLE I

*Effect of phenylglyoxal pretreatment on $Cl^-/^{36}Cl^-$ exchange*

Brush-border membrane vesicles were pre-treated with 4 mM phenylglyoxal at pH 8 for 30 min. The vesicles were washed and preequilibrated for 120 min in a medium consisting of 52 mM TMA-gluconate, 40 mM K-gluconate, 52 mM mannitol, 42 mM Hepes, 35 mM TMA-OH (pH 8.0) that in addition contained 40 mM KCl or 40 mM K-gluconate. Uptake of 4 mM $^{36}Cl^-$ was then assayed after 30 s incubation of the vesicles in a medium of final composition 52 mM TMA-gluconate, 76 mM K-gluconate, 4 mM KCl, 52 mM mannitol, 42 mM Hepes, 35 mM TMA-OH (pH 8.0). The $Cl^-/Cl^-$ exchange activity was calculated by subtracting the $^{36}Cl^-$-influx assayed in the absence of an outward chloride gradient from the total activity measured in the presence of an outward chloride gradient. Values shown represent means ±S.E. for three separate experiments performed in quadruplicate on different membrane preparations and are expressed as percent of the control value measured in the absence of phenylglyoxal.

| Cl influx | 30 s | 2 h |
|---|---|---|
| Control (%) | 100 | 100 |
| Phenylglyoxal-pretreated (% of control) | 32.2±4.3 ($P < 0.01$) | 104.4±6.2 ($P > 0.05$) |

TABLE II

*Effect of DNDS on $Cl^-/^{36}Cl^-$ exchange*

Brush-border membrane vesicles were preequilibrated for 120 min at 20°C in a medium similar to the experiments described in Table I. Uptake of 4 mM $^{36}Cl^-$ was assayed after 30 s in the presence and absence of 2 mM DNDS added to the external solution. Values shown represent means ±S.E. for three separate experiments performed in quadruplicate on different membrane preparations.

| $Cl_i/Cl_o$ (mM): | 0:4 | 40:4 | 40:4 +DNDS |
|---|---|---|---|
| Cl influx (nmol/mg protein per min) | 1.6±0.25 ($P < 0.01$) | 5.1±0.65 ($P < 0.01$) | 2.4±0.34 ($P < 0.01$) |

*Effect of DNDS on brush-border membrane anion-exchange*

In the next experiment, we tested whether DNDS, a reversible inhibitor of anion exchange in erythrocytes [1], could inhibit the $Cl^-/^{36}Cl^-$ exchange system in dog renal brush-border membrane vesicles. An outward gradient of chloride was imposed across the membrane vesicles ($Cl_i/Cl_o = 40:4$ mM ($pH_o = pH_i = 7.5$)) and the effect of 2 mM DNDS was evaluated on $^{36}Cl^-$-influx. As shown in Table II, DNDS significantly inhibited $Cl^-$-gradient-stimulated $^{36}Cl^-$-influx measured at 30 s. In additional experiments not illustrated, it was found that this inhibition was completely reversible. This was tested in a manner similar to Table I. The results demonstrated that pretreatment of brush-border membrane vesicles with 4 mM DNDS for up to 30 min did not affect the actvity of $Cl^-/Cl^-$ exchange (data not shown).

*DNDS-binding proteins in BBM vesicles*

To identify DNDS-binding proteins by affinity chromatography, an affinity matrix was made by immobilizing 8 mM DNDS in acrylamide/bisacrylamide (15%:5%) as described earlier. Vesicles were solubilized in the presence of Triton X-100 and equilibrated with the DNDS affinity matrix. The matrix was washed and the proteins were eluted in the presence of 3 mM DNDS. Coomassie blue stain of the resulting gel is demonstrated in Fig. 1. This revealed that two proteins with molecular masses of 160 and 230 kDa were significantly enriched in the fraction that was eluted from the DNDS-affinity matrix compared to initial solubilized mixture (Fig. 1). In the absence of the immobilized DNDS in the affinity matrix, neither of these two proteins were eluted confirming the specificity of the DNDS-affinity matrix (data not shown).

