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Identification, Characterization and Purification of a 160 kD Bumetanide-binding Glycoprotein from the Rabbit Parotid

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Abstract. We demonstrate the presence of a 160 kD protein in rabbit parotid basolateral membranes that can be labeled with the irreversible sulfhydryl reagent [^{14}C]-N-ethylmaleimide in a bumetanide-protectable fashion. 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 parotid Na^+-K^+-Cl^- cotransporter (*J. Membrane Biol.* **112:**51–58, 1989), 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 pI of this deglycosylated protein is ≈6.4. 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 in this starting extract, this enrichment indicates a high degree of purity of this protein in the 135 kD fraction.

Key words: Loop diuretic — Salivary gland — Fluid secretion — Na^+-K^+-Cl^- cotransport — Acinar cell — Chloride secretion

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Introduction

Much recent experimental effort has been devoted to the study of Na^+-K^+-Cl^- cotransport systems [for reviews, *see* 7, 9, 18]. 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 [23]. Clinically, the cotransporter 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 [7, 9].

Several years ago we provided direct evidence for the presence of Na^+-K^+-Cl^- cotransport activity in basolateral membrane vesicles prepared from the rabbit parotid [26]. In addition, we identified a high affinity ($K_d \sim 3$ μM) bumetanide-binding site in this preparation and provided strong evidence that this site was identical with the bumetanide inhibitory site on the Na^+-K^+-Cl^- cotransporter [24]. In a subsequent study [8], we demonstrated that high affinity bumetanide binding to rabbit parotid basolateral membranes is inactivated by the irreversible sulfhydryl reagent N-ethylmaleimide (NEM). This effect of NEM could be prevented by the presence of bumetanide, indicating the existence of an essential sulfhydryl group at or closely related to the bumetanide-binding site. NEM also reduced ^{22}Na transport via the cotransporter by the same factor as the number of high affinity bumetanide-binding sites [8], again confirming the association of this bumetanide-binding site with the cotransporter.

More recently [25], we demonstrated that the

rabbit parotid bumetanide-binding protein could be partially 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 experiments, we estimated that the bumetanide-binding protein-detergent-lipid complex had a molecular weight ~200 kD and that the molecular weight of the protein itself might be ~160 kD, in good agreement with some earlier estimates of the size of the Na^+-K^+-Cl^- cotransporter in other tissues [11, 12]. Our bumetanide-binding studies also showed that there were approximately 100 pmol of bumetanide-binding sites per mg of rabbit parotid basolateral membrane protein [24]. 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

Materials

Nonidet P-40 was from Boehringer Mannheim (Indianapolis, IN), CHAPS from Calbiochem (La Jolla, CA) and all other materials for electrophoresis, including molecular weight and isoelectric focusing markers were from Bio-Rad Laboratories (Richmond, CA). [^{14}C]-NEM (≈40 mCi/mmol) was obtained from Dupont NEN (Boston, MA). Endoglycosidase F/N-glycosidase F, endoglycosidase H, endoglycosidase D, O-glycosidase and neuraminidase were purchased from Boehringer Mannheim. Bumetanide was a gift from Hoffman-LaRoche (Nutley, NJ). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

Preparation of Parotid Basolateral Membranes and Membrane Extracts

Basolateral membranes (BLM) were prepared from rabbit parotid by a Percoll gradient method, as previously described [26]. Relative 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 × *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% solution of 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 × *g* in a Beckman Airfuge. The supernate from this spin is referred to as the "0.07% Triton extract." We have previously demonstrated that this extract retains most of the bumetanide-binding sites (≥85%) but only 60% of the protein found in the starting BLM [25].

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

BLM (typically 1–3 ml at ≈1 mg protein/ml) were incubated for 1 hr at room temperature (≈25°C) in Buffer A/EDTA containing 100 mM Na gluconate, 95 mM K gluconate, 5 mM KCl and 30 μM bumetanide (in some experiments bumetanide was omitted or chloride was replaced by gluconate—*see* text and figure legends). 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 × *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 × *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 ≈5 mg/ml. Sites previously unreacted 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 room temperature. The BLM were then diluted with 20 ml ice-cold Buffer A/EDTA, centrifuged as above, washed again in Buffer A/EDTA, frozen in Buffer A/EDTA and stored above liquid nitrogen until further use.

