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Purification of Proteins of the Na/Cl Cotransporter from Membranes of Ehrlich Ascites Cells Using a Bumetanide-Sepharose Affinity Column

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Summary. Bumetanide-binding proteins were isolated from membranes of Ehrlich ascites tumor cells by affinity chromatography. An affinity column was constructed with the active moiety of bumetanide as a ligand using 4′-azidobumetanide, a photoactive analogue which inhibits Na/Cl cotransport in Ehrlich cells with high specificity. Covalent binding of the 4′-azidobumetanide with Sepharose was promoted by photolysis. Membranes isolated from Ehrlich cells were solubilized with *n*-octylglucoside. Solubilized proteins retarded by the affinity column were readily eluted by bumetanide. In reducing gels the major proteins eluted by bumetanide were ~76 kDa and 38–39 kDa. There were also two proteins of 32 to 35 kDa eluted in lesser amounts. No proteins retarded by the affinity column were eluted with extensive washing without bumetanide. Furthermore, bumetanide eluted no proteins from a "control" column lacking the specific ligand. Upon rechromatography with bumetanide in solution, bumetanide-eluted proteins were not retarded, but their purity was increased by the retardation of contaminating proteins. Bumetanide-binding protein purified in this manner were characterized further by electrophoresis in nonreducing, nondenaturing gels.

Key Words Na/Cl cotransport · azidobumetanide · bumetanide-Sepharose affinity column · Ehrlich ascites cells · purified cotransporter proteins

Introduction

When Ehrlich ascites tumor cells are shrunken by hyperosomotic challenge, an electroneutral Na/Cl cotransporter is activated which causes osomotically coupled water influx and restoration of cell volume (regulatory volume increase) (Hoffman, Sjøholm & Simonsen, 1983). Earlier Geck et al. (1980) had demonstrated that K-depleted Ehrlich cells are capable of Na/K/2Cl cotransport, the first rigorous demonstration of this type of transport pathway. Even though K may not be coupled to Na and Cl in cotransport during regulatory volume increase in fresh cells (Hoffman et al., 1983), the two processes in Ehrlich ascites cells, both inhibited by furosemide, may be mediated by similar molecular mechanisms. In addition to Ehrlich ascites cells, roles for Na/Cl and Na/K/2Cl cotransport in cell volume regulation have been established in avian red cells (Kregenow, 1981; Haas, Schmidt & McManus, 1982), L cells (Gargus & Slayman, 1980), simian virus-transformed 3T3 cells (Bakker-Grunwald, Ogden & Lamb, 1982), and at the basolateral membrane of frog skin (Ussing, 1985). For recent reviews on the Na/K/2Cl cotransport system and regulation of cell volume, *see* Chipperfield (1986), Siebens (1985), and Hoffmann and Simonsen (1988).

In epithelial cells, Na/Cl cotransport systems, as originally proposed by Nellans, Frizzell and Schultz (1973) for the mucosal membrane of intestinal epithelial cells, have been widely demonstrated and are recognized as playing an important role in transepithelial transport of salt and water (*see* review by Frizzell, Field & Schultz, 1979). In several epithelia the Cl-dependent cation cotransport can be attributed to a Na/K/2Cl cotransport system [for reviews *see* Warnock et al. (1984), Greger (1985) and O'Grady, Palfrey & Field (1987)]. Na/K/Cl and Na/Cl cotransport in epithelial cells have been suggested to be functions of the same pathway, interconvertible depending on cell volume (Eveloff & Calamia, 1986; Eveloff & Warnock, 1987). However, the importance of K-independent Na/Cl cotransport has recently been emphasized (Cremaschi et al., 1987).

The ultimate elucidation of the mechanism of cation-chloride cotransport processes will require the identification and characterization of their molecular constituents. Attempts to date have been based on UV-induced covalent binding of bume-

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tanide or labeling with a photoactive bumetanide derivative. Proteins of 34 kDa (Jørgensen, Petersen & Rees, 1984) and 150 kDa (Haas & Forbush, 1987; 1988) were labeled.

We have taken a different approach to isolating proteins of the cation/Cl cotransporter. The use of a covalent probe necessarily modifies the binding site irreversibly. To circumvent this difficulty and possible problems of reduced specificity of binding of photoactivated probes, we have applied affinity chromatography, a technique which entails reversible binding, using the active moiety of the bumetanide molecule as the ligand.

If bumetanide is to be used as a probe to isolate the cotransport proteins, the drug must bind with high specificity as well as high affinity. We have recently presented evidence for high specificity of binding of bumetanide to the cotransporter of Ehrlich ascites cells (Hoffmann, Schiødt & Dunham, 1986). We measured simultaneously the number of [^{3}H]-bumetanide molecules bound reversibly per cell and inhibition of cotransport caused by the bound bumetanide. Cotransport was measured as net Cl influx in cells subjected to a hypertonic challenge. There was a good linear correlation between bumetanide binding and inhibition of cotransport, and this correlation is evidence for specificity of binding. More recently demonstrations by similar means of specificity of binding of bumetanide to the cotransporter have been made in duck erythrocytes (Haas & Forbush, 1986) and in cultured canine kidney cells (Rugg, Simmons & Tivey, 1986).

The demonstrations of specificity of binding of bumetanide to the cotransporter justify the use of a bumetanide affinity column to attempt to isolate components of the cotransporter. To make a column containing covalently bound molecules with high affinity for bumetanide binding proteins, we used 4′-azidobumetanide (Fig. 1), a photoactive derivative with the azido group in the 4 position in the phenoxy ring (Feit, 1987). This particular position for the azido group was chosen because diuretic activity is retained after several structural changes in this part of the bumetanide molecule (Feit, 1971). Azidobumetanide has diuretic activity in the dog of a potency in the range of the activity of bumetanide itself (P.W. Feit, *unpublished results*). Results presented below show that the 4′-azido derivative of bumetanide, binding reversibly to ascites cells, has about the same affinity and specificity as bumetanide in inhibiting cation/Cl cotransport. All of this supports the use of 4′-azidobumetanide to provide the ligand in an affinity column to isolate bumetanide-binding proteins.

The affinity column was made by reacting azidobumetanide by photolysis to promote covalent binding with Sepharose 4B. This affinity column retained a small fraction of the proteins from preparations of Ehrlich ascites cell membranes solubilized with *n*-octylglucoside. These proteins were readily eluted from the column with bumetanide; with no bumetanide as eluant, the proteins were not eluted. A parallel "control" column with no bumetanide-like ligand did not retain these proteins. These observations constitute the evidence that these proteins are components of the cotransporter.

Materials and Methods

Synthesis of 4′-Azidobumetanide (Potassium Salt)

4-(4-Acetamidophenoxy)-3-Nitro-5-Sulfamoylbenzoic Acid

The starting material, 4-chloro-3-nitro-5-sulfamoylbenazoic acid (Feit, Brunn & Kaergaard-Nielsen, 1970 (70.16 g, 0.25 mol) was dissolved in 0.5 N NaOH (500 ml). 4-Acetamidophenol (75.59 g, 0.5 mol) was added followed by $NaHCO_3$ (63 g, 0.75 mol). The stirred reaction mixture was heated to 85°C for 18 hr. After cooling, and filtration, 4 N HCl (200 ml) and ethanol (250 ml) were added to the cooled filtrate, precipitating 4-(4-acetamidophenoxy)-3-nitro-5-sulfamoylbenzoic acid, which was collected by filtration and air-dried. A sample of the crude material (59.9 g) was recrystallized from aqueous ethanol and dried in air. The reaction product from this step and each succeeding step was checked by thin layer chromatography (plates: silica gel F_{254}, precoated (Merck)); mobile phase: cyclohexane, chloroform, methanol and acetic acid, 10 : 80 : 2.5 : 10. Visualization was by UV and/or I_2 vapor. NMR spectra were also determined; spectral features for all compounds were in accordance with structures. Analytical data were within ±0.25% of the theoretical values, and are indicated by symbols of the elements. The yield from this step was thereby shown to be the analytically pure compound; m.p. 245°C (dec.). Anal. ($C_{15}H_{13}N_3O_8S$) C, H, N.

