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RECONSTITUTION OF ($Na^+ + K^+$)-ATPase PROTEOLIPOSOMES HAVING THE SAME TURNOVER RATE AS THE MEMBRANOUS ENZYME

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Membranous ($Na^+ + K^+$)-ATPase from the electric eel was solubilized with 3-((3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (Chaps). 50 to 70% of the solubilized enzyme was reconstituted in egg phospholipid liposomes containing cholesterol by using Chaps. The obtained proteoliposomes consisted of large vesicles with a diameter of 134 ± 24 nm as the major component, and their protein/lipid ratio was 1.25 ± 0.07 g protein/mol phospholipid. The intravesicular volume of these proteoliposomes is too small to consistently sustain the intravesicular concentrations of ligands, especially K^+, during the assay. The decrease in K^+ concentration was cancelled by the addition of 20 μM valinomycin in the assay medium. The low value of the protein/lipid ratio suggests that these proteoliposomes contain one Na^+/K^+-pump particle with a molecular mass of 280 kDa per one vesicle as the major component. In these proteoliposomes, the specific activity of the ($Na^+ + K^+$)-ATPase reaction was 10 μmol P_i/mg protein per min, and the turnover rate of the ATP-hydrolysis was 3500 min^{-1}, the same as the original enzyme under the same assay condition. The ratio of transported Na^+ to hydrolyzed ATP was 3, the same as that in the red cell. The proteoliposomes could be disintegrated by 40–50 mM Chaps without any significant inactivation. This disintegration of proteoliposomes nearly tripled the ATPase activity compared to the original ones and doubled the specific ATPase activity compared to the membranous enzyme, but the turnover rate was the same as the original proteoliposomes and the membranous enzyme. This disintegration of proteoliposomes by Chaps suggests the selective incorporation of the ($Na^+ + K^+$)-ATPase particle into the liposomes and the asymmetric orientation of the ($Na^+ + K^+$)-ATPase particle in the vesicle.

Introduction

The demonstration of active transport of Na^+ and K^+ in reconstituted ($Na^+ + K^+$)-ATPase proteoliposomes has been considered to be solid evidence that the ($Na^+ + K^+$)-ATPase itself is the Na^+/K^+-pump [1–11]. However, the reported turnover rates of the Na^+-K^+ exchange in the reconstituted vesicles and the associated ATP-hydrolysis are much lower than those of intact cells or the membranous enzyme [8,9]. Jørgensen calculated the quantities of ligand molecules in the intravesicular medium and proposed that this difference in turnover rates occurred through the rapid exhaustion of K^+ in the inside-out vesicles during the enzyme reaction [12].

In this paper, we present an improved method for the reconstitution of ($Na^+ + K^+$)-ATPase into

Abbreviations: Chaps, 3-((3-cholamidopropyl)dimethylammonio)-1-propane-sulfonate; Tes, 2-(tris(hydroxymethyl)-methylamino)ethanesulfonic acid; EP, phosphorylated form of ($Na^+ + K^+$)-ATPase; CDTA, 1,2-cyclohexylenedinitrilotetracetic acid.

liposomes using Chaps and show that the obtained proteoliposomes have a turnover rate of ATP-hydrolysis similar to the original membranous ($Na^+ + K^+$)-ATPase when the K^+ exhaustion in the intravesicular medium is prevented.

Materials and Methods

Liposome preparation. In order to remove inorganic cations and neutral lipids from 'egg lecithin' (Sigma Type IX), 20% (w/v) suspension of 'egg lecithin' in 30 mM Tris-HCl buffer (pH 7.6) was passed through a Dowex 50 (Tris form) column, and then the lipid was extracted with chloroform. The chloroform extract was treated with 10 volumes of acetone, and the precipitated fraction was used as the egg phospholipid in which phosphatidylcholine and phosphatidylethanolamine were the major components and lysophosphatidylethanolamine was a trace component, as identified by TLC. Unless otherwise stated, a homogeneous mixture of egg phospholipid and cholesterol (50 : 8, w/w) was suspended in 25 mM histidine/1 mM EDTA (pH 7.0) containing 50 mg phospholipid/ml. This mixture was sonicated in the presence of mercaptoethanol (10 μl/ml) until it became translucent. The liposomes were obtained in the supernatant after centrifugation (105 000 × *g*, 1 h) of this sonicated suspension.

