Solubilization and Reconstitution of an Amiloride-Inhibited Sodium Transporter from Rabbit Kidney Medulla†

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ABSTRACT: An octyl glucoside extract has been formed from rabbit kidney medulla microsomes from which reconstituted proteoliposomes can be formed by lipid addition and dialysis to remove detergent. These proteoliposomes are capable of amiloride-inhibited $^{22}Na^+$ transport. The amiloride-inhibited Na^+ transport process is complete within 10 min and directly proportional to the vesicle protein concentration. Sodium accumulation by the proteoliposomes has been proven to represent transport by the demonstration that all Na^+ taken up by the vesicles can be removed by the ionophore nigericin. The process has been shown to be specific for amiloride by the demonstration that the effect of amiloride on Na^+ transport could not be reproduced by the similar compound sulfaguanidine nor by pyrazine, 2-pyrazinecarboxamide, 2-pyrazinecarboxylate, or 3-amino-2-pyrazinecarboxylate. The relationship between Na^+ uptake into proteoliposomes and Na^+ concentration was similar to the relationship between Na^+ uptake and concentration observed with medulla microsomes. The concentration of amiloride required for half-maximal inhibition of Na^+ uptake into either proteoliposomes or medulla microsomes was also the same. The evidence seems clear that the protein responsible for amiloride-inhibited Na^+ transport into rabbit kidney medulla microsomes has been extracted from the membranes and incorporated into purified lipid vesicles.

Microsomal fractions, isolated either from the medulla of the rabbit kidney or from the urinary bladder of the tropical toad *Bufo marinus*, have been shown to be capable of amiloride-sensitive Na^+ transport (LaBelle & Lee, 1982; LaBelle & Valentine, 1980). Amiloride (*N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide) is a well-known inhibitor of Na^+ transport into epithelial cells lining either the distal tubules of the mammalian kidney or the urinary bladder of the tropical toad (Bentley, 1968; Stoner et al., 1974). Microsomal fractions from these organs have been isolated to provide starting material for the eventual purification of the amiloride-sensitive Na^+ transporter (LaBelle & Lee, 1982; LaBelle & Valentine, 1980). Such a purified transporter would be useful in the elucidation of the molecular mechanism of the hormone aldosterone, which is currently believed to stimulate epithelial Na^+ transport via effects on the synthesis of this transporter (Sharp & Leaf, 1973; Reich & Scott, 1979; Edelman, 1979).

The development of reconstituted membrane transport systems has provided much information concerning the role of specific purified proteins and lipids in catalyzing such processes (Racker et al., 1975, 1979). Such reconstituted systems have also permitted the partial purification of proteins responsible for certain transport processes (Kasahara & Hinkle, 1976; Carter-Su et al., 1980).

We have been able to produce an octyl glucoside extract of rabbit kidney medulla microsomes from which reconstituted proteoliposomes could be formed that were capable of amiloride-inhibited Na^+ transport. From such a detergent extract of the microsomes, the eventual purification of the amiloride-inhibited Na^+ transporter may be possible.

Materials and Methods

Materials

Amiloride hydrochloride (*N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide) was the generous gift of Dr. Clement A. Stone of Merck Sharp & Dohme Research Laboratories, West Point, PA. Amiloride methanesulfonate was formed by treating amiloride hydrochloride with Dowex 1X4-100 ($CH_3SO_3^-$ form) resin. Nigericin was generously provided by Dr. R. J. Hosley of Eli Lilly, Indianapolis, IN. Hepps,[1] sulfaguanidine, and Dowex 50X8-100 (H^+ form) were obtained from Sigma Chemical Co., St. Louis, MO. $^{22}Na^+$ was obtained from Amersham, Arlington Heights, IL. Asolectin was obtained from Associated Concentrates, Woodside, NY, and octyl β-D-glucopyranoside (octyl glucoside) was obtained from Calbiochem-Behring, La Jolla, CA.

Methods

Rabbit Kidney Medulla Microsomes. Rabbit kidney medulla microsomes were formed by a modification of the procedure of Barnes et al. (1975), as described in LaBelle & Lee (1982). Protein concentrations were measured by the procedure of Lowry et al. (1951).