SDS-PAGE Electrophoresis and Isoelectric Focusing

SDS-PAGE was performed essentially as described by Laemmli [15] 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°C for 2 min in a solution containing 1% SDS, 0.001% bromphenol blue, 3.3% sucrose and 40 mM dithiothreitol (final concentrations) 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).

For isoelectric focusing (IEF) samples were heated at 100°C for 2 min in 100 mM dithiothreitol, 8 M urea, 5% CHAPS, 5% Nonidet P-40 and 2% ampholyte 3/10 (Bio-Rad) then applied to a

Novex pI 3–10 IEF gel (Novex, Encinitas, CA) and run at 100 V for 1 hr, 200 V for 1 hr, and 500 V for 30 min. Prior to staining with Coomassie brilliant blue, gels were soaked in 10% trichloroacetic acid.

Autoradiography and Densitometry

In some experiments (e.g., Fig. 1) autoradiography of 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 NEN) according to the manufacturer's instructions. In other experiments (e.g., Figs. 3 and 6) 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 referred 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), β-mercaptoethanol (100%) and SDS (20%) to yield final concentrations of 40 mM, 1 mM, 1% and 0.5%, respectively, heated at 100°C 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 (*see* Results) were fractionated on the Biorad Model 491 under the same running conditions using a 4% polyacrylamide stacking gel and a 6% polyacrylamide separating gel approximately 5.3 cm in length.

Fractions from preparative electrophoresis were concentrated by ultrafiltration on Centricon-100 or Centricon-30 microconcentrators (Amicon, Beverly, MA).

Protein Determination

Protein was measured by the method of Bradford [1] 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.

Abbreviations

10 mM Tris/HEPES: 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffered with Tris to pH 7.4. EDTA: ethylenediaminetetraacetic acid. BLM: basolateral membranes. NEM: N-ethylmaleimide.

Results

Identification of the Parotid Bumetanide-Binding Protein Using [^{14}C]-NEM Labeling

As mentioned above, we have previously demonstrated [8] that the sulfhydryl reagent NEM irreversibly inactivates the rabbit parotid Na^+-K^+-Cl^- cotransporter and that 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 [^{14}C]-NEM. Briefly stated (*see* Materials and Methods for details), 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 the Na^+-K^+-Cl^- cotransporter ($K_d \approx 3$ μM under these ionic conditions; ref. 24). The membranes were then washed and incubated with [^{14}C]-NEM in the absence of bumetanide to allow reaction of radiolabeled NEM with sites previously protected by bumetanide. The result of this procedure is illustrated in lane *3* of Fig. 1. Here, BLM labeled with [^{14}C]-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 ^{14}C-NEM labeling at an apparent molecular weight of approximately 160 kD in bumetanide-protected BLM (lane *3*) which is virtually absent in bumetanide-unprotected membranes (lane *1*). This is seen even more clearly in lanes *2* and *4* which are the 0.07% Triton extracts of the BLM run in lanes *1* and *3*, respectively. Only five regions of significant [^{14}C]-NEM labeling are seen in these extracts and of these, only the band at 160 kD is protected against unlabeled NEM by

