

Purification and Proteolysis of Vesicles Containing Inside-out and Right-side-out Oriented Reconstituted (Na⁺,K⁺)-ATPase*

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The (Na⁺,K⁺)-ATPase from dog kidney has been reconstituted into egg lecithin vesicles (Goldin, S. M. (1977) *J. Biol. Chem.* 252, 5630-5642). Using sucrose density gradient centrifugation, we have isolated sealed vesicle populations in which the protein molecules have defined orientations. Sealed vesicles sedimented at higher density than unsealed vesicles after equilibration with CsCl. Vesicles containing inside-out oriented enzyme sedimented at lower density than vesicles containing right-side-out oriented enzyme after the internally trapped Cs⁺ had been pumped out during an incubation with Mg²⁺ and ATP. Pools of gradient fractions representing unsealed vesicles and sealed vesicles containing inside-out and right-side-out oriented protein were characterized with respect to orientation and degree of sealing by determination of the ATPase activity, the rate of ATP-dependent Na⁺ uptake, and the inhibition of ATPase activity by ouabain. The accessibilities of sialic acid and of a tryptic site in the vesicle populations were in agreement with the proposed orientations of the protein. The structure of the reconstituted (Na⁺,K⁺)-ATPase was examined by proteolysis with trypsin and chymotrypsin over a range of reconstitution protocols. The fragmentation patterns demonstrate that the cholate-reconstituted enzyme, although functionally competent, differs in structure from the native purified enzyme.

The (Na⁺,K⁺)-ATPase (1, 2) is an integral membrane protein that couples the active transport of Na⁺ and K⁺ across the plasma membrane to the hydrolysis of ATP. All active enzyme preparations consist of two polypeptides, an α chain of $M_r = 100,000$ and a β chain of $M_r = 60,000$.¹ The α chain spans the lipid bilayer because it contains the active site for ATP hydrolysis and the extracellular binding site for ouabain (4, 5). The β chain is exposed extracellularly because it is a glycoprotein. Using the human red blood cell, Sen and Post (6) measured a stoichiometry of 3 Na⁺ and 2 K⁺ transported for each ATP hydrolyzed. Using the purified enzyme from dog kidney, Goldin (7) observed the same stoichiometry of 3 Na⁺/2 K⁺/1 ATP after reconstituting the enzyme into egg lecithin vesicles.

Structural study of the topology of the α and β subunits of the (Na⁺,K⁺)-ATPase requires not only the ability to identify a polypeptide as a component of the ATPase but also the ability to selectively probe the cytoplasmic or extracellular side of membrane-embedded polypeptides. Previous work in this area (8) was carried out using red blood cell ghosts. The red cell membrane was manipulated to produce sealed vesicles that exposed only the cytoplasmic side of the lipid bilayer; the extracellular side was inaccessible from the external medium in the absence of detergents. Since the (Na⁺,K⁺)-ATPase represented an extremely low percentage of the total membrane protein of the red cell (100-500 copies/cell (9)), it was essential to use ouabain-stimulated phosphorylation of the α subunit as a labeling procedure of sufficient sensitivity and selectivity. The necessity for such a label placed restrictions on how much information could be extracted. Thus, analysis in the red cell ghosts was limited to those proteolytic fragments that contained covalently bound ³²P. In order to examine the orientations² of the α and β subunits using proteolysis, one requires a preparation of sided vesicles containing purified (Na⁺,K⁺)-ATPase.

From a microsomal fraction of mammalian kidney, Forbush (10) has isolated membrane vesicles with characteristics consistent with a right-side-out orientation of the ATPase. These vesicles are relatively impermeable to small molecules and, therefore, protect the cytoplasmic side of the protein from external probes. The (Na⁺,K⁺)-ATPase constitutes as much as 50% of the protein in this fraction, based on specific activity. This system is an attractive candidate for studying the cytoplasmic and extracellular locations of portions of the α and β subunits.

In this work we report an alternative approach to the goal of obtaining sealed vesicles possessing (Na⁺,K⁺)-ATPase of defined orientation. Phospholipid vesicles containing inside-out oriented ATPase have been separated from vesicles containing right-side-out oriented ATPase. The sedimentation behavior, transport activities, and sensitivity to neuraminidase of the vesicles are consistent with the suggested orientations of the constituent ATPase molecules. The proteolytic fragmentation patterns of the reconstituted enzyme, prepared

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¹ The apparent molecular weights of polypeptides and peptide fragments are assigned according to Castro and Farley (3), based on the electrophoretic mobilities in 10% SDS gels.

² Orientation is used to describe the location of hydrophilic domains of the (Na⁺,K⁺)-ATPase with respect to the inside and outside of phospholipid vesicles. Inside out refers to the arrangement in which the side of the protein that hydrolyzes ATP is accessible from the exterior of the vesicle. In other words, the cytoplasmic side of the protein, normally protected in intact cells, is exposed and no longer protected in vesicles containing inside-out protein. Inside out also means that the extracellular side, normally exposed in intact cells, is now protected. Conversely, right-side-out oriented protein is positioned so that ouabain can bind but ATP cannot, the same way as in intact cells. With these definitions unsealed membranes cannot be said to possess oriented protein since both sides of the protein are exposed to the same compartment.

in a variety of conditions, are different from those of native kidney membrane enzyme.

EXPERIMENTAL PROCEDURES

Materials—Dog kidneys were generously provided by the Cardiovascular Research Department at the Massachusetts General Hospital. (Na^+ , K^+)-ATPase was prepared as purified membranes using the angle rotor method of Jorgensen (11). The final specific activity was 20–30 μmol of ATP/min/mg of protein at 37 °C and was more than 95% ouabain sensitive. Egg lecithin was prepared from fresh egg yolks as previously described (12). Asolecithin (Associated Concentrates) was washed with acetone (13). Bovine phosphatidylserine (P-L Biochemicals) was used without purification. A lipid fraction of dog kidney was prepared following Folch *et al.* (14). Cortex tissue was blended with 19 volumes of $\text{CHCl}_3/\text{CH}_3\text{OH}$, 2/1 (v/v) containing 15 mM 2-mercaptoethanol. The yellow filtrate was extracted with 0.2 volume of 5 mM MgCl_2 and then dried under vacuum. The yield from 21 g wet tissue, was 330 mg of dried phospholipid. All lipids were suspended by vortexing and homogenization in water containing 5 mM 2-mercaptoethanol and stored under nitrogen at –70 °C. Cholic acid (Nutritional Biochemicals) was decolorized with charcoal and crystallized from 95% ethanol. Octyl glucoside (Calbiochem) was used directly. Ultrapure CsCl was from Alfa, and Tris/ATP (vanadate-free) was from Sigma. [2,4- ^3H]cholic acid, octyl [^{14}C]glucoside, [^3H]glucose, $^{22}\text{Na}^+$, and $^{86}\text{Rb}^+$ were from New England Nuclear. $^{137}\text{CsCl}$ was from the Isotope Laboratory at Harvard University. α -Chymotrypsin, 3 times crystallized, and TPCK-trypsin³ were from Worthington. Both proteases were from bovine pancreas. Neuraminidase (type X) was from Sigma.