Formation of Reconstituted Proteoliposomes. Reconstituted proteoliposomes were formed from rabbit kidney medulla microsomes by a modification of the procedure of Racker (1972). Two volumes of rabbit kidney medulla microsomes was mixed at 0 °C with 1.25 volumes of a solution containing octyl glucoside (8%), sucrose (0.25 M), and NaHepps buffer (50 mM, pH 8.2) to yield a detergent extract with a final protein concentration of 3 mg/mL. This extract was centrifuged for 30 min at 200000*g*, and to 1.6 volumes of the supernatant was added, at 0 °C, 1 volume of a solution containing sonicated soybean phospholipid (asolectin) (35 mg/mL), sucrose (0.25 M), and NaHepps (50 mM, pH 8.2). The phospholipid had been sonicated for 10 min at 22 °C by using a bath-type sonicator from Laboratory Supplies Co., Hicksville, NY (Model GI-255-PI). The mixture of lipid, detergent, and microsomal proteins was then dialyzed for 12–16 h against 1000 volumes of a solution containing sucrose (0.25 M) and NaHepps (32 mM, pH 8.2) at 0 °C. The dialysis procedure removed the octyl glucoside and produced reconstituted proteoliposomes.

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[1] Abbreviation: Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.

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$^{22}Na^+$ Uptake Determination. Na^+ transport into the proteoliposomes was measured by a modification of the method of LaBelle & Valentine (1980). Aliquots of the proteoliposomes were incubated at 22 °C with sucrose (0.25 M), ^{22}NaHepps (0.5 μCi, 10.2 mM, pH 8.2), and either NaCl (0.6 mM) or amiloride hydrochloride (0.6 mM) in a total volume of 0.25 mL. Each incubation mixture was placed on a 1-mL Dowex 50X8 (Tris) 100-mesh column and eluted with sucrose as described (LaBelle & Valentine, 1980). The eluted vesicles were dissolved in 6.5 mL of Aquasol-2 scintillation fluid, and the radioactivity was determined with a Beckman LS-100C liquid scintillation spectrometer.

Analysis of Reconstituted Proteoliposomes. Since the Lowry et al. (1951) protein assay was sensitive to interference by large amounts of phospholipid and by Hepps, the proteoliposome protein concentration could not be determined directly. The reconstituted vesicles were first dialyzed to remove Hepps and then extracted with chloroform/methanol by the procedure of Bligh & Dyer (1959). The lipid partitioned into the chloroform phase while the protein was recovered from the H_2O/methanol phase and from the interface between the phases after the chloroform phase had been removed by pipet and the H_2O/methanol had been removed by evaporation under N_2.

Determination of Octyl Glucoside Concentration. Octyl glucoside was quantitated by a modification of the phenol/sulfuric acid assay for carbohydrate (Dubois et al., 1956). First the octyl glucoside was extracted into ethyl acetate in order to separate it from sucrose and other simple sugars. A 2-mL sample of an aqueous solution containing octyl glucoside was mixed with 2 mL of ethyl acetate, the mixture shaken and centrifuged at low speed, and the aqueous phase extracted with 2 mL more of ethyl acetate. Then the ethyl acetate fractions were combined and added to 4 mL of H_2O and shaken and centrifuged again. The ethyl acetate phase was dried under a N_2 stream, and the solid remaining was dissolved in 0.5 mL of H_2O. Then 0.3 mL of phenol (5%) and 1.8 mL of H_2SO_4 (concentrated) were added to the sample and mixed, and the optical density was measured at 480 nm. This procedure gave the same optical density values for purified octyl glucoside as it did for purified glucose. The ethyl acetate extraction procedure was performed on a sample of sucrose (0.25 M) solution in order to prove that it separated all but undetectable amounts of sucrose from the octyl glucoside, and while the ethyl acetate failed to extract 100% of a known amount of octyl glucoside from an aqueous solution, the recovery was between 70 and 80%. Interference by glycolipid with this assay could be neglected because of the extremely low levels of glycolipid present in the samples analyzed. Interference by glycoprotein could be neglected because it could be demonstrated that nearly all of the glycoprotein in the samples partitioned into the aqueous phase during ethyl acetate extraction. So that it could be determined whether protein or other membrane components could carry much octyl glucoside into the aqueous phase during extraction, a known amount of the detergent (0.7 mg) was extracted into ethyl acetate in both the presence and absence of a mixture containing medulla microsomes (1.4 mg of protein) and asolectin (14 mg). The membranes were incapable of significantly decreasing the amount of octyl glucoside recovered from the ethyl acetate phase.

