Solubilization of Na/K/Cl Cotransporter in Rabbit Parotid Acini and Its Purification-Separation of Bumetanide Binding Protein by ^{14}C-NEM Labeling

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In rat parotid basolateral membranes the presence of a 160 kD protein can be labeled with the irreversible sulfhydryl reagent [^{14}C]-N-ethyl maleimide in a bumetanide-protectable fasion. The previous results suggest that the existence of an esential sulfhydryl group is closely associated with the bumetanide-binding site on the parotid Na/K/Cl cotransporter, provide strong evidence that this protein is a part or all of the parotid bumetanide-binding site. When this protein is treated with endoglycosidase F/N-glycosidase F to remove N-linked oligosaccharides, its apparent molecular weight decreases to 135 kD. The bumetanide-binding protein was purified using two preparative electrophoresis steps. First, a Triton X-100 extract enriched in this protein was run on preparative electrophoresis to obtain fractions containing proteins in the 160 kD range. These were then deglycosylated with endoglycosidase F/N-glycosidase F and selected fractions were pooled and rerun on preparative electrophoresis to obtain a final 135 kD fraction. The enrichment of the bumetanide-binding protein in this final 135 kD fraction estimated from [^{14}C]-N-ethylmaleimide labeling was approximately 48 times relative to the starting membrane extract. Since the bumetanide-binding site represents approximately 2% of the total protein this starting extract, this enrichment indicates a high degree of purity of this protein in the 135 kD fraction.

Key words: loop diuretics, Na/K/Cl cotransporter, parotid acinar cell, non-ionic detergent, chloride secretion.

Introduction

Much recent experimental effort has been devoted to the study of Na/K/Cl cotransporter systems (Geck and Heinz, 1986; O'Grady et al., 1987 Haas, 1989). These transporters have been shown to be responsible for generating the transepithelial chloride fluxes which drive salt and water movements across many absorptive and secretory epithelia, including salivary glands (Turner, 1993). Clinically the transporter is the renal site of action of the commonly prescribed loop diuretic furosemide (Lasix) and its more potent and specific analogue bumetanide. Na/K/Cl cotransporters have also been identified in a variety of nonepithelial tissues where

they are involved in volume regulatory ion fluxes (Geck and Heinz, 1986; Haas, 1989).

It is reported that the direct evidence for the presence of Na/K/Cl cotransport activity in basolateral membrane vesicles prepared from the rabbit parotid (Turner et al., 1986). In addition, a high affinity bumetanide-binding site in this preparation was identified, strong evidence that this site was identical with the bumetanide inhibitory site on the Na/K/Cl cotransporter was provided by Turner et al. (1986). In a subsequent study, it is demonstrated that high affinity bumetanide binding to rabbit parotid basolateral membranes is inactivated by the irreversible sulfhydryl reagent N-ethymaleimide (NEM). This effect of NEM could be prevented by the presence of bumetanide, indicating the existence of an essential sulfhydry group at or closely re-

lated to the bumetanide-binding site. NEM also reduced ^{22}Na transport via the cotransporter by he same factor as the number of high affinity bumetanide-binding sites (George and Turner, 1989), again confirming the association of this bumetanide-binding site with the cotransporter.   .

Furthermore, other reports suggested that the rabbit parotid bumetanide-binding protein could be patially purified by sucrose density centrifugation in the presence of a mild detergent (0.24% Triton X-100). Under these conditions bumetanide-binding activity was preserved. Based on these experiment, we estimated that the bumetanide binding protein-detergent-lipid complex had a molecular weight $\sim$ 200 kD and that the molecular weight of the protein itself might be $\sim$160 kD, in good agreement with some earlier estimates of the size of the Na/K/Cl cotransporter protein in other tissues (Haas and Forbush, 1987, 1988). Our bumetanide-binding studies also showed that there were approximately 100 pmol of bumetanide-binding sites per mg of rabbit basolateral membrane protein. Assuming a molecular weight of 150-200 kD, this indicates that the Na/K/Cl cotransporter represents 1.5-2% of the total basolateral membrane protein, making this preparation a particularly rich source of this protein.

In the present paper we have utilized our previous observation, that the Na/K/Cl cotransporter can be inactivated by NEM in a bumetanide-protectable fashion, to label the rabbit parotid bumetanide-binding protein with [^{14}C]-NEM. The protein identified in this manner has an apparent molecular weight of 160 kD. We also show that this protein is heavily glycosylated with a deglycosylated apparent molecular weight of 135 kD. Finally, we describe a simple method for purifying this protein from rabbit parotid basolateral membranes.

Materials and Methods

Preparation of parotid basolateral membranes and membrane extracts

Basolateral membranes (BLM) were prepared from rabbit parotid by a Percoll gradient method, as previously described (Turner *et al.*, 1986). Rela-

tive to the starting tissue homogenate, the activity of the basolateral membrane marker K$^+$ stimulated p-nitrophenyl phosphatase is enriched approximately 10 times in this BLM fraction, freshly prepared BLM were suspended in Buffer A/EDTA (10 mM Tris/HEPES plus 100 mM mannitol and 1 mM EDTA) containing 100 mM KCl at a protein concentration of 5-10 mg/ml, then fast frozen in aliquots and stored above liquid nitrogen.