Assays—All ATPase and transport activities and ouabain inhibition were measured at 23 °C without adjusting the concentration of alkali cation species in the sample, except as noted; those particular cases usually involved adding Na^+ to the cytoplasmic side of the enzyme or adding K^+ /valinomycin to ensure that K^+ was present at the extracellular side of the enzyme. The ATPase activities and ouabain inhibition were measured by the release of $^{32}\text{P}_i$ from [γ - ^{32}P]ATP (12). Phosphorylation of the (Na^+ , K^+)-ATPase with [γ - ^{32}P]ATP in the absence and presence of K^+ was performed as described (7). Transport activity was measured as trapped isotope by separation of vesicles from external solution on a 10-ml Sephadex G-50 (coarse) column (12). Phospholipid was measured as inorganic phosphate after ashing (15), and the average molecular weight of egg lecithin was taken as 700 (7). Detergent remaining after dialysis was measured using radioactive tracer included in the initial buffers. Protein was determined by the method of Lowry *et al.* (16) in the presence of 1% SDS, and controls showed that lipid did not interfere with this measurement. SDS-gel electrophoresis was performed following Laemmli (17), but the samples were not boiled beforehand. Silver staining was carried out as described (18) using 10% unpurified glutaraldehyde (Kodak) and $\text{AgNO}_3/\text{NaOH}/\text{NH}_4\text{OH}$ and citric acid/formalin mixtures. Vesicle size was measured by phosphate assay and [^3H]glucose trapping after gel filtration on Sepharose 2B (7).

Reconstitution—Cholate dialysis vesicles were prepared as described (7). Lipid was solubilized with cholate, and membrane enzyme was added. The mixture was briefly centrifuged, and the supernatant was loaded into a Bio-Fibers (Bio-Rad) (7) dialysis apparatus. Spectrum Hollow Fibers (Fisher) and Vitafiber 3P10 (Amicon) may be adequate replacements for the discontinued Bio-Fibers; in addition, O'Connell (19) has prepared cholate dialysis vesicles using a flotation step after dialysis in a bag. In all of our preparations, the total initial lipid concentration was 20 mg/ml, and the initial concentration of cholate was 2%. Dialysis removed more than 99% of the cholate. For the Cs^+ permeability and density gradient experiments, the reconstitution buffer was 30 mM imidazole (pH 6.8) containing 175 mM CsCl, 175 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. For the preliminary experiments in characterizing the egg lecithin vesicles and in the later proteolysis experiments on unfractionated vesicles, the standard reconstitution buffer was used: 30 mM imidazole (pH 6.8) containing 250 mM sucrose, 30 mM NaCl, 20 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA (7). The concentrations of NaCl and KCl were varied where indicated in the text.

Octyl glucoside dialysis was performed in essentially the same

manner as for cholate, except that in the reconstitutions using 40 and 80 mM octyl glucoside the mixture was not centrifuged before dialysis. These detergent concentrations were insufficient to solubilize the enzyme from the pellet into the supernatant. Less than 0.1% of the initial octyl glucoside remained after dialysis.

Detergent dilution (20) and freeze-thaw sonication (21) were performed essentially as described with the following modifications. The initial lipid was always 20 mg/ml, and initial protein concentration was 0.7 mg/ml. The initial concentration of octyl glucoside before dilution was 2%. Sucrose was omitted from these reconstitution buffers.

Sucrose Gradients—10% buffer was 30 mM imidazole (pH 6.8) containing 292 mM sucrose (10%), 102 mM KCl, 102 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. 0% buffer was 30 mM imidazole (pH 6.8) containing 175 mM KCl, 175 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. 2% buffer was prepared by mixing 1 volume of 10% buffer with 4 volumes of 0% buffer. Gradients were poured from equal volumes of 10 and 2% buffers. The volume of the gradient was adjusted to compensate for sample size so that the total volume was 5.0–5.2 ml. Prior to loading a sample on top of a gradient, the vesicles were cooled to 4 °C and passed down a 1-ml Sephadex G-50 (coarse) column that had been equilibrated with 0% buffer. This exchange of buffers also served to separate the vesicles from ATP and CsCl. The sucrose gradients were centrifuged in a SW 50.1 rotor at 48,000 rpm ($275,000 \times g$) at 3 °C for 320 min. Fractions (5–7 drops) were collected by puncturing the bottom of the tube. For each ATPase or transport assay, 50 μl of a fraction or pool of fractions were used. The amount of cation transported was calculated from radioactive isotope trapping on the basis of estimates of an average external [Na^+] = 130 and 150 mM for the denser and lighter pools of fractions, respectively. The recovery of phospholipid was always at least 90%.

Digestions—Proteolysis with TPCK-trypsin or chymotrypsin was performed as described in the figure legends. Reactions were quenched with soybean trypsin inhibitor or phenylmethanesulfonyl fluoride and then solubilized in SDS-gel sample buffer. For glycosidase treatment, the samples were adjusted to pH 6.0 with 1 N HCl. Neuraminidase (0.8 milliunit/ μg of ATPase) was added, and the mixture was incubated at 23 °C for 45 min. Reactions were quenched by adding SDS-gel sample buffer.

RESULTS

Characterization of Vesicles Containing Reconstituted (Na^+ , K^+)-ATPase—Purified (Na^+ , K^+)-ATPase was reconstituted into unilamellar egg lecithin vesicles by cholate dialysis (7). The average diameter of the vesicles was about 600 Å as determined by gel filtration on Sepharose 2B. The trapped intravesicular volume per mg of phospholipid was 2.5–3.0 μl /mg. This was measured by equilibration with [^3H]glucose, $^{22}\text{Na}^+$, and $^{86}\text{Rb}^+$ and separation of vesicles from external medium by gel filtration on Sephadex G-50. Equilibration of glucose was achieved by including isotopic label with 1 mM glucose carrier in the reconstitution buffer (7) of 30 mM imidazole (pH 6.8) containing 250 mM sucrose, 30 mM NaCl, 20 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. Equilibration of $^{22}\text{Na}^+$ and $^{86}\text{Rb}^+$ required 3 days at 37 °C (12), indicating a low permeability of these vesicles to alkali cations.

The reconstituted ATPase activity was 20–30 μmol /h/mg of protein when measured at 23 °C in the reconstitution buffer. (Under the same conditions, the ATPase activity of the purified enzyme was 200–300 μmol /h/mg of protein.) The addition of ouabain in the absence of detergent inhibited up to 30% of the ATPase activity. Since ouabain and ATP bind to opposite sides of the protein, this suggests the presence of vesicles that are leaky either to ouabain or to ATP, and, therefore, probably do not efficiently retain alkali cations. The stoichiometries of $^{22}\text{Na}^+$ and $^{86}\text{Rb}^+$ transport, when corrected for this percentage of nontransporting hydrolysis, were approximately 3 Na^+ /2 Rb^+ /1 ATP. Addition of cholate not only stimulated the ATPase activity but also increased the percentage of ouabain-inhibitable ATPase activity. This is consistent with a random orientation of the enzyme. In the

³ The abbreviations used are: TPCK-trypsin, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin; SDS, sodium dodecyl sulfate.

absence of detergent, the level of Na^+ -stimulated, K^+ -discharged phosphorylation of the reconstituted enzyme was 36–38% (2 experiments) of that of the purified (Na^+, K^+)-ATPase. Finally, the observed ATP-dependent efflux of equilibrated $^{86}\text{Rb}^+$ was about 60% of the total trapped isotope. This suggests that more than half of the vesicles contain at least one copy of inside-out oriented ATPase that catalyzes the active efflux of internal Rb^+ .