Results and Discussion

We have been able to form proteoliposomes from a detergent extract of rabbit kidney medulla microsomes that were capable of amiloride-inhibited sodium transport (Figure 1). When

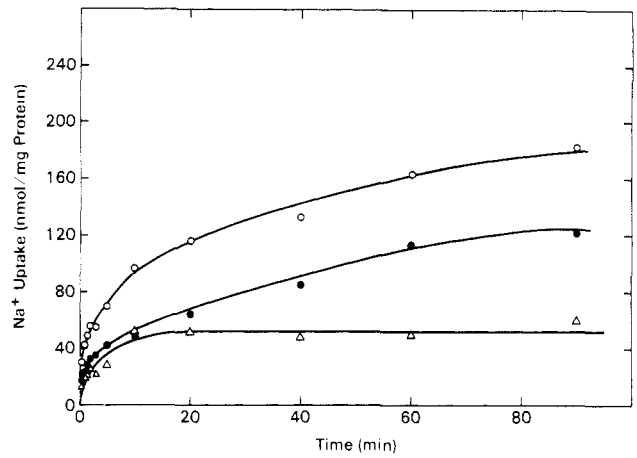

FIGURE 1: Kinetics of $^{22}Na^+$ uptake into proteoliposomes formed from rabbit kidney medulla microsomes. Proteoliposomes were formed from rabbit kidney medulla microsomes by the procedure described under Methods. Aliquots of the proteoliposomes (60 μg of protein) were incubated for the times indicated at 22 °C with sucrose (0.25 M), ^{22}NaHepps (0.5 μCi, 10.2 mM, pH 8.2), and either NaCl (0.6 mM) (○) or amiloride hydrochloride (0.6 mM) (●) in a total volume of 0.25 mL. The incubations were terminated by the application of the incubation mixtures to Dowex columns (1 mL). The columns were eluted with 1 mL of sucrose (0.25 M), and the radioactivity in the eluants was determined. The amiloride-sensitive sodium uptake is also indicated (△), which is the difference between the other two curves.

aliquots of rabbit kidney medulla microsomes were treated with increasing amounts of octyl glucoside (from 0 to 3%), and centrifuged for 30 min at 200000*g*, about 72% of the microsomal protein could be recovered from the pellet as long as the detergent concentration was 0.5% or below (even when the detergent concentration was 0%). Once the detergent concentration was increased above the critical micelle concentration (to 1%) (Baron & Thompson, 1975), then 70–80% of the protein could be recovered from the supernatant after centrifugation. When microsomes treated with concentrations of octyl glucoside of 0.5% or less were centrifuged at high speed, they could be recovered from the pellet with undiminished amiloride-inhibited Na^+ transport activity. However, if the microsomes were treated with more than 0.5% octyl glucoside and centrifuged at high speed, the microsomal fragments recovered from the pellet had lost all Na^+ transport activity. Likewise, if phospholipid was added to the supernatant fractions obtained after octyl glucoside treatment of the microsomes, and the lipid-treated fractions were dialyzed, the vesicles capable of amiloride-inhibited Na^+ transport could only be formed from supernatant fractions obtained after treatment with 1.5% or more octyl glucoside. If the microsomes were treated with increasing concentrations of detergent and centrifuged and both lipid and detergent were added to the supernatants, so that the final detergent concentration was 3%, these supernatants could be dialyzed to form proteoliposomes of high amiloride sensitive activity provided the initial octyl glucoside concentration was 1% or above. This indicated that the formation of reconstituted vesicles was a critical function of both the concentration of octyl glucoside and the presence of added lipid. The ratio of phospholipid to protein employed in the average reconstitution experiment was 7:1. The pH of the reconstitution medium was held at 8.2 by Hepps buffer since unpublished experiments with medulla microsomes indicated that the amiloride-inhibited Na^+ transport activity of such microsomes was optimal at pH 8.2.