On the day of the experiment, an appropriate number of aliquots of frozen BLM were thawed for 30 min at room temperature, diluted 100 times with Buffer A/EDTA and centrifuged at 48,000$\times$g for 20 min. the resulting pellets were taken up in Buffer A/EDTA and stored on ice until use.

A BLM detergent extract was prepared as follows. BLM in Buffer A/EDTA at a protein concentration of 2 mg/ml were diluted 1:1 with a 0.14% Triton X-100 (w/v; Sigma T-6878) also in Buffer A/EDTA. This material was left on ice for 30 min then centrifuged for 5 min at 150,000$\times$g in a Beckman Airfuge. The supernate from this spin is referred to as the 0.07% Triton extract.

[^{14}C]-NEM Labeling of Bumetanide-protectable site

BLM (typically 1-3 ml at $\sim$1 mg protein/ml) were incubated for 1 hr at room temperature ($\sim$25$°$C) in Buffer A/EDTA containing 100 mM Na gluconate, 5 mM KCl and 30 M bumetanide (in some experiments bumetanide was omitted or chloride was replaced by gluconate). Unlabeled NEM (1 mM) was then added and incubation at room temperature was continued for a second hour after which the membranes were diluted 20-fold with ice-cold Buffer A/EDTA and centrifuged at 48,000$\times$g for 20 min. The resulting pellets were resuspended in 10 ml Buffer A/EDTA and left at room temperature for 60 min (to allow for complete dissociation of bound bumetanide). After the addition of 10 ml ice-cold Buffer A/EDTA, the BLM were recentrifuged at 48,000$\times$g for 20 min and resuspended in Buffer A/EDTA containing 100 mM Na gluconate, 95 mM K gluconate and 5 mM KCl at a protein concentration $\sim$5 mg/ml. Sites previously unreac-

ted with NEM in the presence of bumetanide were then labeled by incubating the membranes with 0.17 mM [^{14}C]-NEM for 60 min at a room temperature. The BLM were then diluted with 20 ml ice-cold Buffer A/EDTA, centrifuged as above, washed again in Buffer A/EDTA and stored above liquid nitrogen until further use.

SDS-PAGE Electrophoresis

SDS-PAGE was performed essentially as described by Laemmli (1970) using a 4% polyacrylamide stacking gel and either a 7.5% or a 5-15% (continuous gradient) polyacrylamide separating gel. Samples were heated at 100℃ for 2 min in a solution containing 1% SDS, 0.001% bromophenol blue, 3.3% sucrose and 40 mM dithiothreitol before loading onto the stacking gel. After electrophoresis, gels were fixed and stained in 20% methanol, 7% acetic acid and 0.05% Coomassie brilliant blue R250. The molecular weight markers indicated in the figures are myosin (200 kD), β-galactosidase (116 kD), phosphorylase B (97 kD), bovine serum albumin (69 kD) ovalalbumin (46 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD).

Autoradiography and Densitometry

In some experiments (e.g., Fig. 1) SDS-PAGE gels was carried out using Kodak X-Omat AR film (Eastman Kodak, Rochester NY). In this case gels were presoaked in Enlightening (Dupont NEM) according to the manufacture's instructions. In other experiments, the ^{14}C activity of dried SDS-PAGE gels was scanned using a molecular Dynamics Model 400A PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and images of the resulting autoradiograph printed on a laser printer (Hewlett Packard Laserjet II); these are also reffered to as "autoradiographs" in the text. Coomassie blue stained gels were scanned using a Molecular Dynamics Computing Densitometer. Analyses of scanned autoradiographs and gels were carried out using ImageQuant software supplied with the Molecular Dynamics instruments.

Glycosidase Treatment

Samples (BLM and 0.07% Triton extracts in Buffer A/EDTA, or fractions from preparative electrophoresis in Laemmli running buffer) were diluted with stock solutions of potassium phosphate (400 mM, pH 7.4), EDTA (10 mM), 1 mM, 1% and 0.5%, respectively, heated at 100℃ for 1 min and diluted 3 fold with 3% Triton X-100 in 40 mM potassium phosphate buffer (pH 7.3). Endoglycosidase F/N-glycosidase F was then added in the concentrations indicated in the figure legends and the sample was incubated for 18 hr at room temperature. The reaction was stopped by the addition of 1% SDS and the samples were frozen until further use.

Preparative Electrophoresis

The 0.07% Triton extract was fractionated with a Biorad Model 491 preparative SDS-PAGE electrophoretic apparatus using a 4% polyacrylamide stacking gel and a 5.5% polyacrylamide separating gel approximately 4.3 cm in length. Proteins were separated at 40 mA and 2 ml fractions were collected at a rate of 0.2 ml/min. Approximately 15 mg% of extracted protein from ~25 mg of BLM were applied to a single preparative gel.

Deglycosylated fractions from the above preparative run were fractionated on the Biorad Model 491 under the same running conditions using a 4% polyacrylamide stacking gel and 6% polyacrylamide separating gel approximately 5.3 cm in length.

Fractions from preparative electrophoresis were concentrated by ultrafiltration on Centricon-30 microconcentrator (Amicon, Beverly, MA).

Protein determination

Protein was measured by the method of Bradford (using the Bio-Rad protein assay kit with bovine IgG as the standard. When detergent was present in the sample, the Bio-Rad DC protein assay kit was used instead, with bovine serum albumin as the standard.