The physical and functional characteristics of our preparation of reconstituted (Na^+, K^+)-ATPase are essentially identical to those measured by Goldin (7). We tentatively describe this preparation as consisting of phospholipid in sealed and leaky vesicles and of protein incorporated into sealed vesicles in both inside-out and right-side-out orientations and incorporated into leaky vesicles. The orientation of the protein in leaky vesicles is undefined because the probes, ATP and ouabain, are not excluded from the vesicle interior. The results presented below support this model and are described in that context.

As the first step in the density separation of vesicles containing right-side-out and inside-out oriented protein, the reconstitution buffer was changed to 30 mM imidazole (pH 6.8) containing 175 mM CsCl, 175 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. The use of CsCl in generating a density change is based on previous work (22–24). The relatively high concentrations of CsCl and NaCl were chosen in order to create a significant shift in density and to support adequate enzymatic activity. The initial protein concentration was lowered to 0.4 mg/ml from 1.0 mg/ml while the egg lecithin and cholate concentrations were maintained at 20 mg/ml. This modification was intended to increase the likelihood that any vesicle containing protein would have only one copy of the ATPase and not multiple copies. The rationale for this approach is presented under "Discussion."

Although the specific activity of the ATPase decreased to 10 $\mu\text{mol/h/mg}$ of protein at 23 °C, because of the ionic conditions, the transport stoichiometry was 2 $\text{Cs}^+/\text{1 ATP}$ hydrolyzed. This is consistent with the ability of Cs^+ to substitute for K^+ (25). The average diameter of the vesicles was 600 Å, and the percentage of protein solubilized in 20 mg/ml of cholate and 20 mg/ml of egg lecithin was 40–50%. These results are identical to those obtained with the Na^+/K^+ /sucrose reconstitution buffer and suggest that these reconstituted vesicles are similar to the original preparation.

The permeability of the vesicles to Cs^+ was monitored over an extended period of time since the subsequent procedures depended on the prolonged maintenance of ion gradients. The results of such an experiment are shown in Fig. 1. After equilibration of the vesicles with $^{137}\text{Cs}^+$, the addition of ATP and Mg^{2+} (at 1) generated an efflux of Cs^+ . About 30% of the initially trapped Cs^+ was pumped out in 2 h. A portion of the incubation mixture was removed after 1 h (at 2). The remaining ATP was separated from the vesicles with less than a 2-fold dilution of protein by passage down a 1-ml Sephadex G-50 column. To ensure that the movement of radioactivity accurately reflected the movement of Cs^+ , the column had been equilibrated in buffer containing 175 mM Cs^+ of the same specific activity as the original incubation medium. The passive collapse of the gradient was followed by the influx of $^{137}\text{Cs}^+$. The gradient established by the ATPase appeared to be stable at 4 °C. It was only slowly discharged on incubation at 37 °C (at 3). Furthermore, the vesicles and protein were still intact and functional since the addition of a second charge of ATP and Mg^{2+} (at 4) reproduced the same percentage of Cs^+ efflux with roughly the same time course.

Separation of Sealed Vesicles—The vesicles were fraction-

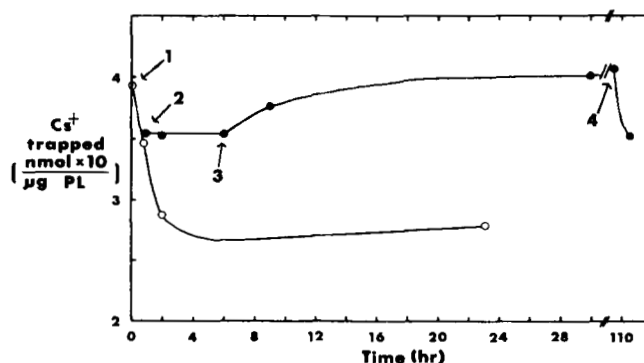


FIG. 1. Active and passive transport of Cs^+ . Reconstituted vesicles (400 μl) that had been equilibrated with $^{137}\text{Cs}^+$ were used to follow the movement of Cs^+ during ATP-supported efflux against a concentration gradient and during passive influx down a concentration gradient. The intravesicular levels of Cs^+ were measured in aliquots of 25 μl as trapped isotope as described under "Experimental Procedures." At 1, Mg^{2+} (7 mM) and ATP (6 mM) were added to the vesicles, and the sample was incubated at 23 °C for 23 h. \circ , represent the changes in internal Cs^+ as a result of active transport. At 2, a portion (300 μl) of the incubation mixture was cooled to 4 °C and loaded on a G-50 column. \bullet , represent the changes in internal Cs^+ in this sample as a result of passive influx, initially at 4 °C. At 3, the vesicles were transferred to 37 °C. At 4, Mg^{2+} (7 mM) and ATP (6 mM) were added to the re-equilibrated vesicles, and active transport was followed. PL, phospholipid.

ated by sucrose density gradient centrifugation. To minimize changes in vesicle size and, therefore, in density, the sucrose gradients were constructed to be iso-osmotic with the high ionic strength reconstitution buffer of 30 mM imidazole (pH 6.8) containing 175 mM CsCl, 175 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. In the gradient buffers, Cs^+ was entirely replaced by K^+ , and, as the sucrose concentration increased, the KCl and NaCl concentrations were correspondingly reduced. The density distribution of the reconstituted (Na^+, K^+)-ATPase vesicles is given in Fig. 2A. Generally, an opaque white band was seen in the gradient at the level of fractions 7–9 while the less dense regions, fractions 11–15, appeared milky, sometimes partially resolved into several white bands and a faintly turbid background. The denser band of phospholipid contained sealed vesicles, and the lighter regions contained unsealed or partially sealed vesicles. In control experiments, if protein was omitted during the reconstitution, the same bimodal distribution of phospholipid was seen. In contrast, if K^+ was substituted for Cs^+ in the reconstitution buffer, these vesicles containing trapped KCl instead of CsCl all sedimented at the level of fractions 14–16 (Fig. 2A) at the top of the gradient. No phospholipid was found in any of the denser fractions of such a gradient. Again, this pattern was not affected by the presence of protein in the reconstitution.

If the vesicles were incubated with ATP and Mg^{2+} prior to centrifugation, some of the phospholipid in fractions 7–9 (Fig. 2A) shifted to the level of fractions 16–19 (Fig. 2B). This behavior suggests that an efflux of Cs^+ , powered by ATP hydrolysis, reduced the density of some of the sealed vesicles. This population of vesicles contained inside-out oriented enzyme. The remainder of the sealed vesicles did not shift because they contained either right-side-out oriented or inactive enzyme. All of the phospholipid-containing fractions of Fig. 2, A and B, also possessed approximately proportional amounts of protein, as judged by silver staining of an SDS gel. This is consistent with the control experiments described above and indicates that the two peaks of phospholipid seen in Fig. 2A do not represent a separation of vesicles containing

