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Asymmetric Incorporation of Na^+, K^+-ATPase into Phospholipid Vesicles

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Purified lamb kidney Na^+,K^+-ATPase, consisting solely of the M_r = 95,000 catalytic subunit and the $M_r \simeq$ 44,000 glycoprotein, was solubilized with Triton X-100 and incorporated into unilamellar phospholipid vesicles. Freeze–fracture electron microscopy of the vesicles showed intramembranous particles of approximately 90–100 Å in diameter, which are similar to those seen in the native Na^+,K^+-ATPase fraction. Digestion of the reconstituted proteins with neuraminidase indicated that the glycoprotein moiety of the Na^+,K^+-ATPase was asymmetrically oriented in the reconstituted vesicles, with greater than 85% of the total sialic acid directed toward the outside of the vesicles. In contrast, in the native Na^+,K^+-ATPase fraction, the glycoprotein was symmetrically distributed. Purified glycoprotein was also asymmetrically incorporated into phospholipid vesicles using Triton X-100 and without detergents as described by R. I. MacDonald and R. L. MacDonald (1975, *J. Biol. Chem.* **250**, 9206–9214). The glycoprotein-containing vesicles were 500–1000 Å in diameter, unilamellar, and, in contrast to the vesicles containing the Na^+,K^+-ATPase, did not contain the 90- to 100-Å intramembranous particles. These results indicate that the intramembranous particles observed in the native Na^+,K^+-ATPase and in the reconstituted Na^+,K^+-ATPase are not due to the glycoprotein alone, but represent either the catalytic subunit, or the catalytic plus the glycoprotein subunit.

Sodium- and potassium-activated adenosine triphosphatase (Na^+,K^+-ATPase, EC 3.6.1.3) is responsible for the active transport of Na^+ and K^+ across the plasma membrane. The enzyme, which has now been purified from a number of different sources, consists primarily of a M_r = 95,000 catalytic subunit and a $M_r \simeq$ 44,000 glycoprotein subunit, which has no known function (for review, see Refs. (1–3)). Recent studies with photoaffinity derivatives of ouabain (4, 5) suggest that the Na^+,K^+-ATPase may also contain a low molecular weight (12,000) "proteolipid" component. Electron microscopic studies (6–9) using thin sectioning, negative staining, and freeze–fracturing indicate that the purified enzyme fractions consist of membranous structures. In an early freeze–fracture study, Van Winkle *et al.* (7) reported that the purified Na^+,K^+-ATPase from the dog renal medulla consisted of vesicular membrane structures with intramembranous particles (95–120 Å in diameter) equally distributed on both fracture faces. Dequchi *et al.* (8) and Vogel *et al.* (9) examined the ultrastructure of rabbit and pig renal Na^+,K^+-ATPase, respectively, and reported that the fractions consisted of cup-shaped membrane fragments with intramembranous particles located primarily on the concave fracture face. The differences in the distribution of the intramembranous particles may reflect the use of different methods for enzyme purification. Van Winkle *et al.* (7) used a Na^+,K^+-ATPase that had been purified by solubilization with deoxycholate (10), whereas the enzymes used by other investigators (8, 9) were purified without solubilization, using the SDS[3]-gradient procedure of Jørgensen (11). Despite the

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differences in ultrastructure, each of the purified Na^+,K^+-ATPase preparations consisted solely of the two major protein subunits, and each preparation contained the intramembranous particles. It therefore seems likely that these intramembranous particles represent aggregates of the catalytic subunit, the glycoprotein subunit, or both subunits.

In the present study, techniques are described for the incorporation of Na^+,K^+-ATPase and of the glycoprotein subunit into defined lipids. Preliminary characterization of the reconstituted proteins indicates that both the Na^+,K^+-ATPase and the purified glycoprotein are asymmetrically incorporated into the lipid vesicles with these techniques, and that only the purified Na^+,K^+-ATPase, which contains the catalytic subunit, exhibits the intramembranous particles.

MATERIALS AND METHODS

Materials. 1,2-Dimyristoyl-SN-glycero-3-phosphocholine (dimyristoyl phosphatidylcholine), 1,2-dipalmitoyl-SN-glycero-3-phosphocholine (dipalmitoyl phosphatidylcholine), 1,2-dioleoyl-SN-glycero-3-phosphocholine (dioleoyl phosphatidylcholine), and 1-stearoyl - 2 - oleoyl - SN - glycero - 3 - phosphocholine (1 - stearoyl-2-oleoyl phosphatidylcholine) were synthesized as described previously (12). Neuraminidase from *Vibrio cholerae* was obtained from Koch Light, Triton X-100 from Rohm and Haas, Bio-Beads from Bio-Rad, trypsin (tosyl-phenylalanine-chloro-methyl-ketone treated) from Serva, and α-chymotrypsin from Boehringer-Mannheim.