4-(4-Acetamidophenoxy)-3-Amino-5-Sulfamoylbenzoic Acid

An aqueous solution (250 ml) of 4-(4-acetamidophenoxy)-3-nitro-5-sulfamoylbenzoic acid (32.2 g, 81 mmol) (pH 8.0, adjusted with 1 N NaOH) was hydrogenated at atmospheric pressure and 22°C by addition of Pd (10%) on carbon catalyst (2.7 g). When the H_2 uptake became negligible, the catalyst was removed by filtration, and the crude 4-(4-acetamidophenoxy)-3-amino-5-sulfamoylbenzoic acid was precipitated by addition of 1 N HCl to pH 2.0. The product was collected by filtration, washed with H_2O and air-dried. A sample of the crude amino acid (27.3 g) was recrystallized from aqueous ethanol to yield the analytically pure compound; m.p. >245°C. Anal. ($C_{15}H_{15}N_3O_6S$) C, H, N.

4-(4-Acetamidophenoxy)-3-n-Butylamino-5-Sulfamoylbenzoic Acid, Sodium Salt, Trihydrate

A solution of 4-(4-acetamidophenoxy)-3-amino-5-sulfamoylbenzoic acid (5 g, 13.7 mmol) in freshly distilled *n*-butanol (7.4 ml, 81

mmol), and methanol (125 ml) was boiled briefly and then cooled to 0°C. $NaBH_4$ (0.775 g, 21 mmol) was dissolved in 1 N NaOH (27.5 ml), diluted with H_2O (275 ml), and cooled to 0°C. The latter solution was added to the cold solution of the amino acid while stirring vigorously. The reaction mixture was kept below 10°C for 16 hr and then allowed to reach 22°C. After the pH was adjusted to 8.0 with 4 N HCl, the methanol was distilled off *in vacuo*. A saturated solution of NaCl (275 ml) was added, and the mixture was kept at 4°C for 16 hr to precipitate the crude sodium salt trihydrate (2.3 g). A sample was recrystallized from H_2O (4.5 ml/g of salt), which yielded the analytically pure 4-(4-acetamidophenoxy)-3-*n*-butylamino-5-sulfamoylbenzoic acid, sodium salt, trihydrate; m.p. > 290°C. Anal. ($C_{19}H_{22}Na$, NO_6S, 3 H_2O), C, H, N.

Ethyl 4-(4-Aminophenoxy)-3-n-Butylamino-5-Sulfamoylbenzoate, HCl

A mixture of 4-(4-acetamidophenoxy)-3-*n*-butylamino-5-sulfamoylbenzoic acid, sodium salt, trihydrate (24.9 g, 50 mmol), ethanol (250 ml), and 4 N HCl (250 ml) was refluxed for 4.5 hr. After evaporation *in vacuo* to dryness, 9 N ethanolic HCl (500 ml) was added, and the resulting suspension was gently refluxed for 4 hr. Cooling, collection by filtration, and drying *in vacuo* yielded crude ethyl 4-(4-aminophenoxy)-3-*n*-butylamino-5-sulfamoylbenzoate, HCl (21.9 g). A sample was twice recrystallized from aqueous ethanol to yield the analytically pure compound: m.p. 277°C (dec.). Anal. ($C_{19}H_{25}N_3O_5S$, HCl) C, H, Cl.

Ethyl 4-(4-Azidophenoxy)-3-n-Butylamino-5-Sulfamoylbenzoate

Concentrated H_2SO_4 (40 ml) was added slowly to a stirred suspension of ethyl 4-(4-aminophenoxy)-3-*n*-butylamino-5-sulfamoylbenzoate, HCl (19.2 g, 43 mmol) in acetic acid (200 ml) while cooling. Then a solution of $NaNO_2$ (27.6 ml of a 10% aqueous solution, 40 mmol) was added dropwise below 8°C. After additional stirring at 8°C for 2 hr, urea (4 g) was added in order to remove unreacted $NaNO_2$. A solution of NaN_3 (4 g, 43 mmol) in H_2O (100 ml) was added and the mixture was allowed to reach 22°C (2 hr). From this stage on, the reaction mixtures and materials were protected from light. The precipitated crude ethyl 4-(4-azidophenoxy)-3-*n*-butylamino-5-sulfamoylbenzoate was collected, washed with H_2O, and dried in air (12 g, m.p. 152–154°C). 10 g of this material were dissolved in ethanol (800 ml), filtered, and the analytically pure azido ester was precipitated from the filtrate by addition of H_2O (800 ml). After collection by filtration, it was washed with H_2O and dried *in vacuo* (7.4 g, m.p. 155.5°C). Anal. ($C_{19}H_{23}N_5O_5S$) C, H, N.

4'-Azidobumetanide, Potassium Salt [4-(4-Azidophenoxy)-3-n-Butylamino-5-Sulfamoyl-Benzoic Acid), Potassium Salt] (Fig. 1)

A mixture of ethyl 4-(4-azidophenoxy)-3-*n*-butylamino-5-sulfamoyl-benzoate (5.63 g, 13 mmol) and 1 N KOH (65 ml) was stirred at room temperature for 5 hr to complete the saponification process (verified by TLC). CO_2 was bubbled in to precipitate the potassium salt of 4-(4-azidophenoxy)-3-*n*-butylamino-5-sulfamoylbenzoic acid. After collection by filtration, the crude material (4.6 g) was recrystallized twice from H_2O (2 ml of H_2O/g of potassium salt) and dried over P_2O_5 *in vacuo* to yield the analytically pure compound (2.98 g). Anal. ($C_{17}H_{18}KN_5O_5S$) C, H, N.

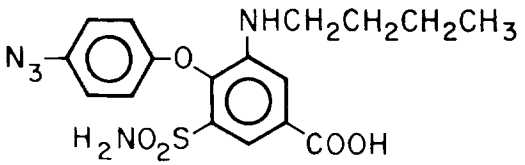

Fig. 1. Structure of 4'-azidobumetanide

Assay of 4'-Azidobumetanide by Reversed-Phase High-Performance Liquid Chromatography

The column was a Hilbar Lichrocart HPLC-Cartridge, LiChrosorb RP-18 (Merck); column length: 125 mm; inside diameter: 4 mm, equipped with a PVDF guard. The mobile phase was 45% acetonitril and 55% 0.1 M KH_2PO_4. The flow rate was 2.5 ml/min. The column was driven with a Mini pump (Milton Roy Co., Rochester, NY). Injection of samples was with a model 7125 loop injector with a 20 μl loop (Reodyne, Cotati, CA). A PYE Unican 4020 UV detector (Philips) with a 3390A reporting integrator (Hewlett Packard) was used (220 nm detection, 1 cm/min chart speed).