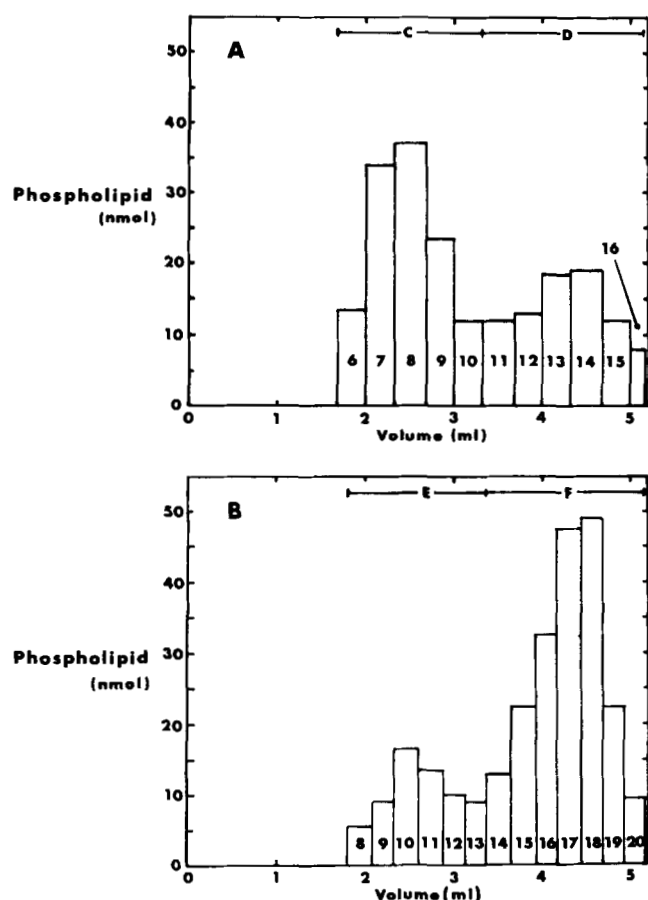


FIG. 2. Phospholipid profiles of sucrose density gradients. The bottom of the gradient is at 0.0 ml, the top at 5.2 ml. Phospholipid was assayed as described under "Experimental Procedures" as nanomoles of P_i in 25 μl of each fraction, and the values were corrected for the background level found in a buffer blank. A, reconstituted vesicles (200 μl) were used directly. B, reconstituted vesicles (200 μl) were preincubated with Mg^{2+} (7 mM) and ATP (6 mM) for 90 min at 23 $^{\circ}\text{C}$.

TABLE I

Transport activity and phospholipid content of gradient fractions

Assays were performed as described under "Experimental Procedures." Values for Na^+ transport are the average of two determinations which were within 10%. Gradient fractions were pooled as shown in Fig. 2, A and B.

Pool	Phospholipid mg	Na^+ uptake		
		+ATP	-ATP	ATP-dependent
C	1.1	2.8	0.3	2.5
D	0.75	0.8	0.3	0.5
E	0.45	0.4	0.4	0.0
F	1.4	2.7	0.4	2.3

protein from vesicles lacking protein.

The fractions from both gradients were collected into pools C, D, E, and F as shown in Fig. 2, A and B, and assayed for phospholipid and ATP-dependent Na^+ uptake. The results are given in Table I. Over half of the sealed vesicles shifted to lighter density after incubation with ATP (compare pools C and E). It can be seen that these vesicles possessed nearly all of the ATP-dependent transport activity, a distinguishing characteristic of inside-out oriented ATPase. Pool D, containing 30–40% of the total phospholipid, displayed a low level of ATP-dependent Na^+ uptake. Since these fractions did contain

protein, this activity is tentatively assigned to partially sealed vesicles; this activity would account for the inhibition of 20–30% of the reconstituted ATPase activity by ouabain in the absence of detergents. It was difficult to determine accurately the degree of sealing and the protein orientation of these partially sealed vesicles, and they were not examined further.

Separation of Sealed Vesicles Containing Inside-out and Right-side-out Oriented (Na^+ , K^+)-ATPase—Since the overlap of partially sealed vesicles and vesicles containing inside-out oriented enzyme undermined the attempt to obtain protein of a restricted orientation, the purification procedure was extended to include two consecutive sucrose density gradients. First, untreated vesicles were centrifuged on a sucrose gradient to produce the phospholipid profile shown in Fig. 3A. The band of sealed vesicles, fractions 8 and 9, was pooled and stored overnight at 4 $^{\circ}\text{C}$. The next day, ATP and Mg^{2+} were added. The vesicles were incubated for 90–120 min at 23 $^{\circ}\text{C}$ and then centrifuged on another sucrose gradient. The phospholipid distribution in the second gradient is shown in Fig. 3B. In control experiments, ATP was omitted from the incubation before the second centrifugation. This resulted in all of the phospholipid appearing at the level of fractions 6–8 in Fig. 3B. Therefore, the movement of vesicles is caused by an ATP-induced efflux of Cs^+ . It is not a result of the sponta-

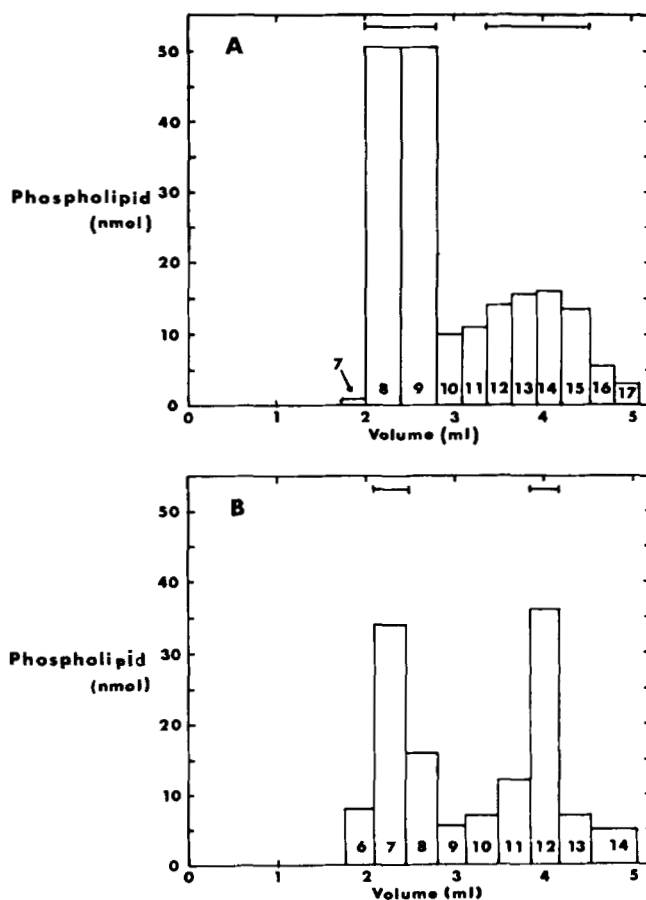


FIG. 3. Phospholipid profiles of consecutive sucrose density gradients. The bottom of the gradient is at 0.0 ml, the top at 5.1 ml. Phospholipid was assayed as described under "Experimental Procedures" as nanomoles of P_i , and values were corrected for a buffer blank. A, reconstituted vesicles (380 μl) were used directly. Phosphate assays were performed on 10 μl of each fraction. B, a portion (500 μl) of the pooled fractions 8 and 9 (from the gradient shown in A) was preincubated with Mg^{2+} (8 mM) and ATP (7 mM) for 90 min at 23 $^{\circ}\text{C}$. Phosphate assays were performed on 20 μl of each fraction.

neous unsealing of some of the vesicles. Nor is it a result of internal Cs⁺ leaking out overnight even though the external medium of the pooled vesicles (fractions 8 and 9 of Fig. 3A) now contains K⁺ and not Cs⁺.