Preparation of Na^+,K^+-ATPase and glycoprotein subunit. Na^+,K^+-ATPase was purified from lamb kidney outer medulla as described previously (10). The isolated glycerol precipitate fraction had an activity of 1120 μmol P_i/h/mg protein, contained 0.9 μmol phospholipid/mg protein, and, as shown in Fig. 1, contained only the catalytic and glycoprotein subunits.

The glycoprotein subunit was isolated by chromatography in SDS on granular hydroxyapatite as previously described (10). The purified glycoprotein gave a single protein band on polyacrylamide gel electrophoresis in SDS (Fig. 1). To remove residual SDS and lipids which are tightly bound, the glycoprotein was delipidated with ethanol (13). To 50 ml of the glycoprotein (1 mg/ml) in 0.01 M Tris–HCl, pH 7.6, and 0.1 mM EDTA, 450 ml ethanol (100%) were added with vigorous stirring. One-tenth volume of 1 M Tris–HCl, pH 7.6, and 10 mM EDTA was then added to precipitate the protein. The glycoprotein was collected by centrifugation, dissolved in 1 mM Tris–HCl, pH 7.6, and dialyzed against the same buffer.

Solubilization of Na^+,K^+-ATPase with Triton X-100. Purified Na^+,K^+-ATPase (1.7 mg protein) was incubated at 0°C with various concentrations of Triton X-100 in a final volume of 3 ml 0.05 M Tris–HCl, pH 7.6. After 30 min, the samples were centrifuged at 150,000*g* for 90 min at 0°C.

Incorporation of Na^+,K^+-ATPase and glycoprotein subunit into lipid. Na^+,K^+-ATPase was incorporated into phospholipid vesicles using Triton X-100 by a method similar to that described by Gerritsen *et al.* (14). The enzyme (1.7 mg protein) was incubated at 0°C for 30 min in 3.0 ml 0.05 M Tris–HCl, pH 7.6, containing 0.25% Triton X-100 and then subjected to ultracentrifugation at 150,000*g* for 90 min at 0°C; 8% of the protein sedimented with these conditions. Dioleoyl phosphatidylcholine (4 mg), dissolved in chloroform, was evaporated to dryness in a 20-ml round-bottom flask and then dried under vacuum for 4 h. Na^+,K^+-ATPase solubilized with Triton X-100 was added to the lipid film and stirred at 0°C for 30 min. The turbid suspension was then treated with 1.0 ml Bio-Beads SM-2 (Bio-Rad) (0.3 g wet beads/ml) for 1 h at 0°C to remove Triton X-100 (15). After removal of the beads, the suspension was subjected to lowspeed centrifugation (9000 rpm, 20 min, 4°C) to remove large multilamellar structures. Less than 5% of the protein added to the film was incorporated into these multilamellar structures. The supernatant fraction was then centrifuged at 150,000*g* for 90 min to sediment the lipid–protein complex. Polyacrylamide gel electrophoresis in SDS of the isolated complex gave a gel pattern indistinguishable from native Na^+,K^+-ATPase. The recovery of glycoprotein as sialic acid in the isolated Na^+,K^+-ATPase–lipid complex was approximately 60%. Glycoprotein–lipid complexes and dioleoyl phosphatidylcholine vesicles containing no proteins were prepared by the Triton X-100 method in an identical manner.

The glycoprotein was also incorporated into lipid vesicles without detergents by the method of MacDonald and MacDonald (16). Phospholipids, dissolved in chloroform, were added to a 20-ml round-bottomed flask and evaporated to dryness. The glycoprotein was added to the lipid film as follows: The protein was dissolved in 1 mM Tris–HCl, pH 7.6, to give 20 mg/ml. To 0.05 ml of this solution were added 3.75 ml methanol and then 7.5 ml chloroform. With these solvent conditions, the glycoprotein remained soluble. The solution was then taken to dryness with the lipids and dried under vacuum for 3 h to remove traces of solvent. The film was hydrated by adding

[3] Abbreviations used: SDS, sodium dodecyl sulfate; PC, phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine.