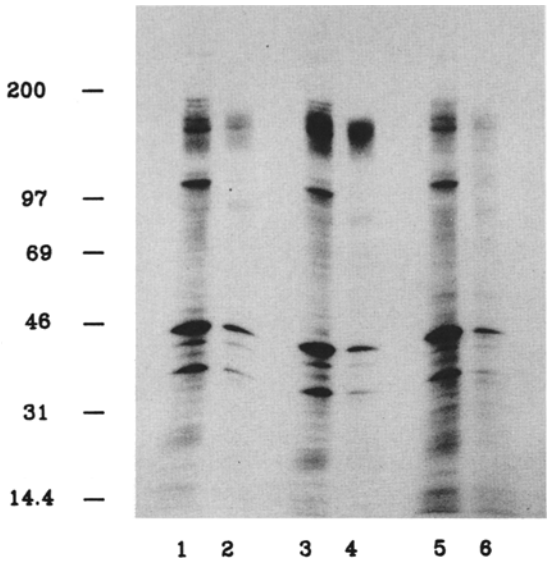

Fig. 1. [^{14}C]-NEM labeling of rabbit parotid BLM. BLM were incubated with unlabeled NEM in the presence of 5 mM chloride and 30 μM bumetanide (lanes *3* and *4*), in the presence of 5 mM chloride but in the absence of bumetanide (lanes *1* and *2*), or in the absence of chloride (replaced by 5 mM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

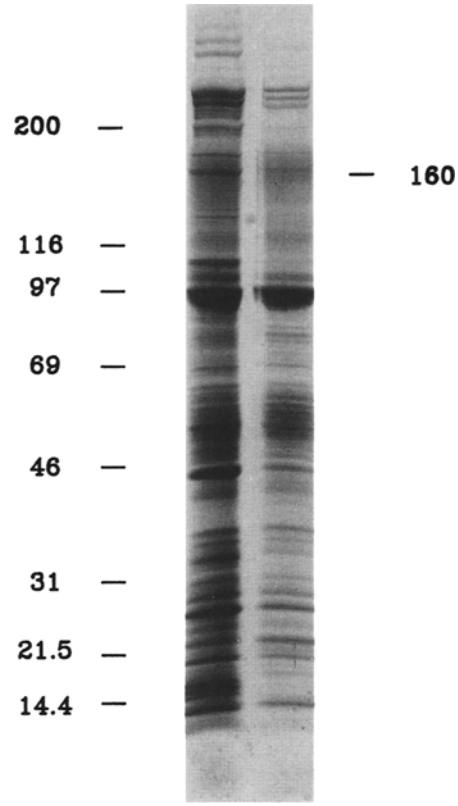

Fig. 2. Photograph of a Coomassie-blue-stained 5–15% SDS-PAGE gel of rabbit parotid BLM (left lane) and their 0.07% Triton extract (right lane).

bumetanide. In 18 experiments of this type, we find an apparent molecular weight of 161 ± 7 kD for this bumetanide-protected band.

Lane *5* of 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 (*see* Fig. 1 legend). Under these conditions, bumetanide is unable to bind to its high affinity site on the Na^+-K^+-Cl^- cotransporter [24] 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 the Na^+-K^+-Cl^- cotransporter. Taken together with our earlier data these results provide strong evidence that 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 [8, 24] and physically [25] in our laboratory, 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 of rabbit parotid BLM (left lane) and their 0.07% Triton extract (right lane) is shown for comparison purposes.

Effects of Glycosidases

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 (*see* Materials and Methods) is shown in Fig. 3. Coomassie-stained gels are shown in the top panel and their corresponding autoradiographs in the bottom panel. In each case, lane *1* corresponds to 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-