When the medulla microsomes were treated with detergent and centrifuged and the supernatant was dialyzed in the absence of added lipid, the Na^+ transport activity of the resulting

Table I: Effect of Nigericin on Na^+ Accumulation by Proteoliposomes[a]

	$^{22}Na^+$ accumulated (nmol/mg of protein ± SD, n = 2)	
	−amiloride	+amiloride
before dilution	31.5 ± 0.26	14.6 ± 0.74
after dilution − nigericin	24.6 ± 2.4	13.4 ± 0.95
after dilution + nigericin	7.43 ± 0.74	2.6 ± 0.45

[a] Proteoliposomes were formed from rabbit kidney medulla microsomes as described under Methods. Aliquots of the proteoliposomes (75 μg of protein) were incubated for 20 min at 22 °C with sucrose (0.25 M), $^{22}Na^+$ (5 μCi, 15 mM), Cl^- (0.6 mM), and sufficient Hepps buffer (pH 8.2) to serve as a counterion for Na^+ both with and without amiloride (0.6 mM) as indicated in a total volume of 0.27 mL. Aliquots (0.04 mL) of the incubation mixtures were either applied directly to Dowex columns or diluted 1:7 with sucrose (0.25 M), with and without nigericin (120 μg/mL), incubated 30 s further, and then applied to Dowex columns.

protein preparation was less than 1% of the Na^+ transport activity of reconstituted proteoliposomes formed by dialysis of the detergent extract in the presence of added lipid. This result indicated that the detergent had thoroughly broken the microsomes into fragments of protein and lipid that were incapable of Na^+ transport after the detergent had been removed. Pure lipid vesicles formed by the dialysis of a sonicated mixture of asolectin and detergent were capable of only 5–10% of the Na^+ transport activity of the reconstituted proteoliposomes, and the Na^+ transport activity of the lipid vesicles was totally insensitive to amiloride. In order to form functional reconstituted proteoliposomes, it was necessary to extract the microsomes with the detergent octyl glucoside and to remove the detergent in the presence of added excess phospholipid. After the octyl glucoside extract of the microsomes had been mixed with asolectin and dialyzed for 16 h, the octyl glucoside concentration of the dialyzed preparation was shown to be less than 0.035%, which was less than 2% of the octyl glucoside concentration before dialysis.

The uptake of $^{22}Na^+$ into the proteoliposomes was almost entirely sensitive to nigericin (Table I). The proteoliposomes were preincubated for 20 min with $^{22}Na^+$, with and without amiloride, and then diluted 1:7 into sucrose (0.25 M) for 30 s. Hardly any $^{22}Na^+$ leaked out of the vesicles after dilution, unless the ionophore nigericin was present in the dilution mixture. Nigericin permitted the $^{22}Na^+$ inside the proteoliposomes to equilibrate with the external solution, so that the final $^{22}Na^+$ concentration inside the vesicles was nearly the optimal 14% of the initial concentration before the 1:7 dilution (Table I). This indicated that the $^{22}Na^+$ accumulation by the proteoliposomes represented transport into membrane vesicles rather than some form of binding (LaBelle & Lee, 1982; LaBelle & Valentine, 1980; LaBelle & Racker, 1977).