Results

Effect of Triton X-100 on Bumetanide Binding to BLM

BLM were treated with the concentrations of Triton X-100 indicated, then [³H]-bumetanide binding was measured either in this detergent-treated material or in the "extract" obtained by centrifuging this material at 150,000×g for 5 min. To allow direct comparision of binding to vesicles and extract, the data are presented as binding per milliliter of sample. First, at Triton concentrations <0.1% there is an apparent "activation" of bumetanide binding over that found in native vesicles. Second, as the Triton concentration increases above its critical micelle concentration (0.02%), there is a dramatic increase in bumetanide binding activity in the vesicle extract, and higher detergent concentrations (0.075%) all of the bumetanide binding activity observed is associated with the extract. In control experiments it has been verified that there is, in fact, no detectable bumetanide binding in the pellets obtained after centrifuging the BLM following treatment with Triton concentrations >0.075 %. Finally, at Triton concentrations >0.05% there is a progressive loss of bumetanide binding activity with increasing detergent concentration. Thus the loss of binding activity at higher Triton X-100 concentrations was not due to a selective failure of nitrocellulose filter to retain the bumetanide binding protein. Owing to its relative convenience we have used the nitrocellulose filtration assay in most of our experiments, and only those results are presented in the studies which follow.

We already showed the protein content of the vesicle extracts obtained at various concentrations of Triton X-100. Note that little additional protein is extracted from the BLM as the Triton X-100 concentration is increased above 0.075%. The 0.07% Triton X-100 was chosen as the starting point for the studies which follow. In additional experiments we have demonstrated that <10% of the bumetanide binding activity found in this extract is lost after centrifugation at 116,000×g×1 hr. Thus this material appears to represent a reasonable compromise between a partial purification and solubilization of the bumetanide-binding protein and its in-
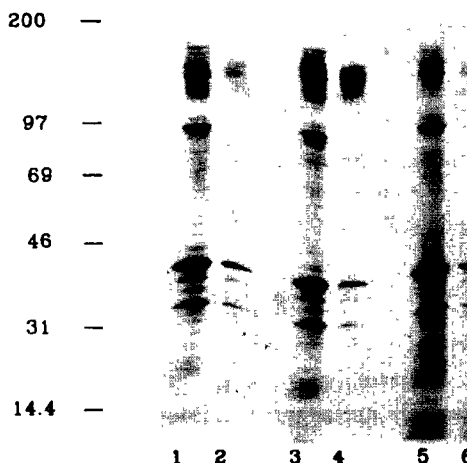

Fig. 1. [¹⁴C]-NEM labeling of rabbit parotid BLM. BLM were incubated with unlabeled NEM in the presence of 5 mM chloride and 30 uM bumetanide (lane 3 and 4), in the presence of 5 mM chloride but in the absence of bumetanide (lane 1 and 2), or in the absence of chloride (replaced by 5 mM gluconate) but in the presence of 30 μM bumetanide (lane 5 and 6), then labeled with [¹⁴C]-NEM. the figure shows an autoradiograph of a 5-15% SDS-PAGE gradient gel of these three membrane fractions (lane 1, 3, and 5) and their 0.07% Triton extracts (lane 2, 4 and 6).

activation by higher concentrations of Triton X-100.

Identification of the parotid bumetanide-binding protein using [¹⁴C]-NEM labeling

As mentioned above, the sulfhydryl reagent NEM irreversibly inactivates the rabbit parotid Na/K/Cl cotransporter and bumetanide, by binding to its high affinity site on the cotransporter, can prevent this effect of NEM. these observations suggested a strategy for labeling the cotransporter, or more accurately its bumetanide-binding moiety, with [¹⁴C]-NEM. Briefly stated, BLM were preincubated with unlabeled NEM in the presence of bumetanide (30 μM) to allow for the reaction of NEM with non-bumetanide protected sites. This preincubation was carried out in the presence of sodium, potassium and chloride (100, 100 and 5 mM, respectively) all of which are required for specific bumetanide binding to Na/Cl cotransporter (K_d 3 μM under these ionic conditions). The membrane were then

washed and incubated with [¹⁴C]-NEM in the absence of bumetanide to allow reaction of radiolabelled NEM with sites previously protected by bumetanide. The result of this procedure is illustrated in lane 3 of Fig. 1. Here, BLM labeled with [¹⁴C]-NEM, as just described, were run on SDS-PAGE and visualized by autoradiography. Control BLM treated identically to those in lane 3 but preincubated with unlabeled NEM in the absence of bumetanide are shown in lane 1. Inspection of these two lanes reveals a striking diffuse band of ¹⁴C-NEM labeling at an apparent molecular weight of approximately 160 kD in bumetanide-protected membranes (lane 1). This is seen even more clearly in lanes 2 and 4 which are the 0.07% Triton extracts of BLM run in lanes 1 and 3, respectively. Only five regions of significant [¹⁴C]-NEM labeling are seen in these extracts and of these, only the band at 160 kD is protected against unlabeled NEM by bumetanide. In 18 experiments of type, we find an apprarent molecular weight of 161±7 kD for this bumetanide-protected band.