In the second gradient the proportion of vesicles in each peak was 50:50. We suggested that fractions 11–13 contained active inside-out oriented enzyme in sealed vesicles and that fractions 6–8 contained active right-side-out oriented enzyme in sealed vesicles. It was also possible that the latter vesicle population contained inactive enzyme.

The four peaks of turbidity from these two gradients were pooled as shown in Fig. 3, A and B, and assayed for Na⁺ transport, ATPase activity, and ouabain inhibition of ATP hydrolysis. The results are given in Table II. In the first gradient, the pool of partially sealed vesicles, fractions 12–15, exhibited a relatively low stoichiometry of Na⁺/ATP = 0.6, and virtually all of the ATPase activity was inhibited by ouabain. This implies that ouabain bound to the extracellular side of enzyme molecules that were also binding and hydrolyzing ATP on their cytoplasmic sides. Clearly, these vesicles must be leaky to either ouabain or ATP, if not both. The pool of sealed vesicles, fractions 8 and 9, showed a coupling of Na⁺/ATP = 1.8, and very little of the ATPase activity was inhibited by ouabain. This implies that few enzyme molecules are capable of binding ATP and ouabain simultaneously and, therefore, that the vesicles are sealed with respect to both ATP and ouabain. These data are in agreement with the expectation that ATP hydrolysis that produces an observable Na⁺ accumulation should be resistant to ouabain. It is unclear to us why the stoichiometry in the sealed vesicle pool is not closer to 3.0.

In the second gradient, the vesicle population of lighter density possessed almost all of the Na⁺ transport and ATP hydrolysis activities. The tight coupling of Na⁺/ATP = 3.0 and the resistance to ouabain suggest that all of the active enzyme molecules are oriented inside out in sealed vesicles. The denser vesicles displayed very low levels of ATPase and Na⁺ transport activities. It was not possible to demonstrate the existence of active right-side-out oriented enzyme. Under various conditions, the addition of cholate always inhibited ATPase activity, probably because the enzyme was being denatured. A similar inhibition of ATPase activity was obtained on adding cholate to the lighter vesicles. This made it difficult to demonstrate a cholate stimulation of the ouabain inhibition of the ATPase activity, as would be predicted for inside-out oriented enzyme. Evidence for the presence of active right-side-out oriented enzyme comes from the cholate stimulation of ATPase activity observed in undiluted unfractionated vesicles. Also, that the inside-out oriented protein

still hydrolyzes ATP and transports Na⁺ indicates that the fractionation procedure does not inactivate the (Na⁺,K⁺)-ATPase.

Although the measured specific activities of ATP hydrolysis decrease during the purification, it should be noted that all of the transport and hydrolysis assays were performed without dilution of the vesicles into a standard assay buffer. Since the ionic compositions of two fractions from different levels of a gradient are dissimilar and since the intravesicular medium has been changed by a Cs⁺ efflux and a Na⁺ influx, it is not possible to quantitatively compare activities. The measurement of stoichiometries was considered valid since the assays were performed on fractions from the same positions in the gradient and were carried out under the same conditions.

Analysis of Oriented (Na⁺,K⁺)-ATPase with Protease and Glycosidase—To complement the evidence for oriented (Na⁺,K⁺)-ATPase presented in previous sections, based on functional properties of the enzyme, the structure of the reconstituted protein was examined. In the α subunit the linear locations of specific tryptic and chymotryptic sites have been determined (3, 26, 27). All of these sites appear to be located on the cytoplasmic side of the membrane (8). The carbohydrate portion of the β subunit was assumed to reside on the extracellular side of the membrane. This section describes experiments in which the sites of hydrolysis, either by trypsin or by neuraminidase, of the reconstituted (Na⁺,K⁺)-ATPase are the same as the sites found in the native protein (3). The exposure of these sites is correlated with the suggested orientations. The last section describes those sites where a clear correspondence between reconstituted and native protein could not be established.

The graded trypsinization of inside-out and right-side-out oriented enzyme is shown in Fig. 4A. In the case of inside-out oriented enzyme (Fig. 3B, fraction 12), the α chain is converted to a doublet (lanes 2, 4, 6) by removal of a small peptide of $M_r = 2000$. This slightly smaller α chain is only faintly visible (at most 10% of initial α staining) when the right-side-out oriented enzyme (Fig. 3B, fraction 7) was exposed to equal amounts of trypsin for the same times (lanes 1, 3, 5). This difference in digestion was reproducibly observed. It was not possible to achieve quantitative conversion because tryptic hydrolysis occurred at other sites to create smaller fragments. In some digestions, the inside-out oriented enzyme yielded only the lower of the two bands while the right-side-out enzyme retained the intact α chain. However, in these cases, digestion had proceeded to the point where staining of the clipped α chain or of the intact α chain was at most half as intense as in the untreated controls. This tryptic site appears to be the same as a cytoplasmic site near the NH₂ terminus of the α polypeptide in the native enzyme (8, 28). It is not possible to eliminate the objection that this difference is merely due to rate of cleavage as opposed to accessibility since a limit pattern was not obtained.

The two other major tryptic sites, which produce fragments of $M_r = 77,000$ or $M_r = 58,000$ and 41,000 (3), were not apparent.⁴ Although the reconstituted enzyme was apparently more susceptible to tryptic cleavage at more sites than the native enzyme (see last section), higher ratios of trypsin/ATPase were generally necessary for digestion. In addition, the resistance of the reconstituted enzyme to chymotrypsin hydrolysis had increased so that little digestion was seen even

TABLE II

Transport and hydrolysis activities of gradient fraction

Assays were performed as described under "Experimental Procedures." Values for Na⁺ transport and ATPase activity are the average of at least two determinations which were usually within 10%. Values for ouabain inhibition are the range of triplicate assays. Gradient fractions were pooled as shown in Fig. 3, A and B. The stoichiometries were calculated by normalizing the activities to the amount of phospholipid. ND, not determined.

Fractions	Specific activity		Na/ATP	Ouabain inhibition
	Na ⁺ transport	ATPase		
	nmol/h/mg lipid			%
Gradient A (12–15)	125	200	0.6	90–100
Gradient A (8, 9)	535	300	1.8	0–10
Gradient B (12)	480	160	3.0	0–10
Gradient B (7)	30	25	1.2	ND

⁴ The presence of contaminating bands in the $M_r = 55,000$ – $65,000$ region of this silver-stained gel made it difficult to see the potential tryptic fragments. But in other gels with much lower levels of contaminants (as in Fig. 4B), it was clear that the major tryptic peptides of the native enzyme were not present in large amounts.