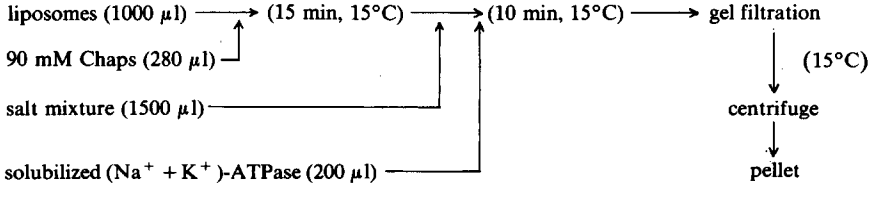
Solubilization of membranous enzyme and reconstitution into proteoliposomes. The ($Na^+ + K^+$)-ATPase-rich membrane fragments from the electric organ of the electric eel, *Electrophorus electricus* [13] were mixed with Chaps solution to final concentrations of 0.5 M NaCl/9 mM Chaps/25 mM histidine/1 mM EDTA (pH 7.0)/1.2 mg protein · ml^{-1}. After incubation for 30 min at 15°C, the non-solubilized residue was removed by centrifugation (105 000 × *g*, 1 h).

For reconstitution of ($Na^+ + K^+$)-ATPase proteoliposomes, Enoch and Strittmatter's procedure [14] was modified as follows: The procedure in Scheme 1 was used unless otherwise stated. After 1 ml of the liposomes described above was incubated with 280 μl of 90 mM Chaps for 15 min at 15°C, 1500 μl of 130 mM NaCl/25 mM histidine/1 mM EDTA (pH 7.0) was mixed, and the solubilized ($Na^+ + K^+$)-ATPase solution (200 μl) was then immediately added. After incubation for 10 min at 15°C, the mixture was applied to a Sephadex G-25 column (1.5 × 30 cm) which had been equilibrated with an eluent, 100 mM NaCl/25 mM histidine/1 mM EDTA (pH 7.0). The reconstituted ($Na^+ + K^+$)-ATPase proteoliposomes were obtained as a pellet from the cloudy fraction of the eluent by centrifugation (105 000 × *g*, 1 h).

Penetration of ^{22}Na into the obtained proteoliposomes was very slow. In 1 h at 0°C or 30°C, less than 10% of the equilibrated amount of Na^+ was found in the intravesicular medium and after incubation for 18 h at 0°C, the penetration of Na^+ reached a plateau.

Preparation for electron microscopy. Negative staining was performed with 2% ammonium molybdate (pH 5.6). One drop of liposome or proteoliposome suspension was placed on a parlodion-coated grid. Immediately after removal of this drop using filter paper, a drop of staining solution was added. This drop was drained off with filter paper, and the preparation was allowed to dry.

Proteoliposomes which had been prepared using Tes buffer (pH 7.4) containing 90 mM NaCl, 10 mM KCl, and 3 mM $MgCl_2$ instead of histidine-EDTA buffer were fixed as a pellet with 2% glutaraldehyde in 25 mM Tes buffer (pH 7.4) containing the above salts for 1 h. The pellet was rinsed with plain buffer and then post-fixed with



Scheme 1. Reconstitution of ($Na^+ + K^+$)-ATPase into liposomes.

2% OsO_4 in the same Tes buffer. Ultra-thin sections were stained with uranyl acetate and lead acetate, and photographed on either a Philips EM410 or a JEOL 100 CX electron microscope.

Assay Procedure. In this study, the concentration of NaCl plus KCl was kept at 100 mM or 110 mM, and the initial concentration of each salt was kept the same in both extravesicular and intravesicular mediums. Magnesium ion and ATP were present only in the extravesicular medium *. As described below, 20 μM valinomycin, a K^+-specific ionophore, was added to the reaction medium to maintain a constant K^+ level inside the vesicle. In order to equilibrate the intravesicular medium to the suitable salt solution, the pellets of proteoliposomes obtained from 1 ml of liposomes were soaked in 1 ml of that salt solution overnight at 0°C. This suspension was directly used for the following assays. In all assays, the reaction time was controlled by a semi-rapid mixing apparatus as previously reported [15].

($Na^+ + K^+$)-ATPase. The ($Na^+ + K^+$)-ATPase activity in the proteoliposomes was measured using [γ-^{32}P]ATP in the presence of 20 μM valinomycin by the phosphomolybdate-extraction method [16] under three conditions: with and without 0.5 mM ouabain for the inside-out proteoliposomes and the inside-out plus unsealed proteoliposomes, respectively, and with 0.2 mM digitoxigenin as the control experiment.

One ml of the typical reaction mixture for the ($Na^+ + K^+$)-ATPase assay contained 50 or 100 μl of the proteoliposome suspension/0.5 mM [γ-^{32}P]ATP/70 mM NaCl/30 mM KCl/20 μM valinomycin/25 mM histidine/5 mM CDTA/10 mM $MgCl_2$/0.5 mM ouabain or 0.2 mM digitoxigenin. The reaction was initiated by the addition of $MgCl_2$, and after 30 s incubation at 37°C, it was quenched with the mixture of $HClO_4/Na_2MoO_4$. The formed phosphomolybdate was extracted with butyl acetate, and its aliquot was counted.