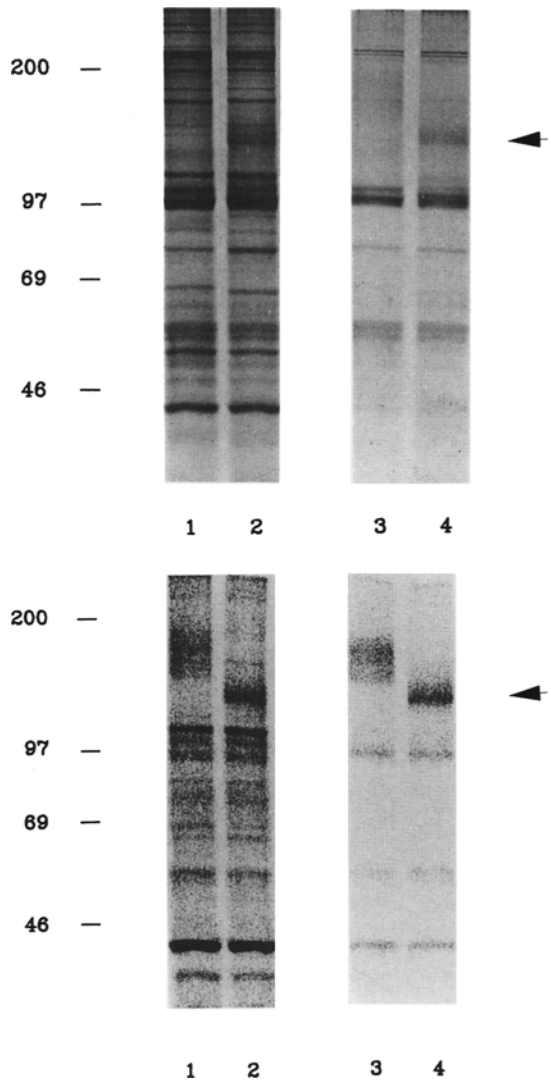

Fig. 3. Effect of deglycosylation on the 160 kD [^{14}C]-NEM-labeled bumetanide-binding protein. BLM were labeled with [^{14}C]-NEM as described for lane *3* of Fig. 1. A portion of these membranes and their 0.07% Triton extracts were then incubated for 18 hr with endoglycosidase F/N-glycosidase F (1 U/mg BLM protein) as described in Materials and Methods. The BLM before and after glycosidase treatment (lanes *1* and *2*, respectively) and their corresponding 0.07% Triton extracts (lanes *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 discussed in the text.

treated 0.07% Triton extract. Focusing first on the autoradiographs (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 toward 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 [4, 20]. In additional experiments (*not shown*), we saw 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 deglycosylated material 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 to 135 kD) and containing little contamination from other non-glycosidase-affected species. These fractions would therefore be expected to be highly enriched in the bumetanide-binding protein.

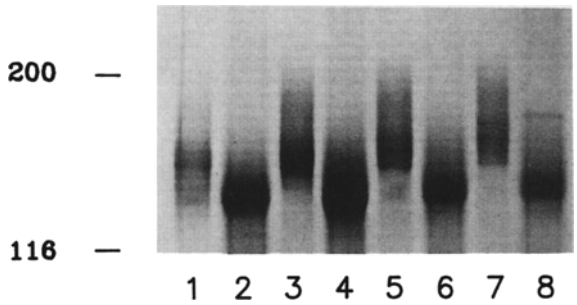

Fig. 4. Purification of the parotid bumetanide-binding protein—samples from the first preparative electrophoresis run before and after deglycosylation. The figure shows a photograph of a Coomassie-blue-stained (dried) 7.5% SDS-PAGE slab gel. Lanes *1, 3, 5* and *7* contain fractions obtained from preparative 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, as described in Materials and Methods) are shown in lanes *2, 4, 6* and *8*, respectively.

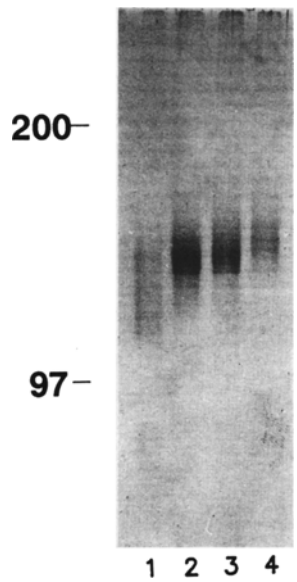

Fig. 5. Purification of the parotid bumetanide-binding protein—samples from the second preparative electrophoresis run. The figure shows a photograph of a Coomassie-blue-stained (dried) 7.5% SDS-PAGE slab gel. Lanes *1–4* show alternate fractions from the second preparative electrophoresis run described in the text.