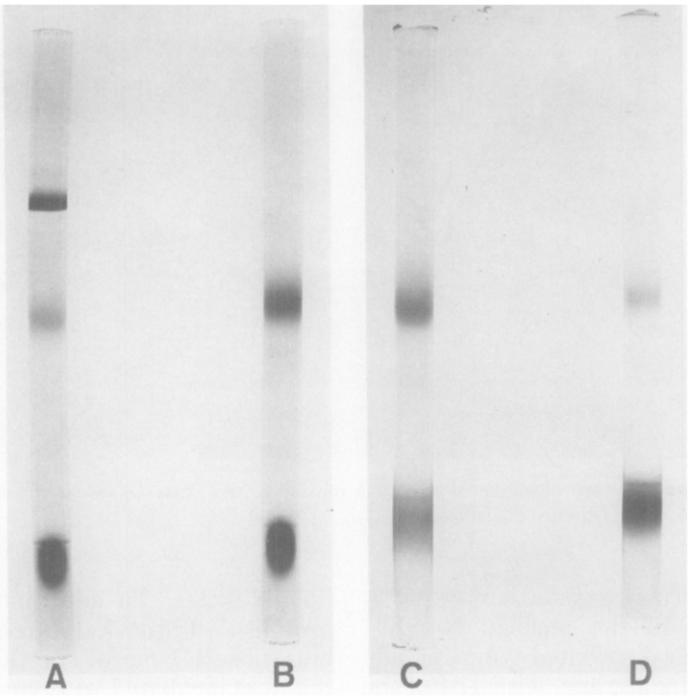

FIG. 1. Polyacrylamide gel electrophoresis of Na^+,K^+-ATPase and of purified glycoprotein subunit. Na^+,K^+-ATPase and glycoprotein subunit were isolated as previously described (10). Electrophoresis was performed on 7.5% acrylamide–0.1% SDS–gels described by Laemmli (25). Gels were stained with either Coomassie brilliant blue (A and C) or periodic/Schiff reagent (B and D). (A) Na^+,K^+-ATPase, 15 μg; (B) 60 μg Na^+,K^+-ATPase; (C) 15 μg glycoprotein; (D) 50 μg glycoprotein. The band at the bottom of the gel corresponds to the dye front.

5 ml of 0.01 M Tris–HCl, pH 7.6, containing 0.1 M NaCl. After a brief (30 s) exposure to bath sonication (this procedure did not affect the lipid–protein ratio, but did improve the yield of complex), the suspension was centrifuged at 10,000*g* for 20 min at 4°C to remove multilamellar structures. The supernatant fraction was then subjected to ultracentrifugation at 150,000*g* for 60 min at 4°C. The pelleted lipid–protein complex was resuspended in 0.3 ml of 0.01 M Tris–HCl, pH 7.6, and 0.1 M NaCl, by vortexing with a glass bead.

Other methods. Protein was determined according to Schacterle and Pollach (17), using bovine serum albumin as standard. To prevent interference by Triton X-100, 0.1 ml of 3% SDS was added to each sample. Sialic acid was determined by the method of Warren (18). Phospholipid-phosphorus was measured by the method of Bartlett (19). Phospholipids were separated by two-dimensional thin-layer chromatography according to Broekhuyse (20). Freeze–fracture electron microscopy was performed as described previously (21). Differential scanning calorimetry was done on a Perkin–Elmer 2 calorimeter, using a scanning rate of 5°C/min (22).

RESULTS AND DISCUSSION

Characterization of Na^+,K^+-ATPase

Freeze–fracture electron microscopy of the purified lamb kidney Na^+,K^+-ATPase preparation revealed vesicular structures with a substructure of particles of approximately 90–100 Å in diameter equally distributed on both fracture faces (Fig. 2). Both the vesicular structure and the equal distribution of the intramembranous particles on the fracture faces are consistent with the previous report by Van Winkle *et al.* (7) of the ultrastructure of dog kidney Na^+,K^+-ATPase, which had been purified by a similar procedure.

Incubation of the purified Na^+,K^+-ATPase with neuraminidase released 47% of the total sialic acid present in the preparation (Table I). Under identical incubation conditions, neuraminidase released 97% of the sialic acid present in the purified glyco-