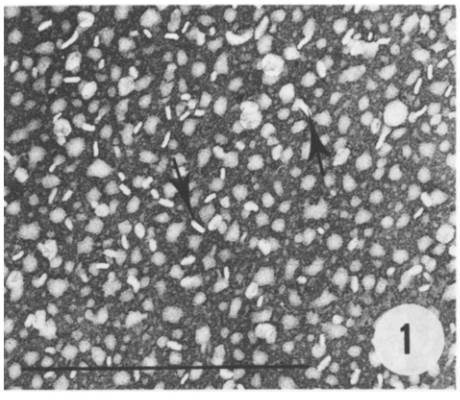
Na^+ transport. For the Na^+-transport assay, the reaction condition was similar to that for the ($Na^+ + K^+$)-ATPase assay, except for using ^{22}Na-labeled ($2 \cdot 10^6$ cpm) 70 mM NaCl and 1 mM ATP instead of unlabeled 70 mM NaCl and 0.5 mM [γ-^{32}P]ATP, respectively. The reaction was quenched by the addition of 60 mM $Tris_3$CDTA after the 30-s incubation. The reaction mixture was passed through a Dowex 50 (Tris form) column, and ^{22}Na inside the proteoliposomes was counted. For the control experiment, the same procedures were performed in the absence of ATP.

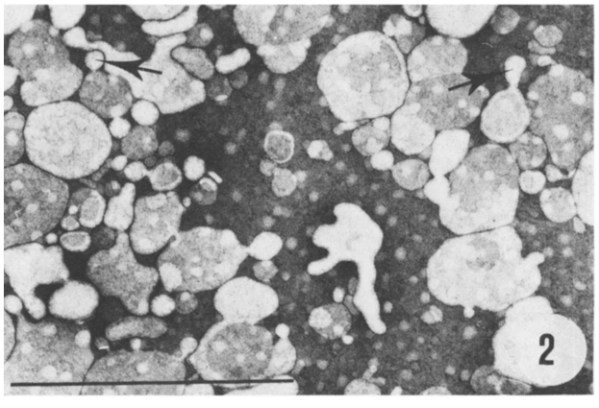
EP level. Assay of the EP was performed by the modified method as previously reported [15]. 1 ml of the phosphorylation medium contained 100 μl of the proteoliposome suspension/100 mM NaCl/10 mM $MgCl_2$/25 mM histidine/5 mM CDTA/2 μM [γ-^{32}P]ATP (pH 7.0). The reaction was initiated by the addition of $MgCl_2$ and quenched with trichloroacetic acid after a 2-s reaction at 25°C. In order to completely precipitate the EP, 0.1 ml of 0.3% bovine serum albumin per 1 ml of the phosphorylation medium was also added simultaneously with the quenching solution. The precipitate of EP in an aliquot of the reaction mixture was collected on a Millipore filter (pore size 0.45 μm) and counted after extensive washing with cold 0.5% trichloroacetic acid/1 mM P_i/0.1 mM ATP. For the control experiment, 2 mM KCl/0.2 mM ADP/20 μM valinomycin was added to the phosphorylation medium.

Protein and phospholipid analysis. Protein in the proteoliposomes was measured by the method of Neuhoff et al. [17] using benzoxanthene yellow H-2495. In the range of 10 ng to 2 μg, the error in the results was 10%, and 85% to 107% of the bovine serum albumin was recovered as the inner standard of the proteoliposomes, with the presence of 5 mg lipid having insignificant effect. Modified methods of Lowry's procedure were used for the other proteoliposomes [4,7,8,10], but these methods were not suitable for the present case due to the presence of large amounts of phospholipid and the low sensitivity of these protein assays.

Phospholipid-P was assayed by the similar method described in the ATPase assay. After digestion of the proteoliposomes or liposomes with $HClO_4/HNO_3$, Na_2MoO_4 and butyl acetate were mixed, and absorbance of the butyl acetate layer

* These concentrations of salts and ATP were not optimum for the ($Na^+ + K^+$)-ATPase reactions, but the higher salt concentrations reduced the yield of proteoliposome formation and the optimum ATP concentration (2–5 mM) increased the blank value in the ATPase assay.