*Effect of DIDS on DNDS-binding proteins*

To determine if either of these two proteins demonstrate similar properties to the Band-3 anion-exchanger

protein, the effect of Band-3 inhibitors [1] on the adsorption of the 160 and 230 kDa proteins was tested. The red blood cell anion-exchanger protein is sensitive to inhibition by disulfonic stilbene derivatives including DIDS [1,2]. To test whether DIDS would block the adsorption of 160 and or 230 kDa proteins to the affinity matrix, an experiment similar to Fig. 3 was performed. The effect of DIDS on the adsorption of the DNDS-sensitive proteins was evaluated by addition of 2 mM DIDS to the solubilized mixture during initial equilibration. The results of this experiment are demonstrated in Fig. 2. The left lane shows the proteins adsorbed to the affinity matrix in the control condition in the absence of DIDS. The right lane illustrates that DIDS blocked the adsorption of several proteins, including the 162 and 230 kDa proteins. These results demonstrate the presence of several DIDS-sensitive proteins in renal brush-border membranes. In addition, they prove the identities of the 160 and 230 kDa proteins as disulfonate-binding proteins.

*Effect of furosemide on DNDS-binding proteins*

Anion exchangers, in general, demonstrate sensitivity to inhibition by several diuretics, including

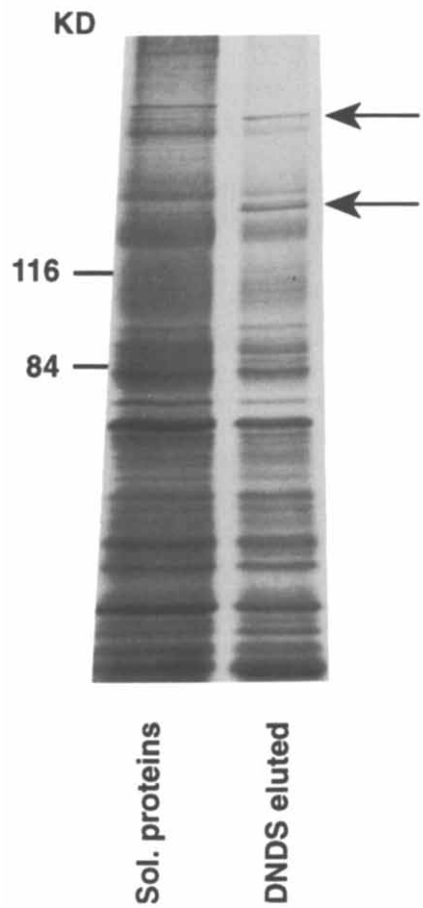


Fig. 1. Identification and purification of DNDS-binding proteins. Left lane: solubilized material. Right lane: DNDS affinity-purified. The arrows point to the partially-purified proteins.

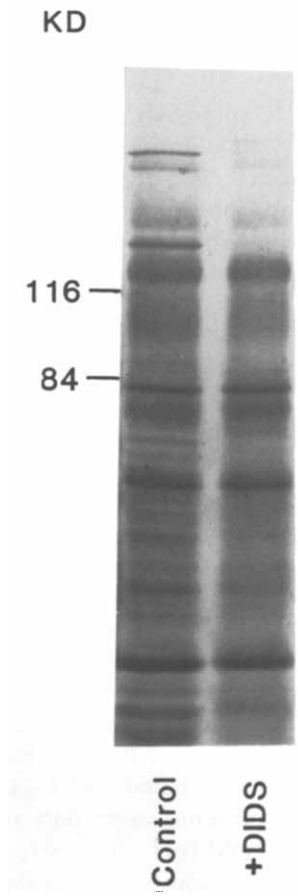


Fig. 2. Effect of DIDS on adsorption of DNDS-binding proteins. Left lane: control. Right lane: adsorption in the presence of 2 mM DIDS.

furosemide [1–3]. Accordingly, the effect of furosemide on the adsorption of the DNDS binding proteins to the affinity matrix was tested in a manner similar to that employed in the experiments described in Fig. 2. As demonstrated in Fig. 3, furosemide (right lane) significantly blocked the adsorption of the 160 kDa protein to the affinity matrix with no significant effects on other proteins.