The effect of time on $^{22}Na^+$ uptake into the reconstituted proteoliposomes is shown in Figure 1. The amiloride-sensitive portion of the Na^+ uptake process reached a maximal equilibrium level very quickly (within 10 min), but the amiloride-insensitive portion of the transport did not reach an equilibrium level within 2 h. Therefore, the incubation times of nearly all of the transport experiments with the proteoliposomes were limited to 2 min, when the ratio of amiloride-sensitive to amiloride-insensitive activity was optimal. The rapid kinetics of the amiloride-inhibited Na^+ uptake were consistent with a transport process. Similar kinetics have been observed by our laboratory while investigating amiloride-inhibited Na^+ uptake into rabbit kidney medulla microsomes and into toad bladder microsomes (LaBelle & Lee, 1982;

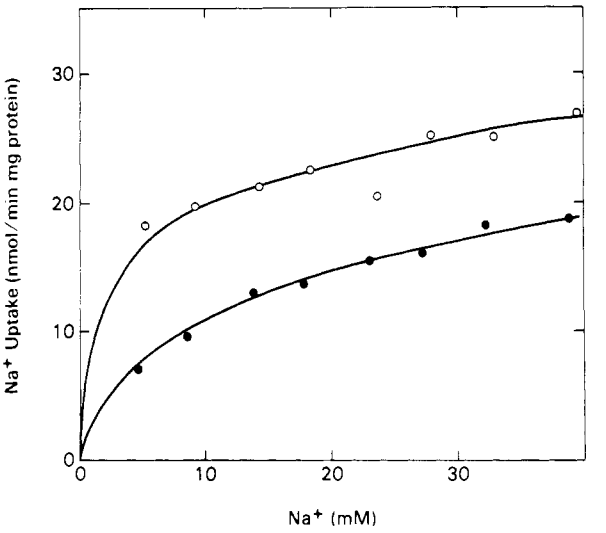

FIGURE 2: Effect of sodium concentration on $^{22}Na^+$ uptake into proteoliposomes from rabbit kidney. Aliquots (30 μg of protein) of proteoliposomes were incubated for 2 min at 22 °C with increasing amounts of ^{22}NaHepps (0.31 μCi/μmol Na^+, pH 8.2) and sufficient sucrose to keep the osmolarity constant, with either amiloride hydrochloride (0.6 mM) (●) or NaCl (0.6 mM) (○), in a total volume of 0.25 mL. The incubation mixtures were applied to Dowex columns as described in the legend of Figure 1. The means of triplicate determinations are shown above.

LaBelle & Valentine, 1980). Other investigators have measured similarly rapid transport processes with membrane vesicles formed from a variety of tissue (Stiernberg & LaBelle, 1981; Kinsella & Aronson, 1980; Cheng et al., 1978; Paris & Ailaud, 1980; Zala & Perdue, 1980; Inui et al., 1980; Hilden & Sacktor, 1979).

The uptake of $^{22}Na^+$ into reconstituted proteoliposomes was shown to be directly proportional to the proteoliposome concentration (data not shown). The Na^+ accumulation could be extrapolated to zero at zero proteoliposome concentration, and the rate of uptake was 25.6 nmol min^{-1} (mg of protein)$^{-1}$ in the absence of amiloride and 13.3 nmol min^{-1} (mg of protein)$^{-1}$ in the presence of amiloride (0.6 mM). The proportionality between Na^+ uptake and proteoliposome concentration was also consistent with a transport process (LaBelle & Lee, 1982; LaBelle & Valentine, 1980; Zala & Perdue, 1980; Fairclough et al., 1979). Sodium uptake also increased as a function of Na^+ concentration (Figure 2), but no K_m or V values were determined since the amiloride-inhibited transport was too rapid to permit the determination of an initial rate (Figure 1). The amount of $^{22}Na^+$ taken up by the vesicles in 2 min in the presence and the absence of amiloride appeared to saturate with increasing Na^+ concentration (Figure 2). The relationship between Na^+ uptake by the proteoliposomes and Na^+ concentration was not identical with the relationship previously reported between Na^+ uptake by medulla microsomes and Na^+ concentration (LaBelle & Lee, 1982). The effect of Na^+ concentration on transport into microsomes was previously determined at pH 7.4 (LaBelle & Lee, 1982), while the effect of Na^+ concentration on transport into proteoliposomes was determined at pH 8.2. When the effect of Na^+ concentration on transport into microsomes was measured at pH 8.2, the amount of Na^+ taken up by the microsomes in the presence and absence of amiloride was shown to be a saturable function of the Na^+ concentration (data not shown).