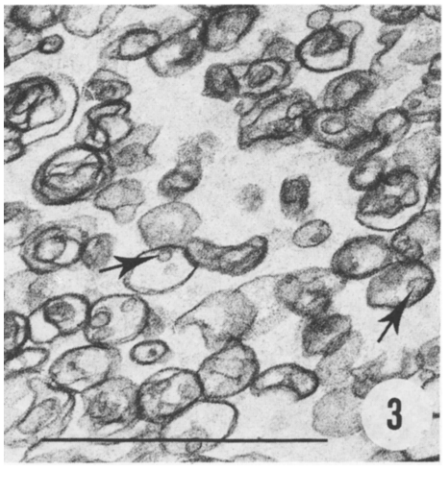


Fig. 1. Electron microscopy of liposomes and proteoliposomes. The preparative methods of the specimen are described in the text. Each bar = 0.5 μm, magnification 73600×. (1) Electron micrograph of negatively stained liposomes. Many of the liposomes are seen en face; however, there are a significant number of others that appear edge on, flattened during the staining process (arrows). (2) Electron micrograph of negatively stained proteoliposomes. There appear to be two major classes of vesicles, small ones having the same dimensions as liposomes seen in Fig. 1-1 and much larger vesicles. At places around the perimeter of many of the larger vesicles, there are small vesicles that appear to have fused with the larger ones (arrows). (3) Electron micrograph of a thin section from a pellet of proteoliposome fixed in sequence with glutaraldehyde and osmium tetraoxide. As in Fig. 1-2, there are two major classes of vesicles. The smaller vesicles (arrows) do not appear to be attached to the larger ones and are more often found within them.

was read at 340 nm for 10–200 nmol phosphorus, or at 400 nm for 0.1–2.0 μmol. Since the proteoliposome or liposome samples except for the phospholipid, contained an insignificant amount of phosphorus, the total phosphorus value equaled the lipid-phosphorus value in these samples.

Results

Electron microscopy

In negatively stained preparations, phospholipid liposomes appeared as either disks or rods (arrows, Fig. 1-1). We believe that the rods and disks are side and face views, respectively, of the same structure, a sphere that was compressed during negative staining. Since the average diameter of the disks was 34 ± 4 nm, which was the same length as the rods, the diameter of the original sphere would be 24 ± 3 nm, which is in agreement with the observed values of other investigators [14].

Preparations of $(Na^+ + K^+)$-ATPase proteoliposomes, either negatively stained or fixed with glutaraldehyde and thin sectioned, are characterized by two classes of vesicles. The larger vesicles appeared as circles with a diameter of 134 ± 24 nm in the negatively stained preparations (Fig. 1-2) or as ellipses with a major ax of 168 ± 25 nm and a minor ax of 116 ± 12 nm in the thin-sectioned preparations (Fig. 1-3). Many of the smaller vesicles with diameters of 24 nm appear to be fusing with the larger ones in negatively stained preparations (arrows, Fig. 1-2) or to be seen inside the larger ones in the thin-sectioned preparations (arrows, Fig. 1-3).

Time-course of ATP hydrolysis

As shown in Fig. 2, the ATP-hydrolysis by

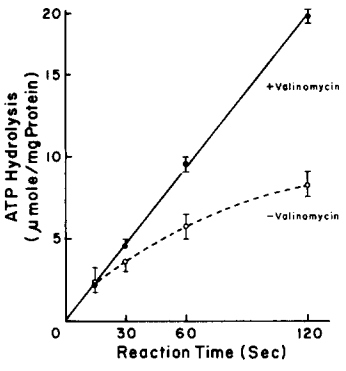


Fig. 2. Time-course of ATP-hydrolysis by proteoliposomes. After $(Na^+ + K^+)$-ATPase proteoliposomes containing 70 mM NaCl/30 mM KCl/25 mM histidine/1 mM EDTA as the internal medium were preincubated in the medium lacking Mg^{2+} with 0.5 mM ouabain or 0.2 mM digitoxigenin for 3 min at 37°C, the enzyme reaction was started by the injection of 10 mM $MgCl_2$. The final concentrations of ligands in the reaction medium were 70 mM NaCl/30 mM KCl/25 mM histidine/5 mM CDTA/0.5 mM [^{32}P]ATP/10 mM $MgCl_2$/0.5 mM ouabain or 0.2 mM digitoxigenin/0.25–0.5 μg of the proteoliposomal protein. The reaction was terminated with 24% $HClO_4$/7% Na_2MoO_4. Assay of the ^{32}P-containing phosphomolybdate is referred to in the text. The difference in P_i formation in the presence of ouabain from that in the presence of digitoxigenin was calculated from triplet assays under both conditions and is shown in this figure. The lines show the results using the medium with or without 20 μM valinomycin, respectively.