Lane 5 if Fig. 1 corresponds to BLM which were treated identically to those in lane 3 except that the preincubation with unlabeled NEM in the presence of bumetanide was carried out in the absence
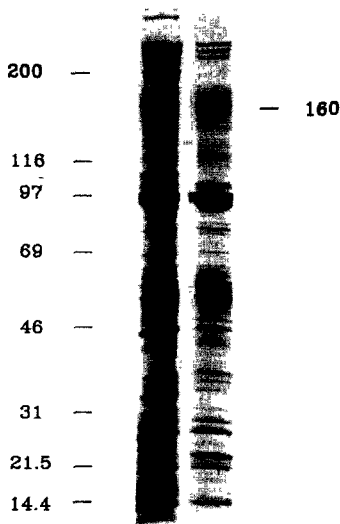
of chloride. Under these conditions, bumetanide is unable to bind its high affinity site on the Na/K/Cl cotransporter and thus would not be expected to provide protection against NEM. The 0.07% Triton extract of these membranes is shown in lane 6. Comparison of lanes 5 and 6 with lanes 1-4 demonstrates that the effect of removing chloride during the preincubation with unlabeled NEM is essentially identical to that of omitting bumetanide. Thus, the ability of bumetanide to protect the 160 kD protein against unlabeled NEM also shows the same chloride dependence which characterizes the binding of bumetanide to Na/K/Cl cotransporter.
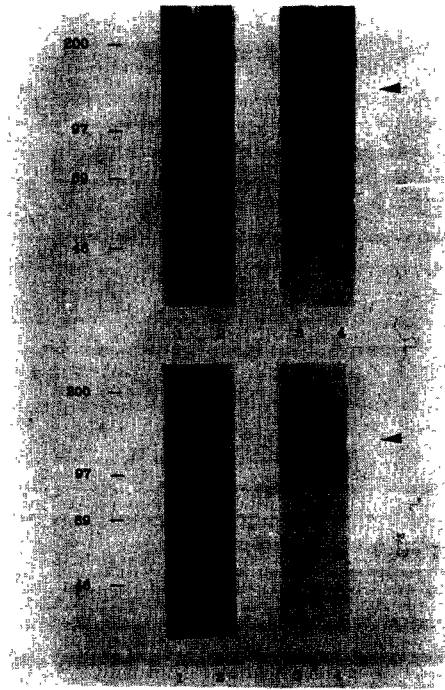

Fig. 3. Effect of deglycosylation on the 160 kD [¹⁴C]-NEM-labeled bumetanide-binding protein. BLM were labeled with [¹⁴C]-NEM as described for lane 3 of Fig. 2. A portion of these membranes and their 0.07% Triton extracts were then incubated for 18 hr with endoglycosidase F/N-glycosidase F (1 U/mg BLM protein). The BLM before and after glycosidase treatment (lane 1 and 2, respectively) and their corresponding 0.07% Triton extacts (lane 3 and 4, respectively) were then run on the same 7.5% SDS-PAGE gel. The top panel shows the resulting Coomassie-blue stained gel and the bottom panel its autoradiograph. The arrow indicates the 135 kD band.
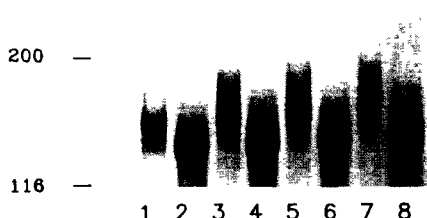

Fig. 2. Photography of a Coomassie-blue-stained 5-15% SDS-PAGE gel of rabbit parotid BLM (left lane) and their 0.07% Triton extract (right lane).

Fig. 4. Purification of the parotid bumetanide-binding protein-samples from the first preparative electrophoresis run before and after deglycosylation. The figure shows a photograph of a Coomassie-blue stained (dried) 7.5% SDS-PAGE slab gel. Lane 1, 3, 5 and 7 contain fractions obtained from preparative electrophoresis of the 0.07% Triton extract carried out as described in the text. These same fractions after treatment with endoglycosidase F/N-glycosidase F (~0.5 U/ml) are shown in lanes 2, 4, 6 and 8, respectively.

Taken together with other previous results provide strong evidence taht the 160 kD protein labeled with [^{14}C]-NEM in lanes 3 and 4 of Fig. 1 is a part or all of the bumetanide binding protein of the rabbit parotid basolateral membrane previously characterized functionally and physically and that this protein is a part or all of the Na/K/Cl cotransporter of this tissue.

In Fig. 2 a photograph of a Coomassie-blue stained gel rabbit parotid BLM (left lane) and their 0.07% Triton extract (right lane) is shown for comparison purposes.