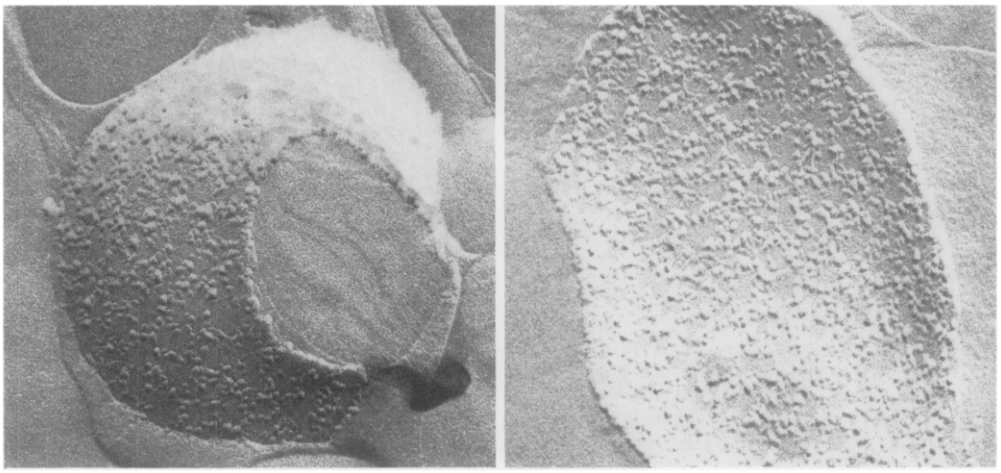

FIG. 2. Freeze–fracture electron micrographs of Na^+,K^+-ATPase. (A) Concave outer fracture face. (B) Convex inner fracture face. Magnification ×110,000.

protein fraction. This suggests that the glycoprotein subunit of the purified Na^+,K^+-ATPase fraction is distributed symmetrically within the lipid membrane, with 47% of the glycoprotein subunits directed toward the outside of the vesicle. Since the concentration (0.35%) of sodium deoxycholate used in the purification procedure does not separate the catalytic and glycoprotein subunits, it is assumed that 47% of the Na^+,K^+-ATPase molecules are directed toward the outside of the vesicle, i.e., only 53% of the enzyme molecules are "right-side-out," with respect to their *in vivo* orientation. The orientation of the purified lamb kidney Na^+,K^+-ATPase is similar to that reported by Goldin (23) for Na^+,K^+-ATPase which had been incorporated into lipid vesicles using sodium cholate.

Neuraminidase treatment of the purified Na^+,K^+-ATPase did not alter the number or the distribution of the intramembranous particles observed with freeze–fracture electron microscopy. As shown in Table I, trypsin and α-chymotrypsin treatment of the purified enzyme released less than 30% of the total sialic acid.

Incorporation of Na^+,K^+-ATPase into Lipid Vesicles

Na^+,K^+-ATPase, solubilized with Triton X-100, was incorporated into phospholipid vesicles. To determine the ratio of protein to Triton X-100 required for solubilization, 1.7 mg of Na^+,K^+-ATPase protein was incubated with various concentrations of detergent. After ultracentrifugation to sediment particulate membrane fragments, the supernatant fractions were analyzed for protein and sialic acid (Fig. 3). With 0.25% Triton X-100 (Triton-to-protein weight ratio of 4.4), 92% of the protein and sialic acid was present in the supernatant fraction. Although 0.05 and 0.10% Triton X-100 only partially solubilized the enzyme, the ratio of protein to sialic acid in the supernatant fraction was constant, indicating that there was no preferential extraction of the glycoprotein subunit. A previous study has also shown that the two subunits are solubilized by Triton X-100 as a unit with a molecular weight 140,000 (26).

The 0.25% Triton X-100 solubilized enzyme was then incorporated into dioleoyl phosphatidylcholine as described under Materials and Methods. The isolated complex contained 2.5 μmol phospholipid/mg protein. Freeze–fracture electron microscopy of the isolated complex showed unilamellar vesicles with diameters of 500 to 1000 Å, and intramembranous particles with diameters of 100 Å on both fracture faces (Fig. 4B). Omitting Na^+,K^+-ATPase from the lipid resulted in similar sized phospholipid vesicles but, as shown in Fig.

TABLE I

ENZYMIC RELEASE OF SIALIC ACID FROM NATIVE Na^+,K^+-ATPase AND Na^+,K^+-ATPase INCORPORATED INTO PHOSPHOLIPID VESICLES[a]

Preparation	Enzyme	Percentage sialic acid removed
Na^+,K^+-ATPase	Neuraminidase (0.05 U)	47
Na^+,K^+-ATPase + Triton (0.0025%)	Neuraminidase (0.05 U)	57
Na^+,K^+-ATPase	Trypsin (10 μg)	17
Na^+,K^+-ATPase	Trypsin (100 μg)	17
Na^+,K^+-ATPase	α-Chymotrypsin (100 μg)	29
Na^+,K^+-ATPase incorporated into DOPC	Neuraminidase (0.05 U)	86

[a] Native Na^+,K^+-ATPase and Na^+,K^+-ATPase incorporated into dioleoyl phosphatidylcholine (DOPC), 0.5 mg protein for each preparation, were incubated with the appropriate enzyme in a final volume of 0.2 ml of 10 mM Tris–HCl, 100 mM NaCl, pH 7.4, for 1 h at 37°C. After incubation, sialic acid was determined (18). The percentage sialic acid removed is based on 100% removed with 0.1 M H_2SO_4.