Standards were prepared the day of use from analytically pure 4'-azidobutanide (potassium salt) dissolved in acetonitrile and subsequently diluted with water 2 : 1 to give a 10 μM solution. This was diluted further with acetonitrile H_2O 2 : 1 as required. In the experiments, stock solutions of azidobumetanide in double strength Ca-free isotonic saline were used. These were diluted 1 : 1 with water before analysis. Cell extracts from azidobumetanide-binding experiments were analyzed directly. All solutions containing azidobumetanide were protected from light.

After a stable baseline was achieved on the recorder, standards and test samples were injected alternately so that at least two injections of a standard were followed by at least three injections of each sample. This ensured identical retention times of standard and the corresponding test sample. Calculations were based on the means of the peak heights of both standard and the corresponding test sample. The detection limits of the assay were 0.05 μM of azidobumetanide with a relatively large amount of bumetanide present, and 0.01 μM with no bumetanide present.

Preparation of Bumetanide-Sepharose Affinity Column

The columns were made from activated thiol-Sepharose 4B (Pharmacia) containing covalently bound glutathione, the thiol groups of which were protected with thiopyridine. Twelve grams of the Sepharose were stirred with 2 l. of water for 2 hr. The Sepharose was collected on a Buchner funnel, resuspended in water, and packed in a glass column (10 × 5.3 cm). The protecting thiopyridyl groups were removed by washing the column with 400 ml of a solution containing 10 mM cysteine, 1 mM EDTA, and 100 mM Na phosphate, pH 8.0. The column was then washed with oxygen-free water until the eluate was free of cysteine, as determined with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (Brocklehurst & Little, 1973). Swollen gel, 50 ml, from the column was mixed with 35 ml distilled water and 2.7 ml of 100 mM Na phosphate, pH 8.0. This was placed in a wide beaker to a

depth of about 1 cm, and was stirred slowly with nitrogen flushing. Azidobumetanide (potassium salt), 120 mg in 2 ml distilled water, was added with protection from direct light. The mixture was then illuminated with a Phillips HFR 125 W lamp from a distance of 12 cm for 5 min, after which approximately 30% of the original, unreacted azidobumetanide could be detected by quantitative TLC. The photoreaction was performed so that azidobumetanide was only partially reacted in order to enhance the probability of specific photochemical activation of the azido group. For the same reason UV light under 313 nm was excluded by using a glass shield. Assays of free sulfhydryl groups in the gel showed that about 85% of these had reacted during the period of illumination. The material was washed exhaustively with water and bubbled with air in an attempt to oxidize the remaining sulfhydryl groups. It was then stored in a solution containing 1 M NaCl, 0.1% Na azide, 0.1 mM PMSF, and 1 mM EDTA. The same solution was used to wash the column between experiments. The gel was packed in columns 180 mm long and 9 mm i.d. (11.5 ml of gel).

A "control" column lacking the specific ligand was constructed by a similar procedure. The washed, swollen gel (50 ml) was illuminated as described above, but without azidobumetanide. After bubbling with air, most sulfhydryl groups remained. These were blocked by reaction with N-ethylmaleimide (NEM) (10 mg of NEM in 0.1 ml ethanol added with stirring, an amount of NEM in twofold excess of the sulfhydryl groups).

Cells

Ehrlich ascites tumor cells (hyperdiploid strain) were maintained in white female mice by bi-weekly intraperitoneal transfer. Eight days after transplantation, the mice were sacrificed and the cells were harvested as described before (Hoffmann et al., 1986) into an isotonic saline containing (mM): NaCl (150), KCl (5), $MgCl_2$ (1), $CaSO_4$ (1), Na_2HPO_4 (1), 2-(*N*-morpholino) propane sulfonic acid (MOPS) (3.3), *N*-tris (hydroxymethyl) methyl-2-aminomethane sulfonic acid (TES) (3.3), *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) (3.3), pH 7.4, and washed two times by gentle centrifugation and resuspension in the same medium.

Isolation of Cell Membranes

The method for this procedure was a slight modification of the one we published recently (Jessen, Sjøholm & Hoffmann, 1986). Freshly harvested and washed cells were suspended at 8% cytocrit in 50 ml of isotonic medium without Mg and Ca and containing 1 mM EDTA. The cells were disrupted by nitrogen cavitation in a cell disruption bomb (Parr Instrument Co.). Cells were incubated at 75 atm (1100 psi) for 15 min with stirring at 4°C. Cell disruption occurred upon sudden reduction of pressure and release of the cells from the bomb. The disrupted cells were released from the bomb into a flask containing sufficient phenylmethylsulfonyl fluoride (PMSF; protease inhibitor; 100 mM stock solution in ethanol, kept at −20°C) to give a final concentration of 0.1 mM. Microscopic observation of homogenates showed that most cells were disrupted, but intact nuclei were present.

To remove intact cells and nuclei, the homogenate was centrifuged at a maximum force of 1500 × *g* for 5 min. Mitochondria were sedimented by centrifugation at 6000 × *g* maximum force for 10 min. Then the membranes were collected by centrifugation at 140,000 × *g* maximum force for 75 min. The membranes were washed twice by centrifugation, first in a solution of (mM): NaCl (140), Tris-HCl (10), EDTA (1), PMSF (0.1), pH 7.5, and second in Tris-HCl (5), EDTA (1), PMSF (0.1), pH 7.5. The enzymes 5'-nucleotidase and alkaline phosphodieterase were used as surface membrane markers. In the membrane preparations, the specific activities of both of these enzymes were 10 times higher than in the whole cell homogenates. The yields of these membrane markers were about 50%. The washed membrane pellets were solubilized in a solution of 2% *n*-octylglucoside, 25 mM NaCl, 5 mM KCl, 25 mM imidazole, pH 7.5 (Na-K-Cl-octylglucoside buffer), at a protein concentration of 10 mg/ml with stirring for 2 hr at 4°C. Insoluble material was removed by centrifugation for 30 min at 140,000 × *g*.

Chromatography of Membrane Proteins in the Bumetanide-Sepharose Affinity Column

The column was kept at 4°C and prewashed with ~25 ml of the Na-K-Cl-octylglucoside buffer. A membrane protein preparation in solution in 8–10 ml of the same buffer, and containing 10–20 mg protein, was applied to the column, collection of 1-ml fractions was begun, and the column was washed with the Na-K-Cl-octylglucoside buffer. The protein emerging from the column was monitored continuously by UV absorbance. The protein not retained by the column emerged as a sharp peak in the first 20–25 fractions. When the absorbance reached background, after 25–30 samples, the column was eluted with the Na-K-Cl-octylglucoside buffer containing bumetanide at 200 μM, ~40 × the $K_{1/2}$ for bumetanide binding.

Polyacrylamide Gel Electrophoresis: Reduced Proteins

To each 1 ml fraction from the column, 70% TCA was added to a final concentration of 10%. The protein was precipitated by incubation for 2 hr at 4°C, and then collected by centrifugation for 10 min at 25,000 × *g*. The pellet was washed twice with acetone by suspension and centrifugation to remove lipid and *n*-octylglucoside. The protein pellet was suspended in 100 μl of Laemmli buffer, a strongly reducing solution (2% Na dodecyl sulfate, 3% dithiothreitol, 30 mM Tris-HCl, 5% glycerol, 0.001% bromphenol blue, pH 6.8; Laemmli, 1970). The mixture was heated at 90°C for 30 min and centrifuged at 120,000 × *g* for 30 min. A small sample of the solution was removed for protein analysis, and the rest was analyzed by polyacrylamide gel electrophoresis. Protein concentrations were determined by the method of Zaman and Verwilghen (1979), a modification of the method of Bradford (1976).