Effects of glycosidase

The somewhat diffuse nature of the 160 kD band identified above gave a preliminary indication that the parotid bumetanide-binding protein might be glycosylated. The effect of treating [^{14}C]-NEM labeled rabbit parotid BLM with a commercially available mixture of endoglycosidase F and N-glycosidase F is shown in Fig. 3. Coomassie-stained gels are shown in the top panel and their corresponding autoradiographys in the bottom panel. In each case, lane 1 correspond BLM before treatment with glycosidase, lane 2 to glycosidase-treated BLM, lane 3 to the 0.07% Triton extract from the BLM in lane 1 and lane 4 to the glycosidase-treated 0.07 % Triton extract. Focusing first on the autoradiographys (bottom panel), it is immediately obvious

that the diffuse band at approximately 160 kD, identified above as the parotid bumetanide-binding protein, is dramatically narrowed and shifted downward lower Mr's after treatment with endoglycosidase F/N-glycosidase F (arrow). In 14 experiments of this type, we find an apparent molecular weight of 134± 6 kD for this deglycosylated protein. Focusing now on the Coomassie-stained gels, we see the appearance of a corresponding protein band at approximately 135 kD in both BLM and their 0.07% Triton extract following glycosidase treatment (arrow). Given the above results, this band is almost certainly the parotid bumetanide-binding protein.

The endoglycosidase F/N-glycosidase F mixture cleaves most common N-linked glycans from glycoproteins (Edler and Alexander, 1982; Plummer *et al.*, 1984). In additional experiments (not shown), we say little or no effect on the ^{14}C-labeled band at 160 kD when the 0.07% Triton extract was treated with endoglycosidase H, endoglycosidase D, O-glycosidase or neuraminidase. Furthermore, no significant difference could be detected between treatment with endoglycosidase F/N-glycosidase alone and endoglycosidase F/N-glycosidase in combination with all of these other glycosidases (data not shown).

Purification of the 135 kD protein

By taking advantage of the facts that (i) the bumetanide-binding protein is relatively abundant in our rabbit parotid basolateral membrane preparation, and (ii) that the apparent molecular weight of the bumetanide-binding protein shifts quite dramatically (25 kD) following deglycosylation (Fig. 3), we have devised a relatively simple procedure for purifying this protein by preparative SDS-PAGE electrophoresis. A 0.07% Triton extract was first run on preparative electrophoresis as described in Materials and Methods. Fractions containing proteins in the 160 kD range were then identified on SDS-PAGE slab gels. These were pooled in lots of 2-3, deglycosylated with endoglycosidase F/N-glycosidase F, and rerun on SDS-PAGE slab gels along with their glycosylated precursors. An example of the results of this procedure is illustrated in Fig. 4. Here the even-numbered lanes contain
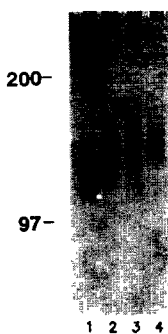
Fig. 5. Purification of the parotid bumetanide-binding protein-sample from the second preparative electrophoresis run. The figure shows a photograph of a Coomassie-blue stained (dried) 7.5% SDS-PAGE slab gel. Lane 1-4 show alternate fractions from the second preparative electrophoresis run described in the text.

deglycosylated materials and the odd-numbered lanes to their left contain the original (pooled) fractions from preparative electrophoresis (note that for practical reasons the same amount of protein has not been run in each lane). Based on these electrophoretic patterns, one can see that the deglycosylated fractions in lanes 4 and 6 are largely made up of protein displaying a dramatic shift in molecular weight following glycosidase treatment (from approximately 160-135 kD) and containing little contamination from other non-glycosidase-affected species. These fractions would therfore be expected to be highly enriched in the bumetanide protein. The enriched samples identified above would typically be pooled and concentrated by ultrafiltration, rerun on preparative electrophoresis as described in Materials and Methods, and the resulting fractions rescreened on SDS-PAGE slab gels. An example of such a rescreening is shown in Fig. 5. In this case, every other fraction from the second preparative run was examined. the fractions in lanes 2 and 3 and the additional fraction which eluted between them would typically be chosen for our final 135 kD protein-enriched preparation. The fractions which preceded the one in lane 2 and followed the one in lane 3 would also be subsequently examined on SDS-PAGE (in this case they were also chosen for inclusion in the final preparation). In five independent preparations follo-

wing the above procedure, the final yield of electrophoretically purified protein prepared by this method was found to be 0.60± 0.09% of the total starting BLM protein.

Preparative electrophoresis was not routinely carried out on [14C]-NEM labeled membranes; however, the results of one such experiment is illustrated in Fig. 6. This figure shows an autoradiograph of an SDS-PAGE slab gel in which BLM (lane 1), their 0.07% Triton extract (lane 2), pooled enriched samples from the first preparative electrophoresis step before (lane 3) and after (lane 4) deglycosylation, and the final purified 135 kD protein (lane 5) were run. It is clear from this figure that the [14C]-NEM labeling we have associated with the parotid bumetanide-binding protein in this study copurifies with our final protein fraction (lane 5). By determining the relative amouns of protein run on this gel from densitometry of Coomassie blue staining, and the relative amounts of radioactivity in the 160 kD band of the 0.07% Triton extract and the 135 kD band corresponding to the final purified protein, it is possible to estimate the enrichment of the [14C]-NEM labeled final purified protein. In this way, we find that the final purified 135 kD protein fraction is enriched approximately 48 times relative to the 0.07% Triton extract.