4A and also as reported by Gerritsen *et al.* (14), there were no particles evident. In the reconstituted Na^+,K^+-ATPase, 86% of the sialic acid was released with neuraminidase, compared to only 47% in the larger vesicular structures of the native enzyme (Table I). This suggests that 86% of the reconstituted Na^+,K^+-ATPase molecules are right-side-out.

Repeated freezing and thawing of the 500–1000 Å vesicles (Fig. 4B) caused the structures to fuse and produced extensive areas of fracture faces (Fig. 4C). The distribution of particles on both fracture faces in the large fused structures was approximately equal. The specific activity of the reconstituted Na^+,K^+-ATPase was only 10% (100 μm P_i/mg/h) that of the native enzyme. This loss of activity could be due (a) to the inactivation of the enzyme by Triton X-100, (b) to the right-side-out orientation of the reconstituted enzyme, where 86% of the phosphorylation sites are located inside the vesicle and are therefore not accessible by ATP, or (c) to the lipid composition of the reconstituted vesicles. The addition of Triton X-100 (0.1–0.2%) or sodium deoxycholate (0.1%) to the reconstituted Na^+,K^+-ATPase did not result in an increase in activity, suggesting that the decreased enzymic activity is not due to the "sideness" of the reconstituted enzyme. Previous studies have indicated that rabbit kidney Na^+,K^+-ATPase (27, 28) and glycophorin (29) and Ca^{2+},Mg^{2+}-ATPase (30) of erythrocytes either preferentially interact with or are activated by negatively charged phospholipids. The decreased activity of this reconstituted Na^+,K^+-ATPase may, therefore, be due to the absence of negatively charged phospholipids, e.g., phosphatidylserine.

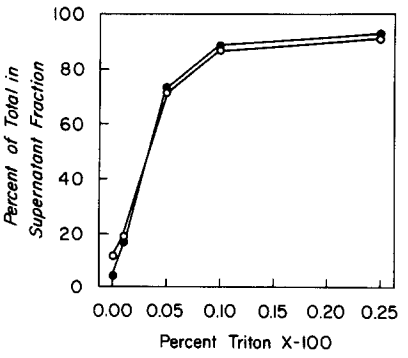

FIG. 3. Solubilization of Na^+,K^+-ATPase with Triton X-100. Na^+,K^+-ATPase (1.7 mg protein) was incubated in 0.05 M Tris–HCl, pH 7.6, with various concentrations of Triton X-100 in a final volume of 3 ml. After incubation for 30 min at 0°C, the samples were centrifuged at 150,000*g* for 90 min at 0°C. The supernatant fractions were then analyzed for protein (– ○ – ○ –) and sialic acid (– ● – ● –) as described under Materials and Methods.