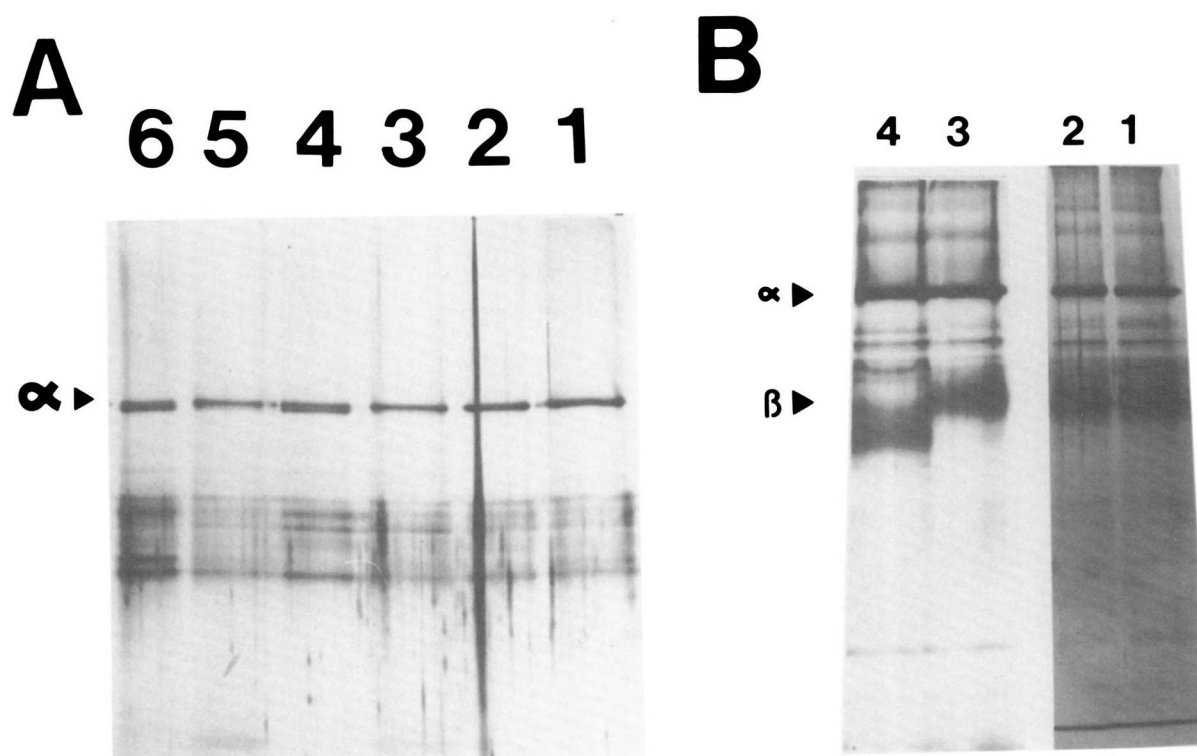


FIG. 4. **Reconstituted (Na^+, K^+)-ATPase treated with trypsin and neuraminidase.** Purified vesicles containing inside-out or right-side-out oriented ATPase were treated with trypsin or neuraminidase. After digestion, samples were electrophoresed on 10% SDS gels, 0.75 mm, and stained with silver as described under "Experimental Procedures." A, each lane contained 200 ng of (Na^+, K^+)-ATPase. Lanes 1, 3, and 5, right-side-out oriented ATPase. Lanes 2, 4, and 6, inside-out oriented ATPase. All samples were incubated for 15 min at 37 °C. Lanes 1 and 2, controls with no protease. Lanes 3 and 4, 100 ng of TPCK-trypsin. Lanes 5 and 6, 500 ng of TPCK-trypsin. B, each lane contained 500 ng of (Na^+, K^+)-ATPase. Lanes 1 and 2, inside-out oriented ATPase. Lanes 3 and 4, right-side-out oriented ATPase. Lanes 1 and 3, controls with no glycosidase. Lanes 2 and 4, neuraminidase. The arrows indicate the positions of intact α and β subunits.

at weight ratios of chymotrypsin/ATPase greater than 1 (data not shown).

Because no specific sites of proteolysis of the β subunit have been located, the carbohydrate residues were examined as an indicator of the orientation of the polypeptide. Treatment of the light and heavy vesicles (from Fig. 3B) with neuraminidase produced the patterns shown in Fig. 4B. The removal of sialic acid (29) markedly increases the electrophoretic mobility of the β chain (30).⁵ The extracellular portion of the (Na^+, K^+)-ATPase was expected to be protected in sealed vesicles with inside-out oriented enzyme, and no shift is seen in lane 2 of Fig. 4B. The vesicles from the denser portion of the gradient (Fig. 3B, fractions 6–8) had been thought to contain right-side-out oriented protein. The observed shift of the β subunit (Fig. 4B, lane 4) is consistent with this orientation.

Reconstitution of the (Na^+, K^+)-ATPase—In order to examine the differences in proteolysis between the native and reconstituted proteins, we wanted to be able to vary the parameters of the reconstitution. Since the preparation of vesicle populations containing oriented protein was carried out under a restricted set of conditions, unfractionated vesicles were used in the following experiments.

In one set of experiments the Na^+ and K^+ concentrations of the standard reconstitution buffer (30 mM imidazole (pH

6.8) containing 250 mM sucrose, 30 mM NaCl, 20 mM KCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA) were varied while the other parameters were kept the same. The lipid used was egg lecithin, the detergent was cholate, and the method of reconstitution was cholate dialysis. This protocol appeared to produce large proportions of sealed vesicles. The ATPase activity depended on the changes in ion concentration and composition, but was mostly resistant to ouabain (Table III, first entry). Vesicles prepared in buffer lacking K^+ were assayed after the addition of K^+ and valinomycin while vesicles prepared in buffer lacking Na^+ were assayed after the addition of Na^+ .

In another set of experiments, the standard buffer was used. Egg lecithin, washed asolectin, phosphatidylserine, and a $\text{CHCl}_3/\text{CH}_3\text{OH}$ (14) fraction of kidney cortex were used as sources of lipid. Cholate and octyl glucoside were used as detergents. Detergent dialysis, detergent dilution (20), and freeze-thaw sonication (21) were tried as methods of reconstitution. In some of these experiments, the NaCl and KCl concentrations were varied in the range 0–50 mM, keeping the total at 50 mM. Earlier work (31) had established that the recovery of ATPase activity depended on the presence of 50 mM ($\text{NaCl} + \text{KCl}$). This had no apparent effect on the formation of sealed vesicles.

The reconstitution of the enzyme in these samples was assayed by the amount of ATPase activity and its resistance to ouabain. The results are given in Table III. Although many

⁵ G. Chin and M. Forgac, unpublished results.

TABLE III

Reconstituted preparations of the (Na⁺,K⁺)-ATPase

Reconstitutions and assays were performed as described under "Experimental Procedures." Values for ATPase and ouabain inhibition are the average of two determinations which were within 10%. PS, phosphatidylserine; OG, octyl glucoside.

Method	Detergent	Phospholipid	ATPase	Ouabain inhibition
			$\mu\text{mol/h/mg}$	%
Dialysis	Cholate	Lecithin	12	25
Dialysis	Cholate	Asolectin	74	80
Dialysis	Cholate	Lecithin/PS, 95/5	19	80
Dialysis	Cholate	Lecithin/asolectin, 50/50	43	80
Dialysis	Cholate	Dog kidney	47	70
Dialysis	OG (40 mM)	Lecithin	30	85
Dialysis	OG (80 mM)	Lecithin	3	100
Dialysis	OG (150 mM)	Lecithin	0	
Dilution	OG	Lecithin	49	90
Dilution	OG	Asolectin	91	95
Freeze-thaw		Lecithin	34	75
Freeze-thaw		Asolectin	41	85

of the changes resulted in samples with much higher ATPase activity than the standard egg lecithin-cholate dialysis preparation, none of them produced well sealed vesicles, judging by the exclusion of ATP and ouabain from the intravesicular space. Resistance of the ATPase activity to ouabain provided evidence that the active enzyme molecules had been incorporated into sealed vesicles.