*Effect of phenylglyoxal on DNDS-binding proteins*

Experiments with red cells Band-3 protein have shown that binding sites for phenylglyoxal and DNDS overlap with one another [1]. To determine if proximal tubule anion exchangers have a similar characteristic, the effect of phenylglyoxal pretreatment of vesicles on DNDS-binding proteins was studied. Membrane vesicles were pretreated with 4 mM phenylglyoxal for 30 min in a manner similar to the experiments described in Table I. Following the treatment, the vesicles were washed, solubilized, and equilibrated with a DNDS affinity matrix. As shown in Fig. 4, pretreatment with phenylglyoxal completely blocked the adsorption of the 160 kDa protein to the DNDS affinity matrix (right lane) compared to control (left lane). Collectively, these

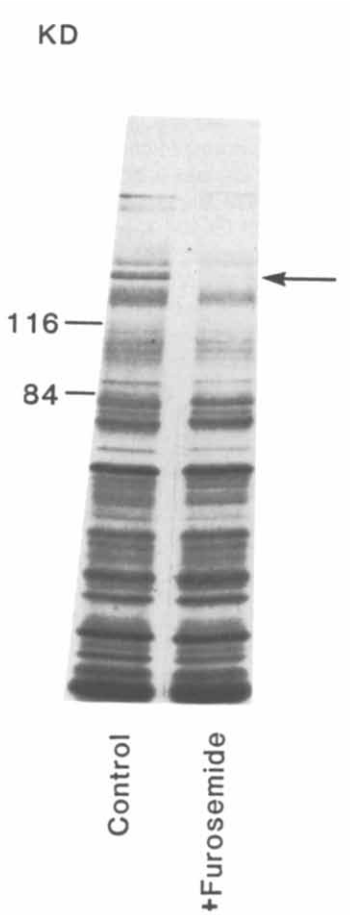


Fig. 3. Effect of furosemide on adsorption of DNDS-binding proteins. Left lane: control. Right lane: adsorption in the presence of 3 mM furosemide. The arrow points to the 160 kDa protein.

results suggest that the 160 kDa protein is a structural candidate for the anion exchanger in luminal membrane of kidney proximal tubules.

*Covalent labeling with [$^{14}$C]phenylglyoxal*

The experiments in Table I demonstrate that phenylglyoxal irreversibly inhibits the proximal tubule anion exchanger. The purpose of the next series of experiments was to determine if any of the proteins eluted from the DNDS-affinity matrix could be covalently labeled with [$^{14}$C]phenylglyoxal. BBM proteins were eluted from a DNDS affinity matrix in the presence of 300 mM $NaHCO_3$ and incubated with 0.2 mM [$^{14}$C]phenylglyoxal for 30 min as described earlier. The reaction was stopped in the presence of excess arginine in cold solution and concentrated in Amicon filters. Proteins were then resolved by SDS-PAGE, and covalent binding of [$^{14}$C]phenylglyoxal was determined by autoradiography. Reaction with [$^{14}$C]phenylglyoxal resulted in covalent labeling of multiple proteins, including the 160 kDa protein (Fig. 5, right lane). The effect of pretreatment of vesicles with phenylglyoxal on [$^{14}$C]phenylglyoxal binding of the DNDS-sensitive proteins was studied. Table I shows that pretreatment of BBM vesicles with 4 mM phenylglyoxal resulted in irreversible inhibition of activity of the proximal tubule anion exchanger. The goal of the next experiment was to test the possibility that pretreatment of BBM vesicles with phenylglyoxal would result in modification of the corresponding binding sites on the brush-border anion exchanger(s). This should prevent the binding of [$^{14}$C]phenylglyoxal to proteins with anion-exchange properties. To test this hypothesis, vesicles were pretreated with 4 mM phenylglyoxal for 30 min. The reaction was stopped according to the protocol. The proteins were washed, solubilized, and equilibrated with the DNDS-affinity matrix. The proteins were eluted in the presence of 300 mM $NaHCO_3$, labeled with [$^{14}$C]phenylglyoxal, resolved on an SDS gel and subjected to autoradiography. The autoradiogram demonstrated that pretreatment with 4 mM phenylglyoxal resulted in selective reduction in labeling of one band (shown in Fig. 5, left lane). The molecular mass of this protein, estimated by comparison to the molecu-