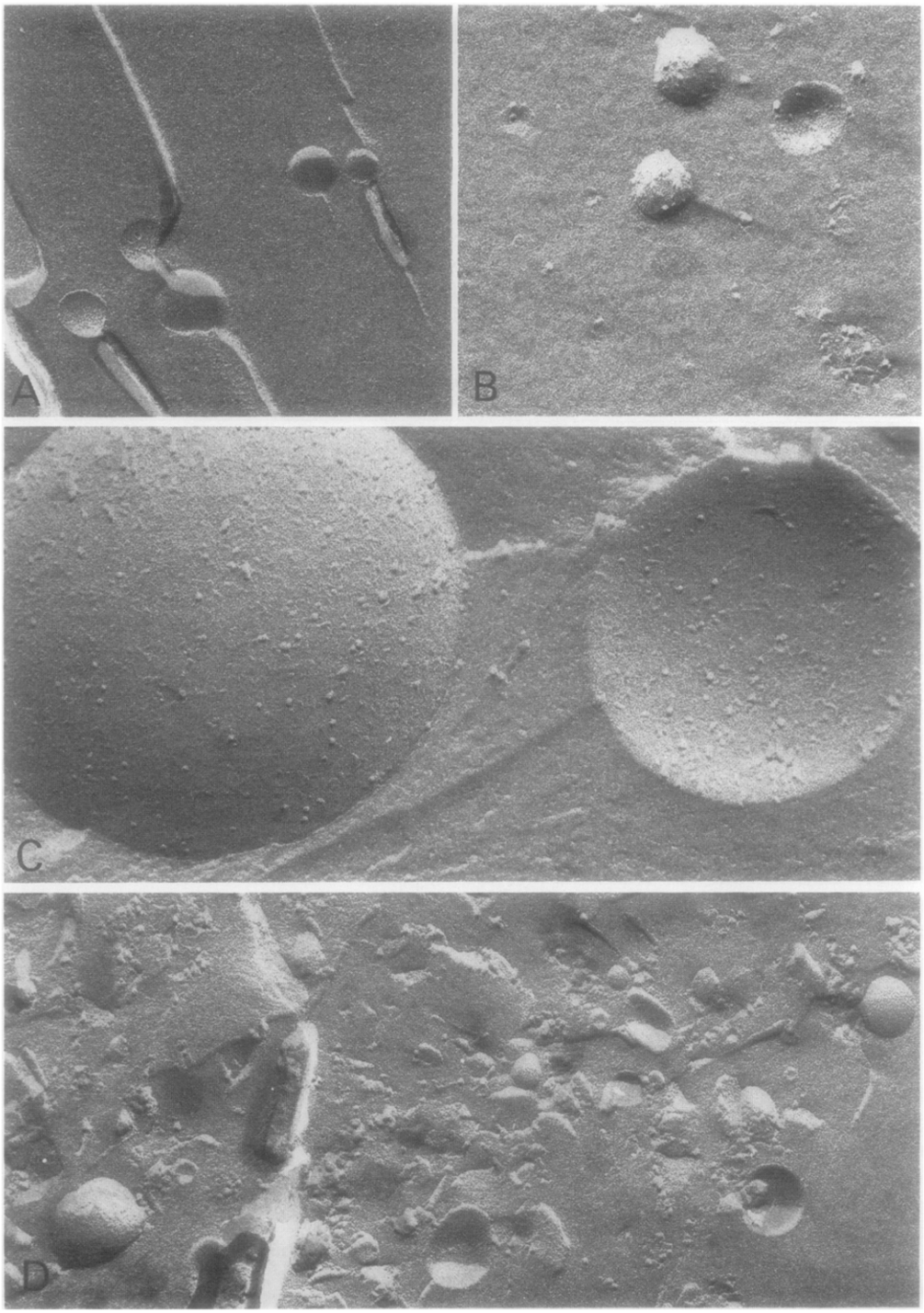

FIG. 4. Freeze–fracture electron micrographs of reconstituted vesicles. Vesicles were prepared from dioleoyl phosphatidylcholine (DOPC) using Triton X-100 as described under Materials and Methods. (A) DOPC alone. (B) DOPC plus Na^+,K^+-ATPase. (C) Vesicles after fusion induced by freezing and thawing of "B". (D) DOPC plus glycoprotein. Magnification ×110,000.

TABLE II

PERCENTAGE OF SIALIC ACID REMOVED BY NEURAMINIDASE FROM GLYCOPROTEIN AND FROM GLYCOPROTEIN–LIPID COMPLEXES[a]

Experiment No.	Preparation	Lipid–protein molar ratio	Percentage sialic acid removed
1	Glycoprotein	—	97
2	Glycoprotein:dioleoyl PC	177	75
3	Glycoprotein:dimyristoyl PC	277	86
4	Glycoprotein:dioleoyl PC	177	95
5	Glycoprotein:dipalmitoyl-1-stearoyl-2-oleoyl PC (1:1 molar ratio)	232	100

[a] The glycoprotein subunit of Na^+,K^+-ATPase was incorporated into phosphatidylcholine (PC) vesicles using Triton X-100 (experiment 2) or by the method of MacDonald and MacDonald (16) (experiments 3–5) as described under Materials and Methods. Each preparation (0.5 mg glycoprotein) was incubated with neuraminidase (0.05 μl) in a final volume of 0.2 ml of 10 mM Tris–HCl, 100 mM NaCl, pH 7.4, for 1 h at 37°C. After incubation, sialic acid was determined (18). The percentage sialic acid removed is based on 100% removed with 0.1 M H_2SO_4.