proteoliposomes was nonlinear against the reaction time. The addition of 20 μM valinomycin (a potassium-specific ionophore), however, made this reaction linear, at least for 2 min, and the slope turned out to be the same as the initial rate of ATP-hydrolysis in the absence of valinomycin. This result suggests that the decrease of K^+ concentration inside the vesicle (in the extracellular medium) causes the slowdown of ATP hydrolysis by proteoliposomes, and the presence of valinomycin supplies the potassium ion from the outside to the inside of vesicles. In this study, the $(Na^+ + K^+)$-ATPase activity in the presence of 20 μM valinomycin is used as '$(Na^+ + K^+)$-ATPase activity'. The change in the valinomycin concentration in this experiment from 10 to 40 μM did not influence $(Na^+ + K^+)$-ATPase activity (data not shown). In the presence of valinomycin, the ratio of the Na^+-ATPase to the $(Na^+ + K^+)$-ATPase of $(Na^+ + K^+)$-ATPase proteoliposomes was less than 10% with the 30–180 s assay, similar to the case of the membranous enzyme (see Table I). The absence of valinomycin reduced the $(Na^+ + K^+)$-ATPase activity with the assay period (e.g., from the initial rate of 10 μmol P_i/min per mg protein to 3.6 μmol P_i/min per mg protein with a 2-min assay). Therefore, the ratio of the Na^+-ATPase to the $(Na^+ + K^+)$-ATPase was increased to more

TABLE I

COMPARISON OF PROTEOLIPOSOMAL $(Na^+ + K^+)$ATPase WITH MEMBRANOUS AND Chaps-SOLUBILIZED PREPARATIONS

The number in each parenthesis shows the number of the sets of experiments. The reaction medium also contained 10 mM $MgCl_2$/25 mM histidine/5 mM CDTA in addition to the list in all experiments. The details of these assays are described in the text.

	Ligands in reaction medium			Proteoliposomal preparation [a]	Membranous preparation	Chaps-solubilized preparation
	NaCl (mM)	KCl (mM)	ATP (mM)			
ATPase (μmol P_i/min per mg protein at 37°C)	100	10	0.5 [b]	9.0–11.3 (8)	13.5–14.9 (2)	9.0–11.0 (3)
	100	0	0.5	0.74 (2)	0.19 (2)	–
EP (nmol/mg protein)	100	0	0.002	2.2–3.2 (3)	3.5–4.2 (2)	3.4 (1)
Turnover rate [c] (min^{-1})				3590–3670 (3)	3350–3620 (2)	3450 (1)

[a] The intravesicular medium was 100 mM NaCl/25 mM histidine/1 mM EDTA.

[b] 20 μM valinomycin was also present.

[c] Ratio of $(Na^+ + K^+)$-ATPase activity in 100 mM NaCl/10 mM KCl/0.5 mM ATP to the EP in the 100 mM NaCl/0.002 mM ATP.

than 20% with a 2-min assay and to more than 80% with a 10-min assay in the case of proteoliposomes if valinomycin was absent. These results suggest that the decrease in K^+ concentration inside the vesicles lowers the values of the (Na^+ + K^+)-ATPase activities in the proteoliposomes.

Effects of cholesterol on reconstitution

The preliminary addition of cholesterol into liposomes proved to be essential in obtaining active ($Na^+ + K^+$)-ATPase proteoliposomes under the present procedure. Ergosterol only partially replaced this cholesterol (data not shown). As shown in Fig. 3, the addition of cholesterol not only increased the incorporation of protein into liposomes but also reduced the level of unsealed proteoliposomes, but the excess cholesterol in the liposomes (1.0%) reduced the incorporation of the protein. As a result, we used the liposomes formed from 50 mg/ml egg phospholipid and 0.8 mg/ml cholesterol in this study.

Na^+ transport

In the preliminary experiments, the addition of 0.5 ml of 60 mM $Tris_3CDTA$ into 1 ml of the transporting mixture quenched the Na^+ transport within 0.5 s, and the ^{22}Na content in the vesicle remained unchanged for 1 h at 37°C or 0°C. Fig. 4 shows the time-course of Na^+ transport in (Na^+ + K^+)-ATPase proteoliposomes. Similar to the (Na^+ + K^+)-ATPase reaction, ATP-dependent Na^+ transport by the proteoliposomes decreased with the reaction time but was kept constant in the presence of valinomycin. Therefore, we measured Na^+ transport in the presence of 20 μM valinomycin.