Discussion

Many researchers have attempted to identify the molecular constituents of the Na/K/Cl cotransporter. Pewitt et al. (1988) have demontrated that, when solubilized calf renal outer medullary membranes are passed over an affinity chromatography column consisting of the bumetanide analogue 4-p-aminobumetanide coupled to Affigel-10, a 160 kD protein is retained and can be subsequently eluted with excess bumetanide. Dunham et al. (1990) and Feit et al. (1988) have also used affinity chromatographic techniques with a bumetanide analogue to identify proteins with molecular weights ~82 and ~39 kD in membranes from Ehrlich ascites tumor cells. Antibodies raised against these proteins strongly inhibited Na/K/Cl cotransport activity (Dunham et al., 1990) and cross-reacted with a 150 kD protein from mouse kidney (Haas et al., 1991). In radiation inac-

tivation studies, Kinne and collaborators (1984) found a target size of 83 kD for sodium transport activity via the cotransporter from the rabbit renal outer medulla.

Jorgensen, Petersen, and Rees (1984) found a 34 kD polypeptide which was photolabeled with [3H]-bumetanide when pig renal outer medullary membranes, preincubated with thw labeled drug, we irradiated with light at 345 nm (an absorptive maximum for bumetanide). O'Grady *et al.* (1987) identified a 6 kD peptide in both winter flounder intestine and bovine kidney that was also photolabeled with [3H]-bumetanide. Using [3H]-azidopiretanide, photoreactive bumetanide analogue, DiStephano *et al.* (1986) have photolebeled a 24 kD protein in isolated pig thick ascending limbs of Henle's loop. Using similiar techniques with another bumetanide analogue, [3H]-4-benzoyl-5-sulfamoyl-3-(3-thenyloxy)-benzoic acid ([3H]-BSTBA), Haas and collaborators have photolabeled 150 kD proteins in dog (Haas and Forbush, 1987) and mouse (Haas *et al.*, 1991) renal medullary membranes and in duck red blood cells (Haas and Forbush, 1988), as well as a 196 kD from the shark rectal gland (Forbush *et al.*, 1992; Lytle *et al.*, 1992).

More recently Lytle *et al.* (1992) have purified the 195 kD protein from shark rectal gland indentified by [3H]-BAPTA labeling and developed a panel of monoclonal antibodies directed against it. With these antibodies they were able to demonstrate that this protein is heavily glycosylated with a deglycosylated apparent weight of 135 kD and that it is localized to the basolateral membrane of the rectal gland secretory cell (Lytle *et al.*, 1992). These antibodies also cross-react with a 170 kD protein from the avian salt gland thought to be the Na/K/Cl cotransporter of that tissue (Torchia *et al.*, 1992). In additional studies, Lytle and Forbush (1992) have shown that phosphorylation of the 195 kD protein in intact rectal gland cells in response to secretagogues closely parallels activation of the Na/K/Cl cotransporter.

In the previous work with the Na/K/Cl cotransporter of the rabbit parotid (Turner and George, 1988), a somewhat different approach has been attempted to purify the solubilized transport protein
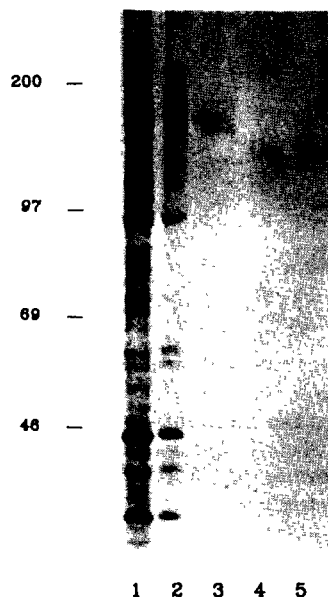

Fig. 6. Purification of the [14C]-NEM labeled bumetanide-binding protein. BLM were labeled with [14C]-NEM as described in Materials and Methods and subjected to the purification procedure for 135 kD bumetanide-binding protein described in the text. The figure is an autoradiograph of an SDS-PAGE slab gel. The samples shown are BLM (lane 1), their 0.07% Triton extract (lane 2), pooled enriched samples from the first preparative electrophoresis step before (lane 3) and after (lane 4) deglycosylation, and the final purified 135 kD protein (lane 5).

by sucrose density centrifugation while preserving its bumetanide-binding activity. In these experiment, we identified a bumetanide-binding protein-detergent-lipid complex with a molecular weight ~ 200 kD we suggested might correspond to a ~160 kD detergent free protein.

At present, it is still not clear what the relationships are among the various proteins enumerated above and Na/K/Cl cotransporter/bumetanide binding protein. It seems reasonable to assume that at least some of this lack of concensus is due to the identification of various protein subunits or degradation products. The work described in this paper, however, strongly correlates with the results of Haas, Lytle and Forbush (Haas and Forbush, 1987, 1988; Haas *et al.*, 1991; Forbush *et al.*, 1992; Lytle and Forbush, 1992; Lytle *et al.*, 1992) outlined

above, while using a completely independent approch. We demonstrate here the presence of a 160 kD protein in rabbit parotid basolateral membrane that can be labeled with [^{14}C]-NEM in a bumetanide-protectable fashion (Fig. 1). The specificity of this labeling and our previous evidence for the existence of an essential sulfhydryl group closely associated with the bumetanide-binding site on the Na/K/Cl cotransporter (George and Turner, 1988) provide strong evidence that this 160 kD protein is a part or all of the parotid bumetanide-binding site. The molecular weight of this protein also agrees well with a number of these earlier estimates from [^{a3}H]-BATBA binding (Haas and Forbush, 1987, 1988; Pewitt et al., 1988; Hass et al., 1991). Furthermore, although the molecular weight of protein identified as the Na/K/Cl cotransporter in the shark rectal gland using [^{a3}H]-BATBA binding is 195 kD (Forbush et al., 1992; Lytle et al., 1992), we show here that the molecular weight of the parotid bumetanide binding protein decrease to 135 kD following deglycosylation (Fig. 3), as does its counterpart in the shark rectal gland. Whether Na/K/Cl cotransporter from other tissues will prove to be as heavily glycosylated as those from these two secretory epithelia remains to be solved.