Characterization of Glycoprotein Subunit

The isolated glycoprotein subunit is a single protein with an approximate molecular weight of 44,000 (10) (Fig. 1). The glycoprotein subunit which was eluted from hydroxyapatite and subsequently from Bio-Gel A-5m contained 10% (w/w) tightly bound phospholipid. Treatment of the glycoprotein fraction with ethanol removed 80% of these lipids. The composition of the extracted phospholipids was as follows: 18.1% phosphatidylcholine, 8.5% lysophosphatidylcholine, 28.4% sphingomyelin, 26.6% phosphatidylserine, and 18.4% phosphatidylethanolamine. The ethanol-delipidated glycoprotein still contained an average of 1 mol phospholipid/mol protein. Based on the average of six separate determinations, the delipidated glycoprotein contained 130 nmol sialic acid/mg protein or 6 mol/mol protein.

Incorporation of Glycoprotein into Lipid Vesicles

To determine the orientation and substructure of the glycoprotein of Na^+,K^+-ATPase, the purified, delipidated glycoprotein was incorporated into phospholipids using the Triton X-100 method described above for the native enzyme. The lipid–protein molar ratio of the isolated glycoprotein–dioleoyl phosphatidylcholine complex was 177 as indicated in Table II. In addition to the Triton method, the glycoprotein was also incorporated into phospholipid vesicles according to the method of MacDonald and MacDonald (16) as described in detail under Materials and Methods. Figure 5 illustrates how the lipid-to-protein ratio in these reconstituted vesicles can be varied by changing the lipid-to-protein ratio in the dried film. The recovery of

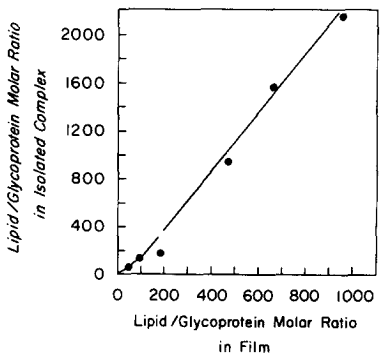

FIG. 5. Incorporation of the glycoprotein subunit of Na^+,K^+-ATPase into vesicles of dimyristoyl phosphatidylcholine. The complexes were prepared and isolated as described under Materials and Methods.