Electrophoresis was carried out in 12% polyacrylamide slab gels (1.5 mm thick) following the method of Laemmli (1970) as modified by Jessen et al. (1986). The gel was made from a degassed solution containing 12% acrylamide, 0.3% bisacrylamide, 375 mM Tris-HCl, 0.1% SDS, 0.03% TEMED, pH 8.8. Polymerization was initiated by adding ammonium persulfate to a final concentration of 0.01%. The solution was covered by isobutanol. A stacking gel was added on the top of the separating gel after washing away the isobutanol with stacking gel solution. The stacking gel contained 4% acrylamide, 0.2% bisacrylamide, 125 mM Tris-HCl, 0.1% SDS, 0.1% TEMED, pH 6.8, and 0.03% ammonium persulphate to initiate polymerization. The electrode buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.8.

The electrophoresis was performed at a constant current of 20 mA.

Molecular weight standards used were: phosphorylase A (92 kDa), bovine serum albumin (67 kDa), glutamate dehydrogenase (53 kDa), ovalbumin (43 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and β-lactalbumin (14 kDa). Immediately after electrophoresis, the gel slab was washed in 50% methanol/10% acetic acid for 1 hour to remove SDS. The gels were stained for protein with 0.25% Coomassie Brilliant Blue R-250 in 50% methanol/10% acetic acid for at least 4 h, and destained in 30% methanol/10% acetic acid. The gels were air-dried in Saran wrap and scanned (with no smoothing) with an UltroScan XL Laser Densitometer (LKB, Bromma, Sweden).

Gel Electrophoresis: Nonreduced Proteins

A few electrophoretic separations were carried out under nominally nonreducing, nondenaturing conditions in Na cholate polyacrylamide gels. The purified bumetanide-eluted proteins were prepared as described above. The volumes of the pooled fractions were reduced by dialysis against dry polyethylene glycol (rather than by TCA precipitation). These samples were dialyzed extensively against a solution containing 400 mM sucrose, 1% Na cholate, 5 mM HEPES, pH 7.8. No Laemmli buffer was used. The gel was a linear gradient polyacrylamide gel, 4–20% (2.6% of the total acrylamide content was bisacrylamide), containing 188 mM Tris (pH 8.8), 0.5% Na cholate, a 5–15% sucrose gradient, and an oppositely directed gradient of TEMED (0.050–0.025%). Polymerization was initiated by adding ammonium persulfate to a final concentration of 0.015%. The gradient gel was overlaid with a 4% polyacrylamide gel which contained the loading wells. This gel contained 0.1% bisacrylamide, 188 mM Tris (pH 8.8), 0.5% Na cholate, 0.1% TEMED, and 0.03% ammonium persulfate. The electrode buffer at the cathode was 30 mM Tris, 192 mM glycine, 0.5% Na cholate, pH 8.8, and at the anode 100 mM Tris, 192 mM glycine, 0.1% Na cholate, pH 8.8. The electrophoresis was carried out at constant current (10 mA) for 20 hr. The gels were fixed, stained and scanned as described above for SDS gels.

Molecular weight standards used were: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and bovine serum albumin (67 kDa). Hydrophilic proteins bind little deoxycholate (Helenius & Simons, 1972), and therefore presumably little cholate (except for thyroglobulin, which binds deoxycholate at 0.13 fraction, wt/wt). Therefore, the electrophoretic mobility of hydrophilic proteins in cholate gels is determined by their native charge and shape, as well their mass. The standards in the cholate gels were run in separate lanes, permitting the observation that their distribution was in the order of their molecular weights. This distribution is a consequence of the sieving effect of the gradient gels, rather than the electrophoretic mobility of the proteins. The evidence for this is the following: the gels were generally run for 20 hr; during an additional 20 hr the positions of the proteins were not substantially advanced. The positions of the standards in the order of the molecular weights of their oligomers indicated that the standards had not been separated into their subunits by reduction of disulfide bonds or by disruption of other intersubunit bonds. For example, ferritin had not been dissociated into its 20 subunits.

Hydrophobic proteins do bind significant amounts of deoxycholate, fractions by weight of 0.3 to 0.7 (Helenius & Simons, 1972). The same is undoubtedly true of cholate. Therefore, in the cholate gels, where distribution of proteins after 20 hr is approximately in order of molecular weight, tentative ranges of molecular weights of membrane (hydrophobic) proteins can assigned by correcting for a fractional weight of 0.3–0.7 of bound detergent.

Coomassie-stained cholate gels were often stained again with silver according to the method of Görg et al. (1985). This procedure was adopted not only because of the greater sensitivity of silver staining, but also because some proteins which stain strongly with Coomassie Blue in SDS gels do not stain well in cholate gels with Coomassie Blue at the same protein concentration.

Materials

N-octylglucoside (1-O-*n*-octyl-β-D-glucopyranoside, Boehringer Mannheim, W. Germany), PMSF (phenylmethylsulfonyl fluoride, Sigma Chemical Co., St. Louis) TEMED (N,N,N′,N′-tetramethylethylenediamine, Sigma). Molecular weight standards for electrophoresis: thyroglobulin and bovine serum albumin (Sigma); ferritin, catalase, and aldolase, (Boehringer-Mannheim); phosphorylase A, glutamate dehydrogenase, ovalbumin, carbonic anhydrase, trypsin inhibitor, glyceraldehyde-3-phosphate dehydrogenase, and β-lactalbumin (LKB). Na cholate, SDS (Na dodecylsulfate), acrylamide and bisacrylamide (Sigma).

Results

The rationale for using an affinity column containing a bumetanide derivative as the ligand was the specificity of binding of bumetanide to the cotransporter in Ehrlich ascites cells (Hoffmann et al., 1986). The rationale for using the 4′-azido derivative (Fig. 1) in the column was twofold. First, the compound could easily be made to bind covalently to the column material by photolysis. Second, the 4 position of the phenoxy ring is not critical to bumetanide's biological activity. Several bumetanide derivatives modified in the 4′ position retain diuretic activity (Feit, 1971). Furthermore, 4′-azidobumetanide itself not only has diuretic activity in dogs (P.W. Feit, *unpublished results*), it is a potent and specific inhibitor of cation/Cl cotransport in Ehrlich ascites cells.

Azidobumetanide Binding to the Cation-Cl Cotransporter

Two types of demonstrations were made. (i) Azidobumetanide can inhibit cotransport reversibly. Cation-Cl cotransport induced by hypertonic challenge as described before (Hoffmann et al., 1983) was measured as net Cl influx in a control preparation and in the presence of azidobumetanide (5 μM) or bumetanide (10 μM). The samples were kept in the dark to prevent covalent binding of azidobume-

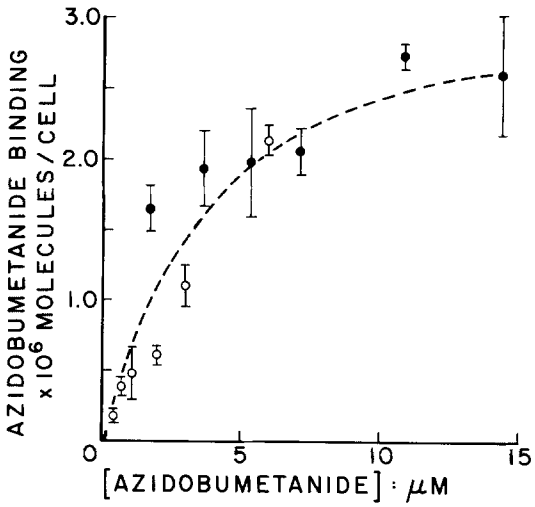

Fig. 2. Binding of azidobumetanide to intact Ehrlich ascites cells, shown as number of molecules bound per cell. Cells were incubated in isotonic media with various azidobumetanide concentrations for 2–4 min in the dark (conditions of equilibrium binding). Cell samples were collected by centrifugation, and trapped extracellular volumes of the cell pellets were determined using [^{3}H]-insulin. Azidobumetanide contents determined as described in the text were corrected for trapped volumes. Numbers of azidobumetanide molecules bound per cell were calculated as described before for [^{3}H]-bumetanide binding (Hoffmann et al., 1986). Results are shown for two separate experiments (open and filled symbols, respectively). Symbols show means ± SD (n = 4)

tanide. The inhibition of net Cl influx was nearly the same with bumetanide and azidobumetanide. To test reversibility of inhibition, an aliquot of cells incubated in azidobumetanide for 2 min (sufficient for equilibrium binding) was washed once by centrifugation before measuring cotransport. The one wash was sufficient to restore influx to 66% of the control value, demonstrating at least partial reversibility of inhibition (after a single wash, the drug probably remained at an appreciable concentration in the medium).