We have made use of the relatively large change in molecular weight of the parotid bumetanide-binding protein following deglycosylation (25 KD) to purify it using two preparative electrophoresis steps (Figs. 4 and 5). It was anticipated that this procedure might be quite successful owing to the relative abundance of the bumetanide-binding protein in the parotid basolateral membrane (with a molecular weight of 135 kD the bumetanide-bind- ing protein is expected to make up approximately 2% of the total protein in the 0.07% Triton extract of the basolateral membrane, the starting material for the first preparative electrophoresis step). This was confirmed by carrying out preparative electrophoresis procedure on [^{14}C]-NEM labeled mem- brane and estimating the enrichment of the ^{14}C- labeled final purified protein (Fig. 6). This enrichment (48 times relative to the 0.07% Triton extract) was close to the theoretical maximum (approx. 50 times), indicating a very high degree of purity of the 135 kD fraction. the yield from this puri-

fication method was also quite good: 0.5% of the total starting basolateral membrane protein, or equivalently 1% of the protein in the 0.07% Triton extract. Using material from this preparative procedure, the avalability of this relatively simple procedure of purifying the parotid bumetanide-binding protein should greatly faciltate future determinations of its biophysical and biochemical properties.

REFERENCES

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. **72**: 248-254, 1978.

Brown, C.D.A., Murer, H.: Characterization of a Na : K : 2Cl cotransport system in the apical membrane of a renal epithelial cell line (LLC-PK1) J. Membrane Biol. **87**: 131-139, 1985.

Castle, A.M., Castle, J.D.: The purification and partial characterization of phospholipase A$_2$, a secretory protein of rabbit parotid gland. Biophys. Acta **666**: 259-274, 1981.

Corecelli, A., Turner, R.J.: Reconstitution of the rabbit parotid basolateral membrane Na/K/Cl dependent bumetanide binding site. Ann. N.Y. Acad. Sci. 1989.

Distephano, A., Wangemann, P., Friedrich, T., Burckhardt, G., Okonomopoulos, R., Englert, H.C. Lang H. J. Greger, R.: Photoaffinity labelling of the Na-K-2Cl carrier. Pfluegers Arch. **406**: R59, 1988.

Dunham, P.B., Jessen, F., Hoffmann E.K.: Inhibition of Na-K-Cl cotransport in Ehrlich ascites cells by antiserum against purified proteins of the cotransporter. Proc. Natl. Acad. Sci. USA **87**: 6828-6832, 1990.

Elder, J.H., Alexander, S.: Endo-β-N-acetylglucosaminidase F: Endoglycosidase from Flavobacterium meningosepticum that cleaves both high-mannose and complex glycoproteins. Proc. Natl. Acad. Sci. USA **79**: 4540-4544, 1982.

Epstein, F.H., Stoff, J.S., Silva, P.: Mechanism and control of hyperosmotic NaCl-rich secretion by the rectal gland of Squalus acanthias. J. Exp. Biol. **106**: 25-41, 1983.

Feit, P.W., Hoffmann, E.K., Schiodt, M., Kristensen, P., Jessen, F.: Purification of proteins of the Na/Cl cotransporter from membranes of Ehrlich ascites cells using a bumetanide-sepharose affinity column. J. Membrane Biol. **103**: 135-147, 1988.

Forbush, B., Haas, M., Lytle, C.: Na-K-Cl cotransport in the shark rectal gland. Regulation in the intact perfused gland. Am. J. Physiol. **262**: C1000-C1008, 1992.

Frizzell, R.A., Field, M., Schultz, S.G.: Sodium coupled chloride transport by epithelial tissues. Am. J.

Physiol. **236**: F1-F8, 1979.

Geck, P., Heinz, E.: The Na-K-2Cl cotransport system. J. Membrane Biol. **91**: 97-105, 1988.

George, J.N., Turner, R.J.: Inactivation of the rabbit parotid Na/K/Cl cotransporter by N-ethylmaleimide. J. Membrane Biol. **112**: 51-58, 1989.

Haas, M.: Properties and diversity of (Na-K-Cl) cotransporters. Annu. Rev. Physiol. **51**: 443-457, 1989.

Haas, M., Dunham, P.B., Forbush, B.: [³H] bumetanide binding to mouse kidney membranes: identification of corresponding membrane proteins. Am. J. Physiol. **260**: C791-C804, 1991.

Haas, M., Forbush, B.: Photolabeling of a 150-kDa (Na + K + Cl) cotransport protein from dog kidney with a bumetanide analogue. Am. J. Physiol. **253**: C243-C250, 1987.

Hjelmeland, L.M., Chrambach, A.: Solubilization of functional membrane proteins. Meth. Enzymol. **104**: 305-318, 1984.