The effect of amiloride concentration on Na^+ uptake into either rabbit kidney medulla microsomes or proteoliposomes formed from such microsomes is detailed in Figure 3. Sodium uptake into either the microsomes or the proteoliposomes was

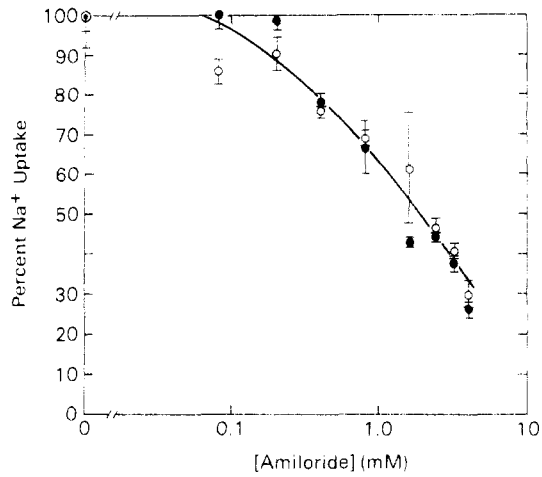

FIGURE 3: Effect of amiloride concentration on $^{22}Na^+$ accumulation by either microsomes or proteoliposomes formed from rabbit kidney. Aliquots of either rabbit kidney medulla microsomes (100 μg of protein) (○) or proteoliposomes (60 μg of protein) (●) formed from the microsomes as described under Methods were incubated for 2 min at 22 °C with sucrose (0.25 M), $^{22}Na^+$ (0.5 μCi, 12 mM), $CH_3SO_3^-$ (4 mM), and sufficient Hepps buffer (pH 8.2) to serve as a counterion for Na^+ in the presence of increasing concentrations of amiloride, in a total volume of 0.25 mL. The incubations were terminated as described in the legend of Figure 1. The Na^+ uptake activity is presented as the percentage of activity observed in the absence of inhibitor vs. log of inhibitor concentration.

50% inhibited by about 1.88 mM amiloride. At the highest amiloride concentration tested (4 mM), the Na^+ uptake was reduced by about 72%. The amiloride concentration required for half-maximal inhibition of Na^+ uptake in our previous study of medulla microsomes (LaBelle & Lee, 1982) was somewhat lower than the K_i observed in the current study (Figure 3). However, the experimental conditions employed in our previous study were different (pH, ionic strength), and the amiloride concentrations used earlier were deliberately kept low (≤0.6 mM) due to the inhibitory effects of Cl^- observed in our study of toad bladder microsomes (LaBelle & Valentine, 1980). Since the amiloride used in our earlier studies was the HCl salt, the amiloride concentration could not be increased above 0.6 mM without increasing the Cl^- concentration to the same extent. Therefore, in the current study, amiloride hydrochloride was replaced by amiloride methanesulfonate which allowed us to use higher amiloride concentrations (up to 4 mM). Control experiments have shown that $CH_3SO_3^-$ itself did not inhibit Na^+ uptake into either kidney microsomes or reconstituted proteoliposomes (data not shown). Therefore, the concentration required for half-maximal inhibition in our previous study was probably erroneous due to our inability to get a correct value for maximal inhibition (LaBelle & Lee, 1982).

Sulfaguanidine failed to reproduce the effects of amiloride on Na^+ transport into proteoliposomes. During a representative experiment, sulfaguanidine (0.8 mM) failed to exert a statistically significant effect on Na^+ uptake into proteoliposomes while the same concentration of amiloride (0.8 mM) inhibited Na^+ uptake by 42%. A higher concentration of sulfaguanidine (4 mM) was shown to inhibit Na^+ uptake by only 18% while the same amount of amiloride (4 mM) inhibited by 78%. The effect of a number of pyrazine derivatives other than amiloride on Na^+ uptake into proteoliposomes was also determined. None of these compounds, which included pyrazine (2.4 mM), 2-pyrazinecarboxylic acid (2.4 mM), 3-amino-2-pyrazinecarboxylic acid (2.4 mM), and 2-pyrazinecarboxamide (2.4 mM), had any effect on Na^+ uptake into proteoliposomes. The inability of sulfaguanidine (which is structurally similar to amiloride) and the other pyrazine derivatives to reproduce the effects of amiloride on the proteoliposomes provided strong evidence that amiloride was interacting with a specific biological system rather than merely exerting a nonspecific effect that any hydrophobic cation might exert (LaBelle & Lee, 1982; LaBelle & Valentine, 1980; Bentley, 1968).