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

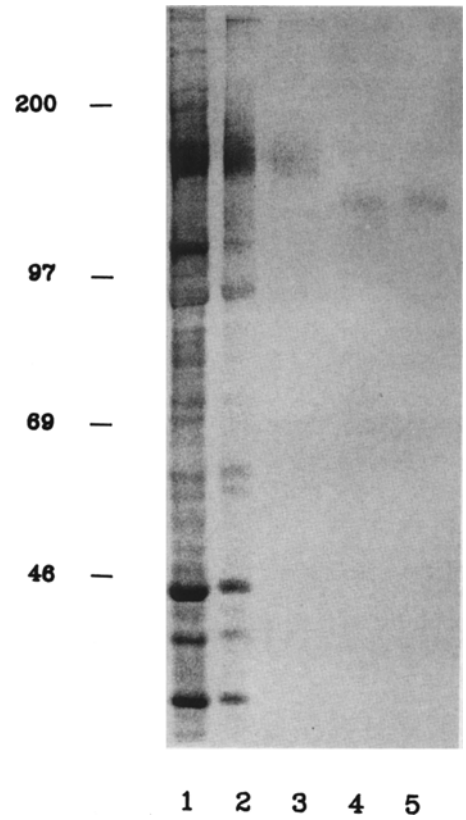

Fig. 6. Purification of the [^{14}C]-NEM labeled bumetanide-binding protein. BLM were labeled with [^{14}C]-NEM as described in Materials and Methods and subjected to the purification procedure for the 135 kD bumetanide-binding protein described in the text. The figure is an autoradiograph of an SDS-PAGE 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*).

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 following 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 [^{14}C]-NEM labeled membranes; however, the result 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 sam-

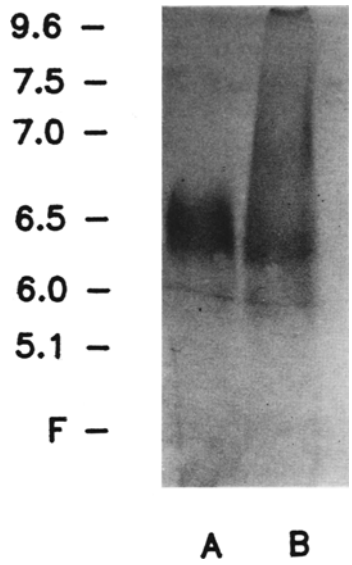

Fig. 7. Isoelectric focusing of the 135 kD purified protein. The figure shows a photograph of a Coomassie-blue-stained isoelectric focusing gel. The final purified 135 kD fraction (lane *A*) and an enriched fraction from the first preparative electrophoresis step before deglycosylation (lane *B*; *see text*) were subjected to isoelectric focusing as described in Materials and Methods.

ples 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 [^{14}C]-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 amounts 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 (*see* Materials and Methods), it is possible to estimate the enrichment of the [^{14}C]-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.

Figure 7 shows a photograph of a pI 3–10 isoelectric focusing gel on which the final purified 135 kD protein was run in lane *A* and an enriched fraction from the first preparative electrophoresis step described above was run in lane *B*. The 135 kD protein migrates as a broad band with pI $\approx$ 6.4.

Amino Acid Composition of the 135 kD Protein

The relative amino acid composition of the 135 kD protein is shown in the Table. When these results are expressed relative to the average amino acid content of all proteins in GenBank (Table), it can be seen that the parotid bumetanide-binding protein is comparatively rich in serine, glutamate/glutamine, tyrosine and histidine and contains relatively little methionine. Comparison with the amino acid content of two other chloride transporting proteins, the cystic fibrosis transmembrane regulator and band 3, revealed no striking similarities (*data not shown*).