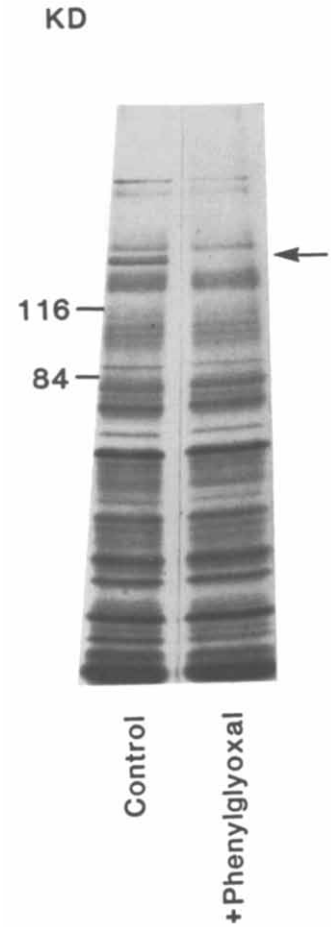


Fig. 4. Effect of phenylglyoxal on adsorption of DNDS-binding proteins. Left lane: Control. Right lane: adsorption in the presence of 4 mM phenylglyoxal. Control in Figs. 2–4 refers to adsorption in the absence of any inhibitors. Both control and experimental groups were eluted in the presence of DNDS. The arrow points to the 160 kDa protein.

lar weight standards, was 160 kDa. Densitometric scanning of the autoradiogram showed that the labeling of no other protein bands was affected by phenylglyoxal pretreatment (data not shown).

*Reconstitution of anion exchanger*

To determine the presence of anion exchange activity in the DNDS eluate, reconstitution of the proteins was carried out. Proteins eluted from the DNDS-affinity matrix were dialyzed overnight and reconstituted into egg lecithin phosphatidylcholine proteoliposomes as described earlier. The resulting liposomes were assayed for anion-exchanger activity by measuring the influx of $^{36}Cl^-$ mediated via $Cl^-$-$Cl^-$ exchange. The influx of 4 mM $^{36}Cl^-$ into the liposomes was assayed at 120 sec in the presence and absence of an outward chloride gradient ($Cl_i/Cl_o = 40:4$ vs. 0:4 mM). As demonstrated in the upper pannel of Table III, an outward chloride gradient stimulated $^{36}Cl^-$-influx into liposomes more than 150% compared to no outward chloride gradient ($P < 0.005$). These experiments were performed in the presence of $K^+_o = K^+_i$ and the potassium ionophore valinomycin to ensure that the increase in $^{36}Cl^-$-influx into the liposomes was not secondary to generation of inside positive membrane potential resulting from outward movement of chloride. The $Cl^-/^{36}Cl^-$ exchange was completely inhibited by 0.5 mM DIDS added to the external solution. In the absence of any protein in the reconstitution media, $Cl^-/^{36}Cl^-$ exchange was not different from the background confirming the presence of anion exchange activity in the proteins that were eluted from the affinity matrix.