(ii) The numbers of azidobumetanide molecules bound per cell under equilibrium conditions were determined with varying azidobumetanide concentrations in the medium. Azidobumetanide in the cell pellets was analyzed by HPLC and corrected for trapped extracellular volume in the pellets using [^{3}H]-insulin (*see* Hoffmann et al., 1986). Figure 2 shows that there was saturation binding, with half maximal binding at ~4 μM, slightly less than the half maximal concentration for [^{3}H]-bumetanide binding under the same conditions (Hoffmann et al., 1986). The number of azidobumetanide molecules bound per cell at apparent saturation was ~2.5 × 10^6. This is nearly the same as the number of bumetanide molecules bound specifically per cell, ~2 × 10^6, as shown by the correlation between inhibition of transport and binding (Hoffmann et al., 1986). Therefore azidobumetanide binds to Ehrlich ascites with an affinity and a specificity similar to those of bumetanide.

These results cannot be offered as proof for the specificity of binding of components of the cotransporter in the affinity column. The reason is that the ligand in the column is no longer 4′-azidobumetanide, but rather some other bumetanide derivative modified in an unknown way at the 4′ position and covalently bound to the column, presumably at thiol groups of the glutathione spacer arms on the Sepharose. Nevertheless, the specificity of inhibition of cotransport by 4′-azidobumetanide is consistent with specificity of binding of cotransporter proteins to the ligand in the affinity column.

Affinity Chromatography of Membrane Proteins in a Bumetanide Column

Membranes of Ehrlich ascites cells were isolated and solubilized in *n*-octylglucoside and applied to the bumetanide-Sepharose affinity column as described in Materials and Methods. The results of a typical experiment are shown in Fig. 3. The column was washed with the Na-K-Cl-octylglucoside buffer; the protein in the eluant was monitored by UV absorbance, and fractions of 1 ml were collected.

Elution with bumetanide (200 μM) in the same buffer was begun at fraction 29, after the level of protein in the eluant had fallen to background. Figure 3*A* shows a scan of a stained electrophoretic gel of fraction 14, containing proteins not retarded by the column. Figure 3*B* shows a scan of a gel of pooled fractions 44–48, containing proteins eluted by bumetanide. Similar amounts of protein were loaded in the two lanes of the gel. Two major protein bands were retained by the affinity column and eluted by bumetanide. These are the crosshatched bands in Fig. 3*B* of approximately 76 and 38 kDa (bands 1 and 2, respectively). A less prominent and complex band of protein was also retained and eluted by bumetanide (stippled band 3) of 33–35 kDa. The positions of bands 1–3 are indicated in Fig. 3*A*, the nonretained proteins. No significant peak of protein is evident at any of these positions.

The patterns of proteins in gels of the preparations of membrane proteins were nearly identical to those in gels of proteins not retained by the affinity column. However, it was possible to detect minor bands in gels of total membrane protein at positions corresponding to bands 1–3 of bumetanide-eluted protein. By contrast these bands were not apparent in gels of nonretained protein (Fig. 3*A*).

It was necessary to make certain that the ligands in the column responsible for retaining the proteins eluted by bumetanide were provided by the photochemical reaction with azidobumetanide. Accordingly, a "control" column lacking these specific ligands was used in an experiment carried out exactly the same way as the one in Fig. 3. Bumetanide eluted no proteins whatsoever from the control column washed with 50 ml of buffer (*results not shown*).

Another experiment was carried out with a bumetanide affinity column in which solubilized membrane proteins were eluted through the column. Instead of eluting with bumetanide, washing with the control buffer continued up to 70 fractions, far beyond the fraction at which elution with bumetanide was usually begun (fraction 29). No protein was recovered from the column after fraction 20, showing that the bumetanide was responsible for elution of proteins normally recovered in these fractions (*results not shown*).

An experiment entailing rechromatography was carried out in an attempt to obtain additional evidence on which proteins are binding to the specific ligands in the column. The strategy was as follows: bumetanide-eluted fractions were pooled and rechromatographed by passage through the affinity column a second time. This sample contained ~200 μM bumetanide in solution, and this would prevent retention of bumetanide-binding proteins in the column. Any proteins retarded by the column at sites other than the fixed bumetanide, and thereby contaminating the bumetanide-eluted proteins, would be retarded by the column during the second passage despite the presence of bumetanide in solution.

During the second run through the column, the UV absorbance of the bumetanide in solution was followed, and four fractions collected at the peak bumetanide concentration were pooled. Their electrophoretic pattern (Fig. 4*C*) was compared to those polypeptides not retained by the column (Fig. 4*A*), and proteins in pooled fractions eluted by bumetanide (Fig. 4*B*). As in Fig. 3, prominent proteins were eluted by bumetanide at ~76 kDa (a more complex band in this experiment) and at 38–39 kDa (Fig. 4*B*). Also as in Fig. 3, a less prominent complex band of 33–35 kDa (band *3*) was eluted by bumetanide. There seemed to be additional protein eluted at ~32 kDa (band *4*) which was not seen in the experiment in Fig. 3; that this band was eluted by bumetanide was made clear only after rechromatography (Fig. 4*C*). The rechromatographed sample (Fig. 4*C*) shows two prominent bands (*1* and *2*), at ~76 and ~38 kDa, as in Fig. 4*B* and in Fig. 3. The contaminating proteins were greatly reduced after