Hoffman, E.K., Schiodt, M., Dunham, P.: The number of chloride-cation cotransport sites on Ehrlich ascites cells measured with [³H] bumetanide. Am. J. Physiol. **250**: C688-C693, 1988.

Haas, M., Forbush, B.: Photoaffinity labelling of a 150-kDa (Na + K + Cl) cotransport protein from duck red blood cells with an analogue of bumetanide. Biochim. Biophys. Acta **939**: 131-144, 1988.

Jorgensen, P.L., Petersen, J., Ress, W.D.: Identification of a Na-K-Cl cotransport protein of Mr 34,000 from kidney by photolabeling with [³H] bumetanide. Biochim. Biophys. Acta **775**: 105-110, 1984.

Kinne, R.K.H.: The Na-K-Cl cotransporter in the kidney. Ann. NY Acad. Sci. **574**: 63-74, 1989.

Kinne, R., Kinne-Saffran, E., Scholermann, B., Schutz, H.: The anion specificity of the sodium-potassium-chloride cotransporter in rabbit kidney outer medulla: Studies on medullary plasma membranes. Pfluegers Arch. **407**: S168-S173, 1988.

Klausner, R.D., von Renswoulde, J., Rivnay, B.: Reconstitution of membrane proteins. Meth. Enzymol. **104**: 340-347, 1984.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685, 1970.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265-275, 1951.

Lytle, C., Forbush, B.: The Na-K-Cl cotransport protein of shark rectal gland. Regulation by direct phosphorylation. J. Biol. Chem. **267**: 25438-25443, 1992.

Lytle, C., Xu, J.C., Biemesderfer, D., Haas, M., Forbush, B.: The Na-K-Cl cotransport protein of shark rectal gland. Development of monoclonal antibodies, immunoaffinity purification, and partial biochemical characterization. J. Biol. Chem. **267**: 25428-25437, 1992.

Melvin, J.E., Kawaguchi, M., Baum, B.J., Turner, R.J.: A muscarinic agonist-stimulated chloride efflux pathway is associated with fluid secretion in rat parotid acinar cells. Biochem. Biophys. Res. Commun. **145**: 754-759, 1987.

Nauntofte, B., Poulsen, J.H.: Effects of Ca²⁺ and furosemide on Cl⁻ transport and O₂ uptake in rat parotid acini. Am. J. Physiol. **251**: C175-C185, 1988.

O'Grady, S.M., Palfery, H.C., Field, M.: Na-K-2Cl cotransport in winter flounder intestine and bovine kidney outer medulla: [³H]-bumetanide binding and effects of furosemide analogues. J. Membrane Biol. **96**: 11-18, 1987.

O'Grady, S.M., Palfrey, H.C., Field, M.: Characteristics and functions of Na-K-Cl cotransport in epithelial tissues. Am. J. Physiol. **253**: C177-C192, 1987.

Petersen, O.H., Maruyama, Y.: Calcium-activated potassium channels and their role in secretion. Nature (London) **307**: 693-696, 1984.

Pewitt, B., Palfrey, H.C., Burnham, C.E.: Affinity purification of a bumetanide binding protein possibly related to the Na/K/Cl cotransporter. FASEB J. **2**: A1725 (Abstr.), 1987.

Pirani, D., Evans, A.R., Cook, D.I., Young, J.A.: Intracellular pH in the rat mandibular salivary gland: The role of Na-H and Cl-HCO₃ antiports in secretion. Pflugers Arch. **408**: 178-184, 1987.

Plummer, T.H., Elder, J.H., Alexander, S., Phelan, A.W., Tarentino, A.L.: Demonstration of peptide: N-glycosidase F activity in Endo-β-N-acetylglucosaminidase preparations. J. Biol. Chem. **259**: 10700-10704, 1984.

Schachman, H.K.: Ultracentrifugation in biochemistry. Academic New York, 1953.

Shaw, G.: Convenient methods for the display, comparison and interpretation of amino acid composition data. Biol. Chem. **267**: 25444-25450, 1992.

Torchia, J., Lytle, C., Pon, D.J., Forbush, B., Sen, A.K.: The Na-K-Cl cotransporter of avian salt gland. J. Biol. Chem. **267**: 25444-25450, 1992.

Towbin, H., Staehelin, T., Gordon, J.: Electrophoretic transfer of proteins polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA **76**: 4350-4354, 1979.

Turner, R.J.: Ion transport related to fluid secretion in salivary glands. In: Biology of the Salivary Glands. K. Dobrosielski-Vergona, eiditor. pp. 105-127. Boca Raton, FL, 1993.

Turner, R.J., George, J.N.: Ionic dependence of bumetanide binding to the rabbit parotid Na/K/Cl cotransporter. J. Membrane Biol. **102**: 71-77, 1988.

Turner, R.J., George, J.N., Baum, B.J.: Evidence for a Na⁺/K⁺/Cl⁻ cotransport system in basolateral membrane vesicles from the rabbit parotid. J. Membrane Biol. **94**: 43-152, 1988.

Young, J.A., Cook, D.I., van Lennep, E.W., Roberts, M.L.: Secretion by the major salivary glands in: Physiology of the Gastrointestinal Tract. (2nd ed.) L. Johnson et al., editors. Raven, New York, 1987.