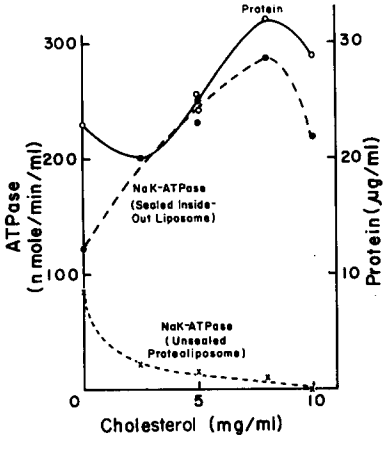


Fig. 3. Effect of cholesterol in liposomes on the ($Na^+ + K^+$)-ATPase proteoliposomes. The liposomes containing the calculated amount of cholesterol were prepared and reconstitution was performed as described in the text. Assay of the (Na^+ + K^+)-ATPase reaction in inside-out proteoliposomes was performed in 100 mM NaCl/10 mM KCl/10 mM $MgCl_2$/0.5 mM [^{32}P]ATP/25 mM histidine/5 mM CDTA/0.5 mM ouabain/20 μM valinomycin (pH 7.0) as the external medium and 100 mM NaCl/25 mM histidine/1 mM EDTA (pH 7.0) as the internal medium. The ($Na^+ + K^+$)-ATPase activity in unsealed proteoliposomes was calculated by subtracting the value for the inside-out proteoliposomes from the value obtained in the absence of ouabain. The details of these ATPase assays and the protein assay are described in the text. Each point denotes the mean value of triple assays and expresses the value against 1 ml liposomes used for the proteoliposome reconstitution.

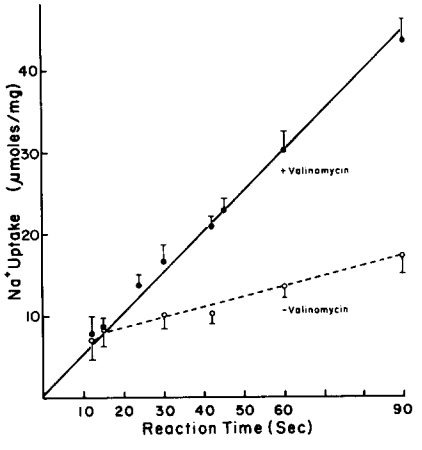


Fig. 4. Time-course of Na^+ transport. After ($Na^+ + K^+$)-ATPase proteoliposomes containing 0.3–0.6 μg protein and 70 mM NaCl/30 mM KCl/25 mM histidine/1 mM EDTA as the internal medium were preincubated in the medium lacking Mg^{2+} for 3 min at 37°C, transport was started by injection of 10 mM $MgCl_2$. The final concentrations of ligands in the reaction medium were 70 mM ^{22}Na-NaCl/30 mM KCl/25 mM histidine/5 mM CDTA/10 mM $MgCl_2$ with or without 1 mM ATP. The reaction was terminated with 0.5 ml of 60 mM $Tris_3CDTA$, and the aliquot was applied to a Dowex 50 column to separate the ^{22}Na in vesicle from that in the external medium. The experimental details are described in the text. The difference in ^{22}Na in vesicles with ATP from that without ATP was calculated from triplet assays under both conditions and is shown in this figure. The lines show the results using the mediums with or without 20 μM valinomycin, respectively.

Yield of the proteoliposomes and their characteristics

The incorporation of solubilized enzyme into liposomes by this procedure was very efficient. From 300 μg of membranous $(Na^+ + K^+)$-ATPase, 70–90 μg of the solubilized enzyme was obtained. Using solubilized enzyme treated in this way and 1 ml of the liposome suspension containing 57 μmol lipid and 8 mg cholesterol, proteoliposomes containing 40–54 μg protein and 31 to 43 μmol lipid-P were obtained according to Scheme I.

Although the specific activity of the proteoliposomal $(Na^+ + K^+)$-ATPase reconstituted by the cholate-dialysis method was only 7% of the starting enzyme [10], that of the present proteoliposomes was higher than the Chaps-solubilized or membranous enzymes, considering that the ATPase activity of only the inside-out proteoliposomes was measured (Table I). However, the turnover rate *, the ratio of $(Na^+ + K^+)$-ATPase activity to the EP level of proteoliposomes, was almost the same as that of the original membranous enzyme.

When NaCl and KCl concentrations were 70 mM and 30 mM, respectively, in the extravesicular and intravesicular mediums, the ATP-dependent Na^+ transport was 30.1–33.2 μmol Na^+/mg protein per min, and the ratio * of Na^+ transport against the ATP-hydrolysis * was 2.8–3.1. On the other hand, the ratio of protein/lipid * in the proteoliposomes was very low (1.17–1.32) compared to the other findings [8,11].

Chaps treatment of proteoliposomes

After treatment of proteoliposomes with various concentrations of Chaps in the presence of 0.5 M NaCl at 0°C, $(Na^+ + K^+)$-ATPase activities and EP levels were measured within 10 min. Concentrations of Chaps up to 50 mM continually increased the $(Na^+ + K^+)$-ATPase activity, and in this range, the increase in EP level was almost parallel to the ATPase activity (Fig. 5). If there was more than 50 mM Chaps, the ATPase activity decreased with the concentration of Chaps.