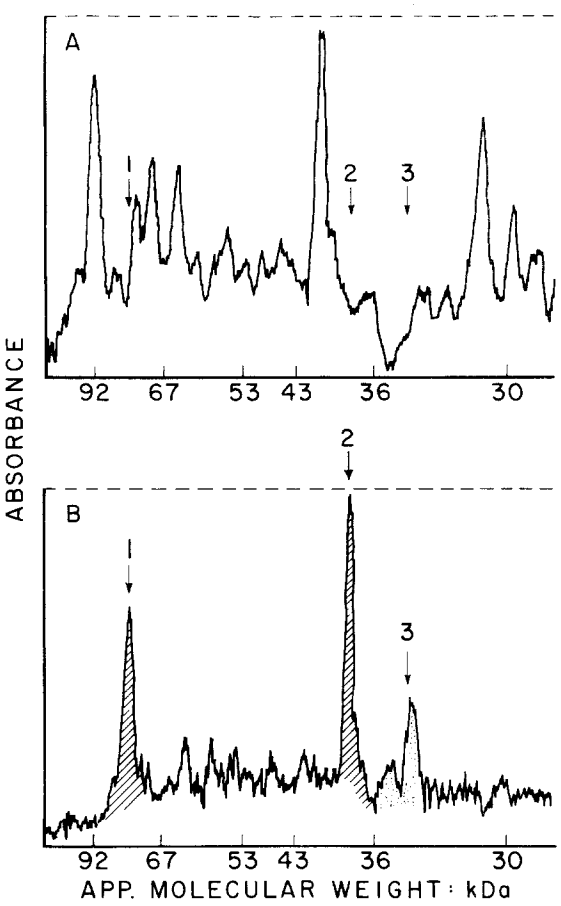

Fig. 3. Scans of stained electrophoretic SDS gels of membrane proteins from Ehrlich ascites cells after chromatography with a bumetanide-Sepharose affinity column; procedures are given in the text. (*A*) Proteins not retained by bumetanide affinity column (fraction 14). (*B*) Proteins retained by the column and eluted by bumetanide (200 μM), added at fraction 29; sample was taken from pooled fractions 44–48. Cross hatched bands (*1* and *2*) and stippled band (*3*) indicate prominent and minor eluted bands, respectively. Positions of the three bumetanide-eluted bands in *B* are indicated in *A*. This experiment is representative of two others with the same results. In this and all subsequent figures, the positions in the gels of the standards is indicated by their apparent molecular weights (kDa)

rechromatography (compare Fig. 4*B* and *C*), showing that this material was not retained by the specific ligands. After rechromatography, band *3* was less complex and band *4* is clearly recognizable as a bumetanide-eluted protein. The positions of the four bumetanide-eluted proteins after rechromatography in Fig. 4*C* (bands *1–4*) are indicated in Fig. 4*B* (where the same proteins are recognized) and Fig. 4*A* (where the proteins are negligible in quantity or absent).

The Table shows the quantitation of the chromatography from the experiment in Fig. 4. Shown are the percents of protein applied to the column

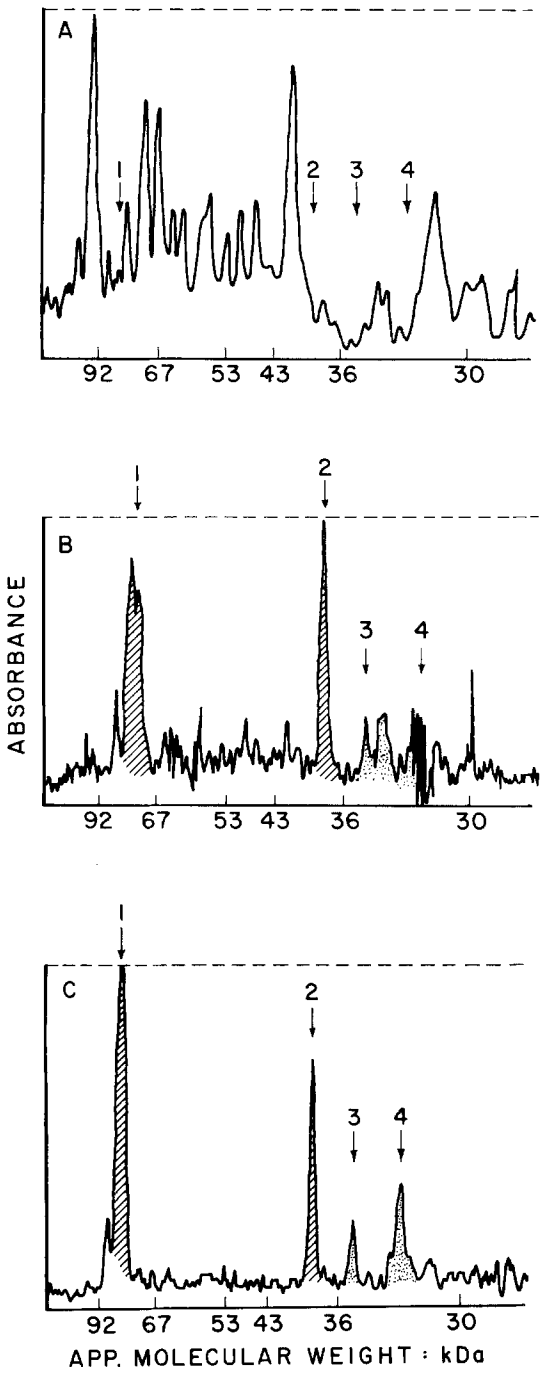

Fig. 4. Scans of SDS gels of solubilized membrane proteins of Ehrlich ascites cells after chromatography and rechromatography with a bumetanide-Sepharose affinity column. (*A*) Proteins not retained by the column (fraction 16). (*B*) Proteins eluted by bumetanide (200 μM added at fraction 29); sample was taken from pooled fractions 45–54. (*C*) Bumetanide-eluted proteins (pooled fractions 45–54) rechromatographed; gel was run of pooled fractions 21–24 obtained during rechromatography. Cross hatching and stippling indicate prominent (*1* and *2*) and minor (*3* and *4*) bumetanide-eluted bands, respectively. Three other experiments carried out in the same way gave the same results

Table. Quantitative aspects, measured and calculated, of chromatography of membrane proteins with a bumetanide-sepharose affinity column

Protein	Percent
1. Not retained by column (recovered in fractions 1–25)	78[a]
2. Eluted by bumetanide	4.0[b]
3. Recovered after rechromatography	0.4[b]
4. Calculated bumetanide binding protein	2.4–4.8[c]

[a] Determined in an experiment like the one in Fig. 4.
[b] Determined in the experiment in Fig. 4.
[c] Calculated from the following values: 2×10^6 bumetanide binding sites per cell and 4.3×10^9 cells/g cell solids (Hoffmann et al., 1986); 0.6 g protein/g cell solids (present study); 10-fold purification of surface membrane during membrane preparation (Jessen et al., 1986; present study); 100–200 kDa range assumed for molecular weight of bumetanide-binding protein. These values yield 1.4×10^{17} bumetanide-binding sites per g protein in the membrane preparation.

which were not retained, which were eluted by bumetanide, and which were recovered after rechromatography. The Table also shows the results of a calculation of the fraction the membrane preparation expected to be collected as bumetanide-binding (or bumetanide-eluted) protein. As indicated in the Table, the calculation was based on the number of bumetanide-binding sites per cell, the amount of protein per cell, and the purification factor of membrane proteins during the preparation. A range of molecular weights was assumed for the protein of the bumetanide-binding site from 100 to 200 kDa. The amount of bumetanide-binding protein recovered after rechromatography was about an order of magnitude less than the calculated percent bumetanide-binding protein.

The proteins in Figs. 3 and 4 eluted by bumetanide were all identified by electrophoresis after solubilization in a denaturing and strongly reducing buffer. Therefore, the polypeptides isolated may be subunits of a larger protein formed by disulfide or other intersubunit bonds. On the other hand, the isolated polypeptides may be separate proteins. In this case, it would be useful to be able to isolate them in their native state in order to study their functional properties separately. Accordingly, bumetanide-binding protein was investigated by a new technique in cholate gels, which are nominally nonreducing and nondenaturing. Protein was isolated by chromatography and rechromatography just as in the experiment in Fig. 4. First it was shown in a reducing SDS gel that exactly the same proteins were purified as in Fig. 4 (*results not shown*). The

purified protein was also prepared in a nonreducing, nondenaturing buffer containing Na cholate (*see* Materials and Methods). Electrophoresis of the protein showed one doublet protein band, as shown by the scan of the stained gel in Fig. 5*A*. The distribution of the standards was in order of the molecular weights of their oligomers.