Table. Amino acid composition of the 135 kD protein*

Amino acid(s)	Relative content (nmol/nmol Glx)	Content/Genebank
Asx	0.683	1.051
Glx	1.000	1.378
Ser	0.855	1.678
Thr	0.310	0.742
Arg	0.305	0.809
His	0.189	1.199
Lys	0.407	1.018
Pro	0.256	0.693
Met	0.086	0.525
Ile	0.280	0.748
Leu	0.564	0.864
Val	0.369	0.821
Ala	0.452	0.851
Phe	0.242	0.892
Tyr	0.273	1.234

* Enriched deglycosylated samples from the first preparative electrophoresis step (*see text*) were run on a 9% SDS-PAGE slab gel (instead of the second preparative electrophoresis step) and the band corresponding to the purified deglycosylated 135 kD protein was cut out and sent to the W.M. Keck Foundation (New Haven, CT) for total amino acid analysis. Glycine content could not be reliably estimated and cysteine content was not measured. The results (in nmol/sample) have been normalized to the content of Glx, or converted to nmol % and expressed relative to the nmol % for that amino acid averaged over all proteins in GenBank [21], as indicated. The results are the averages of two independent determinations carried out in duplicate.

Discussion

A number of laboratories have attempted to identify the molecular constituents of the Na^+-K^+-Cl^- cotransporter. Pewitt, Palfrey and Burnham [19] have demonstrated 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 and collaborators [3, 5] 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 [3] and cross-reacted with a 150 kD protein from mouse kidney [10]. In radiation inactivation studies, Kinne and collaborators [14] found a target size of 83 kD for sodium transport activity via the cotransporter from the rabbit renal outer medulla.

Jorgensen, Petersen, and Rees [13] found a 34 kD polypeptide which was photolabeled with [^{3}H]-bumetanide when pig renal outer medullary membranes, preincubated with the labeled drug, were irradiated with light at 345 nm (an absorptive maximum for bumetanide). O'Grady et al. [18] identified a 6 kD peptide in both winter flounder intestine and bovine kidney that was also photolabeled with [^{3}H]-bumetanide. Using [^{3}H]-azidopiretanide, a photoreactive bumetanide analogue, DiStephano et al. [2] have photolabeled a 24 kD protein in isolated pig kidney thick ascending limbs of Henle's loop. Using similar techniques with another bumetanide analogue, [^{3}H]-4-benzoyl-5-sulfamoyl-3-(3-thenyloxy)-benzoic acid ([^{3}H]-BSTBA), Haas and collaborators have photolabeled 150 kD proteins in dog [11] and mouse [10] renal medullary membranes and in duck red blood cells [12], as well as a 195 kD protein from the shark rectal gland [6, 17].

More recently Lytle et al. [17] have purified the 195 kD protein from shark rectal gland identified by [^{3}H]-BSTBA 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 [17]. 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 [22]. In additional studies, Lytle and Forbush [16] 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 our own earlier work with the Na^+-K^+-Cl^- cotransporter of the rabbit parotid [25], we took a somewhat different approach by attempting to purify the solubilized transport protein by sucrose density centrifugation while preserving its bumetanide-binding activity. In these experiments, we identified a bumetanide-binding protein-detergent-lipid complex with a molecular weight ~200 kD which 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 the Na^+-K^+-Cl^- cotransporter/bumetanide binding protein. It seems reasonable to assume that at least some of this lack of consensus is due to species and/or tissue differences or 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 (6, 10–12, 16, 17) outlined above, while using a completely independent approach. We demonstrate here the presence of a 160 kD protein in rabbit parotid basolateral membranes 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 [8] 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 [^{3}H]-BSTBA binding [10–12, 19]. Furthermore, although the molecular weight of protein identified as the Na^+-K^+-Cl^- transporter in the shark rectal gland using [^{3}H]-BSTBA binding is 195 kD [6, 17], we show here that the molecular weight of the parotid bumetanide binding protein decreases to 135 kD following deglycosylation (Fig. 3), as does its counterpart in the shark rectal gland [17]. Whether Na^+-K^+-Cl^- cotransporters from other tissues will prove to be as heavily glycosylated as those from these two secretory epithelia remains to be determined.

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-binding 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 the preparative electrophoresis procedure on [^{14}C]-NEM labeled membranes 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—*see* Results) 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 purification method was also quite good: 0.6% 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, we were able to establish the pI of the 135 kD protein (≈6.4; Fig. 7) and its amino

acid composition (Table). The availability of this relatively simple procedure for purifying the parotid bumetanide-binding protein should greatly facilitate future determinations of its biophysical and biochemical properties.

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