Moreover, this Chaps treatment of proteoliposomes changed the ouabain sensitivity of the $(Na^+ + K^+)$-ATPase reactions. Without Chaps treatment, the ATPase of proteoliposomes was inhibited less than 10% with 0.5 mM ouabain, whereas the treatment with 15 mM Chaps caused 60% inhibition of $(Na^+ + K^+)$-ATPase activity with 0.1 mM ouabain. Treatment with more than 25 mM Chaps caused 95% inhibition of $(Na^+ + K^+)$-ATPase activity with 0.1 mM ouabain.

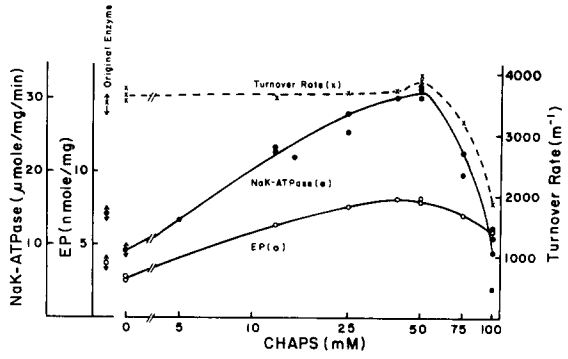


Fig. 5. Chaps treatment of $(Na^+ + K^+)$-ATPase proteoliposomes. The proteoliposome suspension (30 μg protein/ml) containing 100 mM NaCl/25 mM histidine/1 mM EDTA as the intravesicular medium was mixed with an equivolume of 1 M NaCl/25 mM histidine/1 mM EDTA (pH 7.0) containing CHAPS as cited in the figure. After a 2–10 min treatment at 0°C, 0.2 ml of the mixture was diluted with 0.7 ml of the $(Na^+ + K^+)$-ATPase assay medium or the phosphorylation medium lacking Mg^{2+}, and ATP-hydrolysis or phosphorylation was started by the addition of 0.1 ml of 0.1 M $MgCl_2$. The assay procedures are described in the text. Each point denotes the mean value of triple assays in each set. The turnover rate, the ratio of $(Na^+ + K^+)$-ATPase activity to EP level, was calculated from both assay results obtained from the same sample.

These results indicate that:

(1) the Chaps treatment of proteoliposomes probably disintegrates the proteoliposomes and increases the accessibility of ligands to their sites on the $(Na^+ + K^+)$-ATPase molecule;

(2) the reconstitution and disintegration of the $(Na^+ + K^+)$-ATPase proteoliposomes by Chaps does not harm the $(Na^+ + K^+)$-ATPase molecule since there were no decreases in either the turnover rate of ATP hydrolysis or in its specific ATPase activity; actually, there was an enhancement of the specific activity.

* The turnover rate, the ratio of transported Na^+ to hydrolyzed ATP, and the protein/lipid ratio were calculated from the results obtained from the same samples.

Discussion

The $(Na^+ + K^+)$-ATPase has been incorporated into liposomes using several procedures. Among them, a cholate-dialysis procedure is the most widely used [1,2,4,5,7,8,10] for reconstitution of other membrane enzymes, and a freeze-thaw sonication method has been used in several studies [6,9,11]. The present reconstitution procedure is an application of Enoch and Strittmatter's method for large liposome formation [12]. The use of Chaps instead of cholate or deoxycholate permits reconstitution without serious inactivation of the enzyme and provides proteoliposomes with diameters of 134 ± 23 nm.

As Jørgensen has calculated, the decrease of K^+ concentration in the intravesicular medium by the ATPase reaction causes serious problems for measuring ATPase activity and Na^+ transport in proteoliposomes with such a small size. When the K^+ concentration is 10 mM, the K^+ content in the intravesicular medium of the present proteoliposomes is only 3000–10000 ions in each vesicle (Fig. 6). Since the turnover rate of $(Na^+ + K^+)$-ATPase is reported to be 8500 min^{-1} under the optimal condition [12], and two potassium ions are transported during the hydrolysis of one ATP molecule, the suitable assay period is only 1.5–6.0 s for the proteoliposomes containing one Na^+/K^+-pump particle in each vesicle, and after that, these activities are reduced. The actual time course of ATP-hydrolysis and Na^+ transport in proteoliposomes (Figs. 2 and 4) supported this estimation even though suboptimal conditions for the Na^+/K^+-pump were used (the turnover rate was 3600 min^{-1}). The difficulties with such short period assays can be overcome by the addition of valinomycin into the reaction medium (valinomycin doesnot influence the $(Na^+ + K^+)$-ATPase activity of the membranous preparation). Karlish and Pick [9] reported the activation of ATP-dependent ^{22}Na-uptake into the $(Na^+ + K^+)$-ATPase proteoliposomes by valinomycin plus *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone, but they did not employ these ionophores for the assays of the proteosomal $(Na^+ + K^+)$-ATPase activity or the Na^+ transport.