In the cholate gel, the bumetanide-binding protein was distributed between the 232 and 158 kDa standards, at about 175 kDa. If it is a hydrophobic protein binding 0.3–0.7 cholate, wt/wt, then the molecular weight of the bumetanide-binding protein in the cholate gel is between 100 and 135 kDa. The band of bumetanide-eluted protein in the experiment in Fig. 5*A* was cut out of a parallel unstained cholate gel, eluted and run in a reducing SDS gel. As shown in Fig. 5*B*, the scan of this gel shows a single band of the same molecular weight as band *1* in Figs. 3 and 4. No other significant bands were observed, demonstrating that protein in the cholate gel (Fig. 5*A*) is not a hetero-oligomer of the 2 to 4 bumetanide-binding proteins observed in Figs. 3 and 4. The upper limit of the range of estimates of the mean molecular weight of the protein in the band in Fig. 5*A*, ~135 kDa, is consistent with a dimer of band *1*, the only protein in Fig. 5*B*.

In the experiment in Fig. 5*A* and in two others like it, it was noted that some protein material did not enter the gel. In one experiment, this material was eluted and run in a reducing SDS gel, and a single protein band was observed of about 38 kDa (*results not shown*), band *2* in Figs. 3 and 4. Thus the bumetanide-binding site consists of two main proteins or of a protein which dissociates into subunits in Na cholate buffer, despite the nonreducing, nondenaturing conditions of this buffer. Bands *3* and *4* were not observed in the SDS gels of proteins eluted from cholate gels.

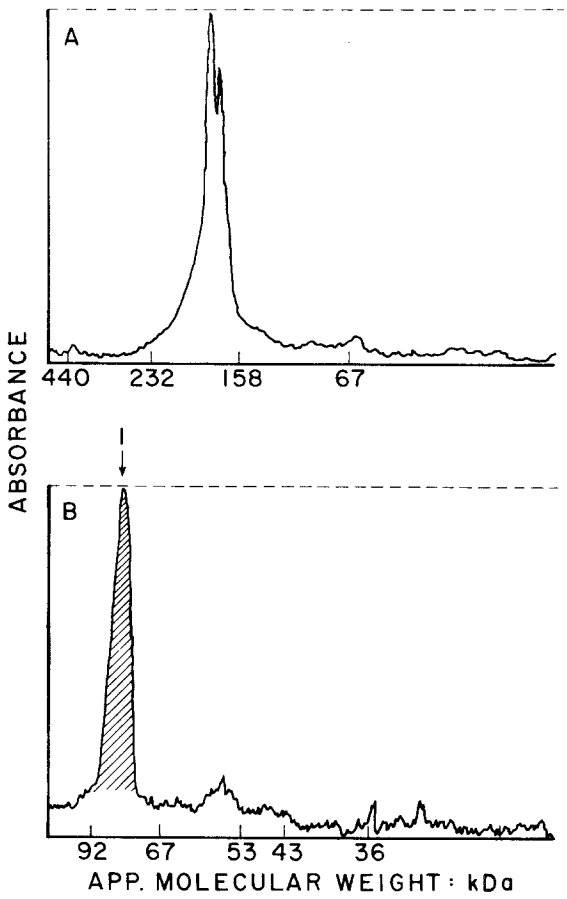

Fig. 5. (*A*) Scan of a Na-cholate gel of bumetanide-eluted proteins solubilized under nondenaturing, nonreducing conditions. Protein was isolated as in Fig. 4 (chromatography and rechromatography). It was then solubilized in a Na-cholate buffer rather than the usual SDS-dithiothreitol buffer (*see* Materials and Methods). Coomassie Blue-stained gel was silver stained before scanning. The results are representative of three other experiments carried out in the same way. (*B*) Scan of an SDS gel of material eluted from the major band of a companion gel to that shown in *A*

Covalent UV Induced Binding of [^{3}H]-Bumetanide

Finally, we repeated an experiment like the one Jørgensen et al. (1984) carried out on pig kidney membranes. We labeled Ehrlich ascites cells with [^{3}H]-bumetanide by UV irradiation, solubilized the isolated membranes, separated the proteins by electrophoresis in SDS gels, and identified bumetanide-binding proteins by counting gel slices. In confirmation of Jørgensen et al. (1984), many proteins were covalently labeled with [^{3}H]-bumetanide; most prominent was a broad band at 31–34 kDa. We tested for saturable binding by labeling at three concentrations of [^{3}H]-bumetanide: 6, 12, and 24 μM. A portion of the broad band (34–33 kDa) showed saturable binding. In addition there were suggestions of saturable binding to polypeptide bands at 42 and 28 kDa. Our conclusions from three experiments of this kind were, first that UV-induced covalent binding of bumetanide may give indications of candidate proteins for the cotransporter. However, there is so much low affinity binding which is presumably nonspecific (i.e., not to the cotransporter) that this approach may not be a useful one for the isolation of proteins of the cotransporter. The 34-kDa polypeptide labeled by [^{3}H]-bumetanide may be the same as band *3* eluted by bumetanide. If so, it is not clear why bands *1* and *2*, 76 and 38 kDa, the main polypeptides isolated by affinity chromatography, were not covalently labeled by [^{3}H]-bumetanide. Finally,

the use of proteins isolated using covalently binding probes is somewhat limited because the binding site for the ligand, presumably a catalytically important site, is irreversibly modified.

Discussion

Using affinity chromatography, we have isolated proteins which are likely to be constituents of the Na/Cl cotransporter of Ehrlich ascites cell membranes. The evidence is based on the photochemically provided ligand in the column and on the method of elution of the proteins from the column. The ligand was formed by the photochemical reaction of 4′-azidobumetanide with thiols of Sepharose. The photoactive 4 position of the phenoxy ring of the bumetanide derivative is undoubtedly responsible for all of the binding of the ligand in the column. As stated above, derivatives of bumetanide modified in the 4′ position retain diuretic activity, and 4′-azidobumetanide not only has diuretic activity but also inhibits cotransport in Ehrlich ascites cells and binds to these cells with affinity and specificity similar to bumetanide's. Therefore a bumetanide affinity column constructed using azidobumetanide is likely to bind specifically constituents of the cotransporter. Our results with solubilized membrane proteins in a bumetanide-Sepharose affinity column show the following: (i) Proteins retained by the column are specifically eluted by bumetanide. (ii) A "control" column lacking the photochemically provided specific ligand retains no proteins which can be eluted by bumetanide. (iii) The proteins cannot be eluted from the column in detectable amounts if bumetanide is not present in the eluant.

The two major polypeptides retained by the column and eluted by bumetanide were of molecular weights ~76 and ~38 kDa, determined by electrophoresis under reducing conditions. This was a highly reproducible result. Furthermore, upon rechromatography with bumetanide in the mobile phase, the proteins were not retained by the column and emerged with less contaminating protein (presumably retarded nonspecifically by the column in the presence of bumetanide in the eluant). The stoichiometric relationship between these two proteins was approximately 1 : 1 in six different experiments.

Consistently smaller amounts of two polypeptides of 32–35 kDa eluted by bumetanide were observed after rechromatography. Perhaps these smaller proteins were associated with the cotransporter, but are present in lesser amounts in the gels because they are not a part of the bumetanide binding site, and therefore less readily retarded by the affinity column and/or are less readily eluted by bumetanide.