The purified kidney enzyme consists of unsealed membrane discs (32) that do not spontaneously seal into vesicles. One explanation of the ouabain-sensitive ATPase activity is that these reconstitution procedures simply attach lipid to pre-existing membranes. This results in a (Na⁺,K⁺)-ATPase that has not been solubilized and that exposes both cytoplasmic and extracellular sides to the external medium. Such a preparation is unsuitable for proteolysis. Measurement of ATP-dependent uptake was insufficient since this assay fails to reveal the presence of unsealed enzyme. Ouabain inhibition of ATPase activity was used as a relatively simple measure of the degree of sealing in a reconstituted preparation. Most of the samples consisting of large percentages of unsealed, and possibly still native, membranes were not considered.

Proteolysis of Reconstituted (Na⁺,K⁺)-ATPase—Reconstituted (Na⁺,K⁺)-ATPase preparations that contained appreciable quantities of ouabain-resistant ATPase activity were subjected to proteolysis. Fig. 5, A and B, shows the differences in the proteolytic fragments obtained with the cholate dialysis vesicles and kidney membranes.

In the reconstituted samples, chymotryptic digestion of the α polypeptide (Fig. 5A, lanes 3, 5, 6, 8) proceeded slowly; even in an equal weight ratio to substrate, chymotrypsin did not greatly reduce the staining in the $M_r = 100,000$ region. The samples shown had been prepared in buffer containing 350 mM NaCl, 50 mM NaCl, or 50 mM KCl. Inhibition of the protease was not the explanation since controls (and see Fig. 5B and Ref. 3) demonstrated digestion of kidney membrane ATPase under the same ionic conditions. The major chymotryptic product, $M_r = 77,000$, of the reconstituted (Na⁺,K⁺)-ATPase did appear to correspond to that of the native enzyme (compare Fig. 5A, lane 3 to Fig. 5B, lane 2).

Trypsin attacked the α chain of the reconstituted enzyme at many sites (Fig. 5A, lanes 2, 4, 7, 9), producing a ladder of bands of $M_r = 55,000$ –80,000 in addition to bands of lower M_r . This occurred in buffers containing 350 mM NaCl, 50 mM NaCl, or 50 mM KCl. In contrast, the patterns derived from the tryptic digestion of purified kidney membranes are shown

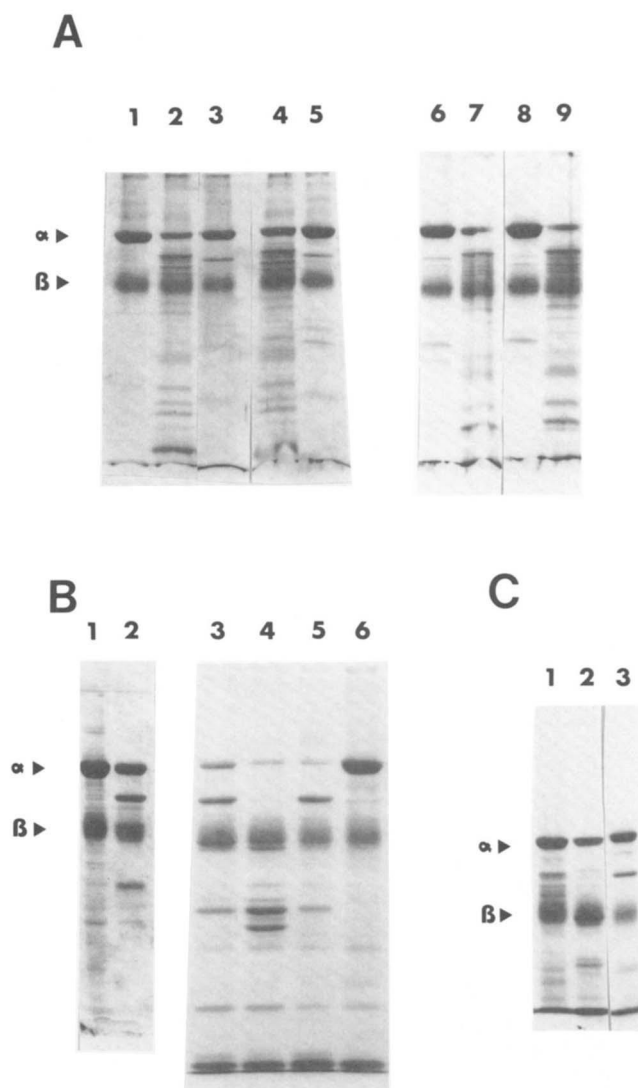


FIG. 5. Proteolysis of the (Na⁺,K⁺)-ATPase. Portions of five experiments are shown, and the Coomassie blue-stained gels have been arranged to align the α subunit. Each lane contained 20 μg of ATPase. A, all samples were reconstituted by cholate dialysis, digested, and run on 10% SDS gels. The irregular staining near the dye front is due to phospholipid which was: lanes 1–7, egg lecithin; lanes 8 and 9, egg lecithin/phosphatidylserine, 95/5 (w/w). The ionic conditions were: lanes 1–3, 350 mM NaCl; lanes 4 and 5, 50 mM NaCl; lanes 6–9, 50 mM KCl. No digestion, lane 1. Trypsin: lanes 2 and 4, 0.5 μg for 5 min at 23 °C; lanes 7 and 9, 2 μg for 10 min at 23 °C. Chymotrypsin: lanes 3 and 5, 8 μg for 30 min at 37 °C; lanes 6 and 8, 4 μg for 60 min at 37 °C. B, purified kidney membranes were digested and run on 10% SDS gels. The ionic conditions were: lanes 1 and 2, 30 mM NaCl + 20 mM KCl; lane 3, 50 mM NaCl; lane 4, 50 mM KCl, lanes 5 and 6, no NaCl or KCl. No digestion, lanes 1 and 6. Trypsin: lanes 3–5, 1 μg for 10 min at 37 °C. Chymotrypsin: lane 2, 8 μg for 30 min at 37 °C. C, egg lecithin samples reconstituted by octyl glucoside dialysis were digested in 50 mM KCl and run on 8% SDS gels. No digestion, lane 1. Trypsin: lane 2, 1 μg for 10 min at 37 °C. Chymotrypsin: lane 3, 4 μg for 60 min at 37 °C.

in Fig. 5B. As previously established (3, 26, 27) tryptic attack on the Na⁺ form of the enzyme (lanes 3 and 5) produces a major fragment of $M_r = 77,000$, and attack on the K⁺ form (lane 4) produces fragments of $M_r = 58,000$ and 41,000. Whether these sites are hydrolyzed in the reconstituted enzyme is difficult to visualize. It appears that reconstitution has eliminated the preferential sensitivity of only a few tryptic sites.

The digestion pattern of one of the samples that possessed a high level of ouabain-sensitive ATPase activity is shown in Fig. 5C. In this buffer containing 50 mM KCl, trypsin produced the $M_r = 58,000$ and 41,000 fragments (the former is somewhat obscured by the β subunit), and chymotrypsin produced the $M_r = 77,000$ peptide. These patterns were characteristic of the preparations listed in Table III as containing active ATPase in membranes that were unsealed with respect to ouabain or ATP.