When the octyl glucoside extract of the kidney microsomes was stored at 0 °C for increasing periods of time, reconstituted proteoliposomes could be formed from this extract by phospholipid addition and dialysis even after 19 h of storage, and the amiloride-inhibited Na^+ transport activity of these vesicles was not significantly diminished. The proteoliposomes, themselves, were stable for up to 24 h after the conclusion of dialysis. These data indicate that purification procedures can be used to begin to fractionate this extract. Such experiments are currently in progress.

The above experiments provide convincing evidence that the amiloride-inhibited Na^+ transport protein has been extracted from rabbit kidney medulla microsomes and reincorporated into functionally reconstituted proteoliposomes.

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References

Barnes, L. D., Hui, Y. S. F., Frohnert, P. P., & Dousa, T. P. (1975) *Endocrinology (Philadelphia) 96*, 119–129.

Baron, C., & Thompson, T. E. (1975) *Biochim. Biophys. Acta 382*, 276–285.

Bentley, P. J. (1968) *J. Physiol. (London) 195*, 317–330.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Carter-Su, C., Pillion, D. J., & Czech, M. P. (1980) *Biochemistry 11*, 2374–2385.

Cheng, L. C., Rogus, E. M., & Zierler, K. (1978) *Biochim. Biophys. Acta 513*, 141–155.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Edelman, I. S. (1979) *J. Endocrinol. 81*, 49p–53p.

Fairclough, P., Malathi, P., Preiser, H., & Crane, R. K. (1979) *Biochim. Biophys. Acta 553*, 295–306.

Hilden, S. A., & Sacktor, B. (1979) *J. Biol. Chem. 254*, 7090–7096.

Inui, K., Tillotson, L. G., & Isselbacher, K. J. (1980) *Biochim. Biophys. Acta 598*, 616–627.

Kasahara, M., & Hinkle, P. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 396–400.

Kinsella, J. L., & Aronson, P. S. (1980) *Am. J. Physiol. 238*, F461–469.

LaBelle, E. F., & Racker, E. (1977) *J. Membr. Biol. 31*, 301–315.

LaBelle, E. F., & Valentine, M. E. (1980) *Biochim. Biophys. Acta 601*, 195–205.

LaBelle, E. F., & Lee, S. O. (1982) *Biochim. Biophys. Acta 685*, 367–378.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Paris, S., & Ailaud, G. (1980) *Biochim. Biophys. Acta 601*, 630–639.

Racker, E. (1972) *J. Biol. Chem. 247*, 8198–8200.

Racker, E., Knowles, A. F., & Eytan, E. (1975) *Ann. N.Y. Acad. Sci. 264*, 17–33.

Racker, E., Violand, B., O'Neal, S., Alfonzo, M., & Telford,

J. (1979) *Arch. Biochem. Biophys. 198*, 470–477.
Reich, I. M., & Scott, W. N. (1979) *Mt. Sinai J. Med. 46*, 367–377.
Sharp, G. W. G., & Leaf, A. (1973) in *Handbook of Physiology* (Orloff, J., & Berliner, R. W., Eds.) Section 8, pp 815–830, American Physiological Society, Washington, DC.
Stiernberg, J. S., & LaBelle, E. F. (1981) *Biochem. Biophys. Res. Commun. 103*, 759–766.
Stoner, L. C., Burg, M. B., & Orloff, J. (1974) *Am. J. Physiol. 227*, 453–459.
Zala, C. A., & Perdue, J. F. (1980) *Biochim. Biophys. Acta 600*, 157–172.