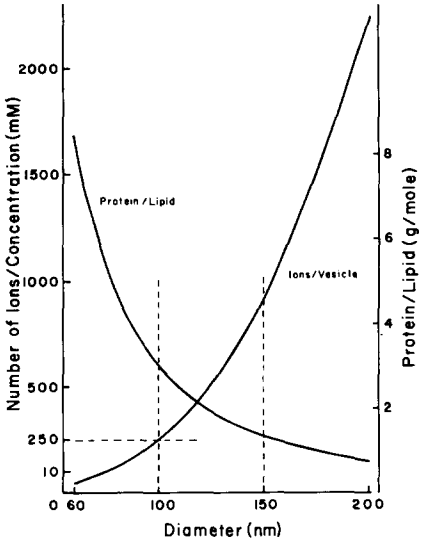


Fig. 6. Number of intravesicular ions per vesicle and ratio of protein against phospholipid calculated as a function of the diameter of a $(Na^+ + K^+)$-ATPase proteoliposome. Calculations are based on bilayer thickness of 3.7 nm, a phospholipid specific volume of 1.253 nm^3/molecule [24], a molecular mass of 280 kDa per Na^+/K^+-pump particle [12].

Precise comparison of the enzyme activities of the present proteoliposomes with the values previously reporeted is difficult because these previous studies were performed without care of the ligand exhaustion in the intravesicular medium. But the specific activity of the present $(Na^+ + K^+)$-ATPase proteoliposomes is 10 μmol P_i/mg protein per min; this value is about 12-times more than the reported value of 0.8 μmol/mg per min [10]. The turnover rate of $(Na^+ + K^+)$-ATPase proteoliposomes (the ratio of $(Na^+ + K^+)$-ATPase/EP) in the presence of valinomycin had similar values to that of the membranous $(Na^+ + K^+)$-ATPase. When the concentrations of Na^+ and K^+ are the same between the intravesicular and extravesicular sides, Na^+ transport by the proteoliposomes was more than 30 μmol Na^+/min per mg protein and was 10-times more active than the reported values [4,6,8,9]. Furthermore, the ratio of the transported sodium ion to the hydrolyzed ATP was about 3, the same as the previously reported values in the red cell ghost [18–21]. Finally, the $(Na^+ + K^+)$-ATPase proteoliposomes can be disintegrated into the membranous enzyme or into the leaky vesicles by Chaps and in both instances the turnover rate was similar to that of the original membranous enzyme.

The specific activity of the $(Na^+ + K^+)$-ATPase reaction in the proteoliposomes is about 60% of the original membranous preparation. The specific activity in proteoliposomes disintegrated with 40–50 mM Chaps is near two-times that of the membranous preparation or three times that of the proteoliposomes. This result suggests that the $(Na^+ + K^+)$-ATPase particles in the solubilized preparation are selectively incorporated into the liposomes just as they are during sarcoplasmic reticulum reconstitution [22], and their orientation may be asymmetrically arranged in the inside-in form, contrary to a previous report [4,8,10]. This asymmetric orientation of the $(Na^+ + K^+)$-ATPase particle may be related to the preferential orientation of the hydrophilic glycoprotein part on the outside of the vesicle as in the case of cytochrome oxidase [23].

Another characteristic of these proteoliposomes is the low value of the protein/lipid ratio, 1.17–1.32 g protein/mol lipid. The reported values are 1.5–50.2 in the proteoliposomes by cholate-dialysis [8], and 3–9 in those prepared with the freeze-thaw sonication procedure [11]. As described above, one Na^+/K^+-pump particle can transport about 280 potassium ions per second under optimum conditions so that each proteoliposome vesicle must contain more than 2500 potassium ions for the 1-s assay. Therefore, the diameter of the proteoliposome should be more than 100 nm if the K^+ concentration is to be 10 mM (Fig. 6). The larger uniform proteoliposomes are more suitable for studying the Na^+/K^+-pump, but the diameter of the available $(Na^+ + K^+)$-ATPase proteoliposomes is less than 150 nm. In this diameter range, the protein/lipid ratio is between 3 and 1 g protein/mol lipid. Contamination with small starting liposomes, which is revealed in the present preparation by electron microscopy, as well as large liposomes which do not contain $(Na^+ + K^+)$-ATPase particles, is unavoidable, and both undoubtedly reduce the protein/lipid ratio even further.

Unless denatured enzyme is also contained in the proteoliposomes, high values of the protein/lipid ratio suggest that either there is more than one $(Na^+ + K^+)$-ATPase particle per vesicle or the size of the vesicles is relatively small. Under these circumstances:

(1) the exhaustion speed of the intravesicular ligand becomes too rapid;

(2) the Na^+ inside the vesicle might overaccumulate as a result;

(3) the rate of ATP-hydrolysis and Na^+ transport might decrease rapidly during their assays.

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