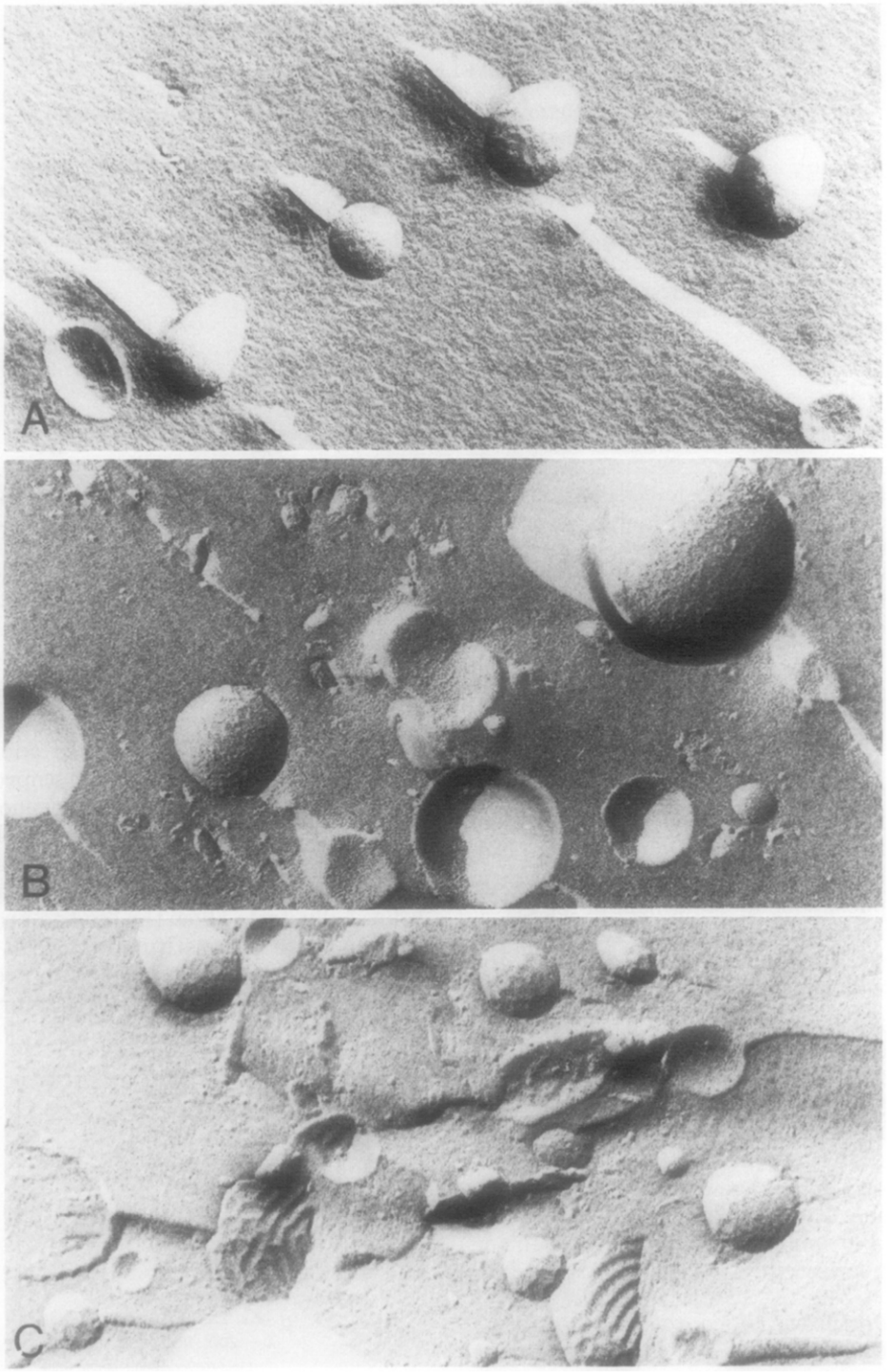

FIG. 6. Freeze–fracture electron micrographs of the glycoprotein subunit of Na^+,K^+-ATPase incorporated into phospholipid vesicles. (A) Dimyristoyl phosphatidylcholine (lipid/protein molar ratio 277). (B) Dioleoyl phosphatidylcholine (lipid/protein molar ratio 177). (C) A 1:1 molar ratio of dipalmitoyl phosphatidylcholine and 1-stearoyl-2-oleoyl phosphatidylcholine (lipid/protein molar ratio 232). The complexes were prepared by the method of MacDonald and MacDonald (16). For further details see Materials and Methods. Magnification ×110,000.

the glycoprotein obtained in these complexes was 30–40%, with the remaining glycoprotein either free in the 150,000*g* supernatant fraction or associated with phospholipid vesicles which did not sediment under conditions of ultracentrifugation.

It is important to note that if the glycoprotein were added to the phospholipid after the lipid had been hydrated, 95% of the lipid was sedimented by low-speed ultracentrifugation. However, there was no glycoprotein associated with the lipid. Furthermore, the glycoprotein did not sediment by ultracentrifugation unless it was associated with lipid.

Table II shows that in the glycoprotein–phospholipid complexes, obtained from either the Triton method or the method of MacDonald and MacDonald (16), greater than 75% of the total sialic acid was released with neuraminidase. This result suggests that there is an asymmetric distribution of sialic acid in the glycoprotein–phospholipid complexes. This result is in contrast to the native enzyme where only approximately 50% of the sialic acid is released. Since Triton X-100 was used to form the phospholipid–protein complexes, it could be argued that the Bio-Beads did not remove all of the Triton and that the results were due to residual Triton which was associated with the complex. To rule out this possibility, Na^+,K^+-ATPase was incubated with 0.0025% Triton X-100 and the percentage of sialic acid removed with neuraminidase was determined. As shown in Table I, the addition of this amount of Triton had little effect on the removal of sialic acid, indicating that the native Na^+,K^+-ATPase vesicles were not leaky to neuraminidase.

Freeze–fracture electron microscopy of the glycoprotein–phospholipid complexes revealed vesicles of 500–1000 Å diameter (Figs. 4D and 6). However, in contrast to the native Na^+,K^+-ATPase (Fig. 2), or to the reconstituted Na^+,K^+-ATPase (Fig. 4), no well-defined intramembranous particles were present. This indicates that the intramembranous particles observed in the native Na^+,K^+-ATPase are due either to the catalytic subunit alone or to the catalytic plus glycoprotein subunits, but not to the glycoprotein alone.

The definitive experiment for the determination of the molecular composition of the intramembranous particles would be the incorporation of the purified catalytic subunit into phospholipid vesicles. Due to the insolubility of the lipid- and detergent-free catalytic subunit, however, this has not yet been accomplished.

There is no direct evidence that the reconstituted glycoprotein is present in these vesicles as an integral membrane protein. Preliminary results of differential scanning calorimetry of the glycoprotein–containing vesicles of dimyristoyl phosphatidylcholine indicate that the protein does not alter the lipid phase transition temperature, but it does significantly reduce the enthalpy change of this phase transition. In the case of glycophorin, this has been taken as a strong indication that the protein is interacting with the lipids by means of hydrophobic forces (31).

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