Self-Association of Myelin Basic Protein: Enhancement by Detergents and Lipids†

Ross Smith

ABSTRACT: Self-association of basic protein has been proposed to be of functional significance in central nervous system myelin. In aqueous solution this protein self-associates, previous data being consistent with the formation of dimers, which then undergo an indefinite isodesmic self-association [Smith, R. (1980) *Bichemistry 19*, 1826–1831]. As this protein is membrane bound in vivo, we have now examined the effects of amphiphiles on the self-association equilibria. Contrary to the expected effects, at low molar ratios dodecyl sulfate, deoxycholate, Triton X-100, and lysophosphatidylcholine increased protein intermolecular attraction. The anionic detergents led to partial precipitation even at 1:1 protein:detergent molar ratios whereas the zwitterionic lipid and the nonionic detergent exerted less pronounced effects. Sedimentation velocity and equilibrium measurements have been used to define quantitatively the effects of lysophosphatidylcholine. The sedimentation coefficient increases up to a lipid:protein ratio of 4:1 and then remains constant up to a ratio of 12:1. The sedimentation equilibrium data suggest that the mode of protein–protein interaction is the same as in the absence of lipid but with substantially increased association constants. The dimerization constant is increased from 1.20×10^2 M^{-1} to 1.0×10^3 M^{-1} and the isodesmic association constant from 3.4×10^4 M^{-1} to 1.2×10^5 M^{-1}. The effects of detergents on myelin basic protein are compared with the effects on other proteins, and the implications for the state of the protein with myelin are discussed.

Interest in the self-association of the basic protein of myelin has been promoted by the suggestion that dimers of this protein may stabilize the structure of central nervous system myelin (Smith, 1977b, 1978; Braun, 1977; Rumsby, 1978). The self-association data (Smith, 1980) are best described by a scheme involving initial dimer formation followed by an indefinite isodesmic association of dimers. It can, however, readily be argued that studies of the protein in aqueous solution are of limited relevance to its behavior in vivo. For example, one clear objection is that in vivo the protein does not exist alone but in intimate contact with lipids that may influence the properties of the protein, particularly its self-association. This objection does not diminish the force of the thermodynamic argument that a full description of the state of the protein in myelin must take into consideration self-association of free protein molecules but emphasizes that such equilibria may represent only a small perturbation on the behavior of the largely lipid bound protein.

We have previously observed that although the protein is monomeric at high dodecyl sulfate concentrations it is dimeric at lower concentrations, suggesting that specific protein–protein interactions may be retained even in the presence of moderate detergent concentrations (Smith & McDonald, 1979). The self-association equilibria have now been reexamined in the presence of low detergent concentrations. We have found that binding of just one or two amphiphile molecules per protein molecule suffices to markedly increase the association and that the protein–protein interactions persist over a wide range of amphiphile concentrations.

Materials and Methods

Many of the methods have been outlined in an earlier publication (Smith, 1980): essential details only are set out here. Basic protein was prepared from fresh bovine white matter (Smith, 1977a,b). The protein was fractionated by chromatography on carboxymethylcellulose (Deibler & Martenson, 1973), and only the major peak, which eluted last, was kept. This fraction contained protein that had undergone no posttranslational modification, except for methylation of the arginine residue at position 109.

Sedimentation equilibrium experiments were performed on a Beckman Model E ultracentrifuge with interference optics. Aliquots of 150 μL of solvent and of solution were used in a double-sector cell with a capillary-type synthetic-boundary centerpiece and sapphire windows. In experiments where the meniscus was not depleted at equilibrium the actual concentration in the cell was determined in a subsequent synthetic-boundary experiment. Prior to the sedimentation experiments the protein was dissolved in buffer containing 0.1 M phosphate, 0.2 M sodium chloride, and 2 mM sodium azide and then dialyzed against this buffer for 48 h at 4 °C. The initial protein concentration in the sedimentation equilibrium experiments ranged from 0.3 to 2.5 g L^{-1}.

The apparent partial specific volume (ϕ') of the protein in detergent solution was calculated by using the equation (1 –

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