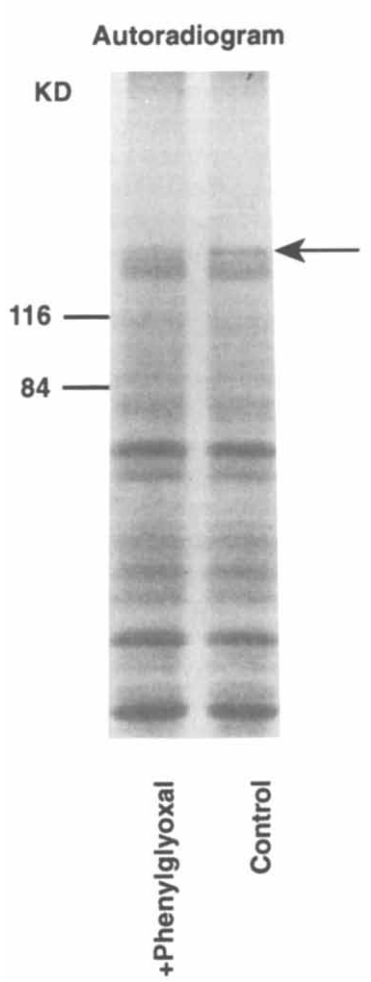


Fig. 5. Covalent labelling of DNDS binding proteins by [$^{14}$C]phenylglyoxal. The autoradiograms were obtained by treatment of DNDS column eluates with 0.2 mM [$^{14}$C]phenylglyoxal for 30 min at pH 8.0. Right lane: control (no pretreatment with any chemicals). Left lane: phenylglyoxal pretreatment. The arrow points to the 160 kDa protein.

TABLE III

*$^{36}Cl^-$-influx in the proteoliposomes*

The proteins were eluted from control and phenylglyoxal-protected affinity matrices and reconstituted into the liposomes. The liposomes were pre-equilibrated for 120 min at 20°C in a medium that consisted of 100 mM mannitol, 10 mM Hepes, 8 mM TMA hydroxide (pH 8.0) that in addition contained 60 mM K-gluconate and 40 mM KCl or contained 100 mM K-gluconate. $^{36}Cl^-$-influx in the liposomes was measured 120 s after 1:10 dilution and incubation of the liposomes in a medium of final composition 96 mM K-gluconate, 4.3 mM KCl, 100 mM mannitol, 10 mM Hepes, 8 mM TMA hydroxide (pH 8.0). Each datum represents the mean ± S.E. for three separate experiments performed on different reconstitution preparations. Unit: nmol/mg protein per min.

| $Cl_i/Cl_o$ (mM): | 0:4 | 40:4 | 40:4 +DIDS |
|---|---|---|---|
| Control | 12.5 ± 1.5 | 29.5 ± 2.3 | 10.1 ± 2.2 |
| Phenylglyoxal-protected | 10.3 ± 1.3 ($P > 0.05$) | 16.9 ± 1.7 ($P < 0.01$) | 8.9 ± 1.2 ($P > 0.05$) |

*Effect of phenylglyoxal pretreatment on anion-exchange activity*

The experiments in Table III (upper pannel) show that BBM membrane proteins eluted from a DNDS-affinity matrix contained anion exchange activity. To determine if the 160 kDa protein is an anion exchanger, the strategy was to delete this protein from the eluted mixture by phenylglyoxal pretreatment and reconstitute the remaining proteins into liposomes. Diminution in the anion-exchange activity in the liposomes would strongly suggest that the 160 kDa protein is an anion-exchanger candidate. Vesicles were pretreated with 4 mM phenylglyoxal for 30 min. The proteins were then solubilized, equilibrated with DNDS-affinity matrix, and eluted in the presence of 300 mM $NaHCO_3$ as described earlier. The proteins were reconstituted and activity of the anion-exchange system was monitored by assaying the influx of $^{36}Cl^-$-mediated $Cl^-/Cl^-$ exchange. As demonstrated in the lower pannel of Table III, pretreatment of BBM vesicles with phenylglyoxal significantly inhibited $Cl^-/Cl^-$

exchange activity in the liposomes reconstituted with the proteins eluted from the DNDS affinity matrix. This strongly argues that the 160 kDa protein is a likely anion exchanger.