There have been several other recent attempts to isolate components of cation/Cl cotransporters. The Na/K/2Cl cotransporter has been partially purified from pig kidney, the criterion for recovery having been reconstitution, in preliminary experiments, of transport activity in artificial lipid vesicles (Burnham, Karlish & Jørgensen, 1985). No attempt was made to characterize the proteins of the transport system.

The other attempts to identify proteins of cotransporters have employed the strategy of photoactivated covalent binding of tritiated loop diuretics to membranes of mammalian kidney, followed by identification of the labeled proteins after electrophoretic separation. Bumetanide, a potent high ceiling loop diuretic (Feit, 1971), inhibits cation-Cl cotransport with high affinity in avian erythrocytes (Palfrey, Feit & Greengard, 1980) and in all other systems tested. Jørgensen et al. (1984) used near ultraviolet light to provoke covalent binding of [^{3}H]-bumetanide to pig kidney membranes. A number of membrane polypeptides were labeled, but the most prominent one, and the one with highest apparent affinity, was a polypeptide of 34 kDa. Unaccountably, labeling was carried out in Cl-free medium, and Cl is a promoter, if not a requirement, for bumetanide binding to dog kidney membranes (Forbush & Palfrey, 1983). No attempt was made to establish directly that the 34-kDa protein is a component of the cotransporter, i.e., that the binding of [^{3}H]-bumetanide had the specificity attributed to it. Indeed, the specificity of covalent bumetanide binding after UV-induced molecular disruption can be expected to be lower than the specificity of reversible binding of intact bumetanide.

In a more complete study, Haas and Forbush (1987) used a tritiated photoactive compound of the bumetanide series, [^{3}H]-4-benzoyl-5-sulfamoyl-3-(3-thenyloxy)benzoic acid (BSTBA), to label dog kidney cortex membranes. This analogue of bumetanide, described by Nielsen et al. (1975), has potent diuretic activity. Haas and Forbush (1987) showed that BSTBA competes with [^{3}H]-bumetanide in reversible binding and that BSTBA itself binds reversibly with high affinity to the membranes. Photoactivated covalent binding of [^{3}H]-BSTBA to several proteins was observed, but binding to a 150-kDa protein had characteristics suggesting that this protein might be a part of the cotransporter. Among these characteristics was the requirement for the simultaneous presence of Na,

K, and Cl for binding. It is not clear from the description of the electrophoretic separation of proteins performed in this study if it was carried out under reducing and denaturing conditions; presumably the Laemmli (1970) method was used and the proteins were denatured (molecular weights were estimated). Haas and Forbush (1988) have recently carried out a similar study with similar results: [^{3}H]-BSTBA labeled a protein of 150 kDa from duck red cells.

Our results differed from those of Haas and Forbush (1987, 1988) in that in their studies a single large protein was found and we identified several smaller ones. The difference in results could be due to the difference in cellular systems. The cotransporter in Ehrlich cells may not be identical to the cotransporters in dog kidney or duck red cells. In Ehrlich cells the bumetanide affinity appears to be considerably lower than in the other two systems, and other cell types as well (Hoffmann et al., 1986; Haas & Forbush 1987, 1988; O'Grady et al., 1987). Perhaps in correlation with the difference in affinity, there may be a difference in the substrates transported. Under the conditions we employ, the bumetanide-inhibitable flux activated by hyperosmotic challenge in Ehrlich cells appears to be Na/Cl cotransport (Hoffmann et al., 1983). In duck red cells the corresponding cotransporter is clearly for Na/K/Cl. In dog kidney it is probably the same, though definitive proof is not available.

Attention has been called to a large difference between the turnover numbers of the cotransporters of Ehrlich cells and duck red cells. In Ehrlich cells it is 50 Cl ions/site · sec (Hoffmann et al., 1986), and in duck red cells it is 4000 Na ions/site · sec (Haas & Forbush, 1986). It has been suggested that this large difference is related to the different transport processes mediated by Na/Cl and Na/K/Cl cotransporters (Haas & Forbush, 1986; O'Grady et al., 1987). However, two other Na/K/Cl cotransporters have been shown to have turnover numbers closer to that of the Na/Cl transporters of Ehrlich cells than that of the Na/K/Cl cotransporter of duck red cells. Rugg et al. (1986) estimated a range of turnover numbers of 120–850 K ions/site · sec for the Na/K/Cl cotransporter of Madin Darby canine kidney cells in culture. O'Donnell and Owen (1988) found a turnover number of about 70 K ions/site · sec for the Na/K/Cl cotransporter of vascular smooth muscle cells from rats. Thus, while Na/Cl and Na/K/Cl cotransporters may differ in their bumetanide affinities and in the molecular weights of their proteins, turnover number may not be a diagnostic difference.

In our study and in those of Haas and Forbush (1987, 1988), bumetanide analogues were employed, in ours as a specific reversibly binding ligand in an affinity column, and by Haas and Forbush as a photoactivated covalently binding probe. As outlined above, the advantages of our approach are: (i) the binding of the ligand may be more specific since the structure of the ligand need not be altered to induce binding; (ii) the bumetanide binding site is not irreversibly modified. This latter point is a particularly important advantage of our approach if reconstitution of function is to be attempted.

The length of the spacer arm is important in successful affinity chromatography (*cf.* Pimplikar & Reithmeier, 1986). In the affinity column we used, the ligand is undoubtedly located primarily or entirely at the end of the glutathione spacer arm of the Sepharose, where it can interact with proteins passing through the column. Another important factor in affinity chromatography is the concentration of ligand in the column. The concentration of sulfhydryl groups in the swollen sepharose gel, 1 mM, provides only an upper limit for the ligand concentration. If this was the ligand concentration, it may be lower than the optimum for affinity chromatography, held to be ~15 mM (Pimplikar & Rethmeier, 1986).

Affinity chromatography has been an important technique in biochemistry for many years (Venter, 1982). There are a few examples of its application in the isolation of components of transport systems. For example, Pimplikar and Reithmeier (1986) successfully used affinity columns with stilbene derivatives as ligands to isolate the anion exchanger from red cells. Weber et al. (1985) have reported some success in isolating the glucose transporter by affinity chromatography. Finally Cherksey et al. (1987) have used a furosemide affinity gel to isolate proteins from Ehrlich ascites cells which are part of a K channel. This protein identified by electrophoresis in a reducing gel had polypeptides of 95 and 49 kDa. These were clearly of different molecular weights from the two major proteins (76 and 38 kDa) we isolated using the bumetanide affinity column.

Proteins are often retained on affinity columns by nonspecific binding. If this happens with a few proteins from a complex mixture, it could confound the conclusions. In the present study, however, both binding and elution are by specific ligands, and furthermore, binding was prevented upon rechromatography by bumetanide in solution. These considerations lead to the conclusion that the proteins isolated have specific affinity for bumetanide, and are therefore likely to be involved in cotransport. Of course, the only proof that the proteins we have isolated are constituents of the cotransporter will

come from the successful reconstitution of cotransport function after incorporation of the proteins into artificial membranes. Since it is possible to separate the two major bumetanide-binding proteins using cholate gels, it should be possible to test reconstitution of function of the native proteins both together and separately.

Frank Sløk assisted with the HPLC analyses. Technical assistance in the synthesis of azidobumetanide was given by Lene Bjork Nielsen, Anne Schluter, and Vibeke Jørgensen. Dr. Caroline Enns, Syracuse University, and Dr. Svend Olaf Anderson, August Krogh Institute, provided valuable advice. We are grateful to them all. The work was supported by grants from the Danish Natural Science Research Council (to EKH, PBD, and PK) and from the U.S. Public Health Services, NIH (DK-28290, DK-33640 to PBD).

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