## Discussion

More than 50% of the filtered load of chloride is reabsorbed in the proximal tubule. Little is known about the mechanism of chloride reabsorption in this nephron segment. However, one important mechanism involves anion exchange. This is accomplished by exchanging one chloride for an anion. Different isoforms of anion exchangers have been identified in the kidney proximal tubule. These include: chloride/formate [3–6], chloride/oxalate [5,7], chloride/hydroxyl [9–11], and, possibly, chloride/bicarbonate exchangers. Imposition of an outward chloride gradient across brush-border membrane vesicles induced uphill influx of [$^{14}$C]formate compatible with a chloride/formate exchanger [3–5]. In intact proximal tubule of rabbit kidney, addition of physiologic concentrations of formate to the luminal and peritubular solutions increased the rate of NaCl absorption by 60% [6]. These results are also consistent with a chloride/formate exchanger in kidney proximal tubule. Also, in brush-border membrane vesicles isolated from rabbit kidney cortex, imposition of an outward chloride gradient induced an above equilibrium influx of oxalate suggesting the presence of chloride/oxalate exchanger [5]. The physiologic significance of chloride/oxalate exchange is not known. The presence of chloride/hydroxyl exchange in brush-border membranes remains controversial [9–11]. In the intact perfused tubule, addition of chloride to the luminal solution did not affect cell pH. However, after inhibition of the basolateral pH-regulating processes with the disulfonic stilbene SITS, addition of chloride to the lumen caused cell acidification, suggesting the presence of chloride/hydroxyl or bicarbonate exchange [6]. It is believed that chloride/bicarbonate exchange does not play a significant role in chloride absorption in proximal tubule [3]. Given the results of the above studies, it is evident that much remains to be learned about the mechanism(s) of chloride reabsorption in kidney proximal tubule.

Using a SITS-affinity column and canine brush-border membrane vesicles, Pimplicar et al. isolated a 130 kDa protein with binding affinity for two stilbene derivatives, DIDS and BADS [27]. Subsequent studies suggested that the 130 kDa protein is not an anion exchanger. This is not surprising, since there are multiple disulfonic binding proteins in brush-border membranes. In those studies, the interaction of 130 kDa protein with other known inhibitors of renal or RBC anion exchanger such as furosemide, or phenylglyoxal was not tested. Moreover, the presence of anion exchange activity in the eluted protein mixture was not evaluated.

In the present studies, a 160 kDa protein with binding affinity for DIDS and furosemide has been identified. In addition, the 160 kDa protein covalently binds to phenylglyoxal. The effect of phenylglyoxal on DNDS-sensitive proteins demonstrated that the binding sites for phenylglyoxal and DNDS on the 160 kDa protein overlap with one another. This is very similar to red cell anion exchanger. Furthermore, reconstitution of the proteins deficient in 160 kDa protein into liposomes demonstrated decreased anion-exchange activity. This strongly suggests that the 160 kDa protein is a likely brush-border anion exchanger. It is possible that phenylglyoxal pretreatment could have decreased $Cl^-/Cl^-$ exchange activity by modifying the anion exchanger without interfering with the DNDS binding site. Such would be the case if the DNDS and phenylglyoxal binding sites did not overlap with one another. However, Fig. 5 demonstrated that, except for the 160 kDa protein, pretreatment of vesicles with phenylglyoxal did not result in modification of any other protein. This suggests that while there are several brush-border membrane proteins with phenylglyoxal binding site, the 160 kDa protein is the only one with both phenylglyoxal and DNDS binding sites. Moreover, if pretreatment with 4 mM phenylglyoxal, a concentration that siginificantly inhibits anion exchange activity in liposomes, was due to interaction of phenylglyoxal with a protein other than the 160 kDa band, covalent labeling of that protein with [$^{14}$C]phenylglyoxal should have been prevented. However, the autoradiogram in Fig. 5 shows that pretreatment of brush-border membrane vesicles with 4 mM phenylglyoxal did not inhibit the binding of [$^{14}$C]phenylglyoxal to any other protein.

In conclusion, the results of these experiments strongly suggest that a partially-purified 160 kDa protein is a likely structural component of the renal brush-border anion exchanger. Further purification of the 160 kDa protein followed by reconstitution should provide us with significant information regarding the functional identity, regulatory, and molecular characteristics of proximal tubule anion exchanger(s).

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## References


1 Passow, H. (1986) Rev. Physiol. Biochem. Pharmacol. 103, 162–222.
2 Jennings, M.L. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 397–430.
3 Aronson, P.S. (1989) Annu. Rev. Physiol. 51, 419–441.
4 Karniski, L.P. and Aronson, P.S. (1985) Proc. Natl. Acad. Sci. USA 82, 6362–6365.
5 Karniski, L.P. and Aronson, P.S. (1987) Am. J. Physiol. 253, F513–F521.
6 Alpern, R.J. (1987) J. Clin. Invest. 79, 1026–1030.
7 Kuo, S.M. and Aronson, P.S. (1986) FASEB J. 2, A753.
8 Talor, Z., Gold, R.M., Yang, W.-C. and Arruda, J.A.L. (1987) Eur. J. Biochem. 164, 695–702.
9 Warnock, D.G. and Yee, V.J. (1981) J. Clin. Invest. 67, 103–115.
10 Cassano, G., Stieger, B. and Murer, H. (1984) Eur. J. Physiol. 400, 309–317.
11 Ives, H.E., Chen, A-Y. and Verkman, A.S. (1986) Biochim. Biophys. Acta 863, 91–100.
12 Cox, J.V., Moon, R.T. and Lazarides, E. (1985) J. Cell. Biol. 100, 1548–1557.
13 Alper, L.S. (1991) Annu. Rev. Physiol. 53, 549–564.
14 Takahashi, K. (1968) J. Biol. Chem. 243, 6171–6179.
15 Bjerrum, P.J., Quagliariello, E and Palmieri, F. (eds.) (1983) in Structure and function of membrane proteins, pp. 107–115, Elsevier, Amsterdam.
16 Bjerrum, P.J., Wieth, J.O. and Borders, C.L. (1983) J. Gen. Physiol. 81, 453–484.
17 Zaki, L. and Julien, T. (1985) Biochim. Biophys. Acta 818, 325–332.
18 Stipani, L., Zara, V., Zaki, L., Prezioso, G. and Palmieri, F. (1986) FEBS Lett. 325, 282–286.
19 Evers, C., Hasse, W., Murer, H. and Kinne, R. (1978) Membr. Biochem. 1, 202–219.
20 Soleimani, M., Bergman, J.A., Hosford, M.A. and McKinney, T.D. (1991) J. Clin. Invest. 86, 1076–1083.
21 Soleimani, M., Bizal, G.L., McKinney, T.D. and Hattabaugh, Y. J. (1992) J. Clin. Invest. 90, 211–218.
22 Uchida, T. and Filburn, C.R. (1984) J. Biol. Chem. 259, 12311–12314.
23 Blackshear, P.J. (1984) Methods Enzymol. 104, 237–255.
24 Laemmli, U.K. (1970) Nature 227, 680–685.
25 Levy, D., Gulik, A., Seigneuret, M. and Riguad, J-L. (1990) Biochemistry 29, 9480–9488.
26 Levy, D., Bluzal, A., Seigneuret, M. and Riguad, J-L. (1990) Biochim. Biophys. Acta 1025, 179–190.
27 Pimplicar, W.P. and Reithmeier, R.A.F. (1988). J. Biol. Chem. 263, 4485–4493.