DISCUSSION

The cholate dialysis procedure for reconstituting the (Na^+, K^+)-ATPase (7) produces sealed vesicles containing randomly oriented protein molecules. In order to obtain vesicles containing proteins of a single orientation, it is desirable to have few vesicles that contain more than one protein molecule. At least half of the multiple copy vesicles would have protein molecules of both orientations. An estimate of a suitable initial protein/lipid ratio was calculated by assuming that the incorporation of protein could be described by a Poisson distribution.⁶

From the average vesicle size, the area occupied by an egg lecithin molecule (33), and the molecular weight of egg lecithin (7), we calculated the average vesicle mass 1.9×10^7 daltons. From the amounts of protein and lipid solubilized by cholate, the assumption of 3×10^5 daltons for a functional protein unit (1), we estimated that at an initial protein/lipid = 0.4 mg/ml/20 mg/ml = 0.02, about 20% of the vesicles might contain multiple copies. Many of the vesicles would be devoid of protein, while most of the remainder would have only a single copy.

In order to determine the applicability of this model, we used $^{86}\text{Rb}^+$ and $^{137}\text{Cs}^+$ as markers of intravesicular volume and asked how much of the total internal isotope could be pumped out. Each vesicle that lost its trapped isotope was assumed to possess at least one molecule of inside-out oriented enzyme. The percentage of vesicles containing one or more copies of inside-out oriented (Na^+, K^+)-ATPase was calculated. The observed percentages of efflux, 60% at initial protein/lipid = 1.0 mg/ml/20 mg/ml and 30% at initial protein/lipid = 0.02, are approximately the same as predicted, 66 and 35%. Because it is possible that not all of the internal Cs^+ was pumped out from vesicles containing inside-out oriented enzyme and because the range of vesicles sizes is broad (7), this agreement may be fortuitous. Nevertheless, these experiments suggest that the protein molecules are widely distributed among the vesicles.⁷

The results on isotope efflux also support the hypothesis (7) that most of the reconstituted enzyme is uniformly functioning at lower specific activity. At an initial protein/lipid = 0.02, the reconstituted preparation contains an average of one functional protein unit per vesicle. The random orientation of the protein molecules implies that at most half of the vesicles will incorporate inside-out oriented enzyme. If all of

these enzyme molecules are able to pump out all of the internally trapped $^{137}\text{Cs}^+$, we would expect to measure a loss of 50% of the initially trapped isotope. Since the observed decreased is 30%, this suggests that at least 60% of the reconstituted enzyme is capable of active transport. An alternative interpretation of the lower specific activity is a small fraction of fully active enzyme and a large fraction of inactive enzyme. The complex tryptic patterns (Fig. 5A) could then be assigned to partially denatured protein. However, this interpretation predicts much lower percentages of efflux. It is also inconsistent with earlier data (7) showing Na^+ -stimulated phosphorylation and K^+ -stimulated dephosphorylation of most of the reconstituted enzyme molecules. Our measurement of the level of phosphorylation of the reconstituted enzyme, combined with the 2-fold stimulation of ATPase activity by cholate, suggests that 75% of the reconstituted enzyme molecules are capable of being phosphorylated and dephosphorylated.

The description of purified sealed vesicles containing inside-out or right-side-out oriented protein relies on three different sets of experiments: the sedimentation behavior of reconstituted vesicles, the functional assays on these gradient pools, and the digestions by trypsin and neuraminidase of the ATPase subunits.

The presence of sealed vesicles containing inside-out oriented enzyme is suggested by the ATP-generated decrease in density that is associated with Cs^+ efflux from the vesicle interior. This hypothesis is supported by observation of a ouabain-resistant ATPase activity and an ATP-dependent Na^+ transport. Protection of the extracellularly disposed carbohydrate of the β subunit and sensitivity of a cytoplasmic tryptic site on the α chain are also consistent with an inside-out orientation of the protein.

The presence of sealed vesicles containing right-side-out oriented enzyme is suggested by the cholate stimulation of ATPase activity seen in undiluted vesicles. The denser peak of vesicles (Fig. 3B, fractions 6–8) was found to be sealed and to contain protein, yet did not hydrolyze ATP or take up Na^+ . Finally, the protection of a cytoplasmic tryptic site on the α subunit and the exposure of the extracellular carbohydrate on the β subunit support this assignment of orientation.

It should be noted that a common criterion of specific orientation, when some previously inaccessible site becomes exposed after the addition of detergent, could not be applied. This was because the effects of detergent, in our hands, could not be ascribed only to breaching a permeability barrier but also could be explained by solubilization and denaturation of the protein. The inhibition of ATPase activity by cholate, at low protein concentrations and extremely low protein/lipid ratios, made it impossible to observe either detergent activation of ouabain inhibition or detergent activation of ATP hydrolysis. The amount of detergent required to permeabilize a membrane almost exclusively composed of lipid is relatively large compared to the concentration of protein in the membrane. At low concentrations of protein in solution, it may not be possible to solubilize so much lipid without denaturing the protein.

The structure of the protein in the reconstituted vesicles is different from the structure of the native protein as determined by proteolysis. Trypsin hydrolyzes many more peptide bonds in the α subunit after reconstitution. Thus, the accessibility of tryptic sites could not be used as a criterion of orientation. Although it might be argued that any alteration would be unlikely to translocate a cytoplasmic site across to the extracellular side, a previously cryptic site in the extracellular domain might become exposed during the reconsti-

⁶ We do not conclude that this is actually the way in which protein molecules are incorporated into phospholipid vesicles, but a Poisson distribution model generates quantitative predictions that are in reasonable agreement with experimental data, for example, the percentages of equilibrated isotope that can be transported out of the vesicles.

⁷ Attempts at affinity chromatography of reconstituted vesicles were unsuccessful; ATP-agarose and wheat germ agglutinin-Sepharose were tried. Virtually none of the reconstituted protein bound to the column under conditions which allowed specific binding of up to 70% of the protein in the purified membranes. The presence of only one or a few binding sites per 2×10^7 -dalton vesicle may preclude a tight interaction.

tution procedure. This possibility is consistent with preliminary experiments suggesting that both inside-out and right-side-out oriented enzyme exhibit tryptic fragmentation, but at relatively high amounts of protease.⁵ We think that the altered patterns are not derived from a fraction of denatured protein but reflect a change in the structure of the protein as a result of reconstitution.

In a study using cholate-reconstituted enzyme, Karlsh and Pick (34) determined the effect of trypsin digestion on the kinetics of (Na^+, K^+)-ATPase inactivation. Digestion of the reconstituted enzyme in the K^+ form produced a single exponential loss of activity, and digestion of the Na^+ form produced a biphasic loss. This was identical to results of Jorgensen (27) obtained with purified kidney membranes. On the other than, in their reconstituted vesicles, the K^+ -induced fluorescence change (35) was no longer apparent. This suggests that the conformation of the reconstituted (Na^+, K^+)-ATPase is not completely congruous to that of the native protein. No data on the fragments produced by proteolytic digestion of the reconstituted enzyme was presented.

O'Connell (19) has used both the reconstituted vesicles of Goldin (7) and the membrane vesicles of Forbush (10) in a structural study of the α subunit. The area of protein exposed cytoplasmically was determined to be unequal to the area exposed on the extracellular side; the ratio of the areas was the same for the two preparations. This indication of similarity before and after reconstitution is not inconsistent with experiments reported here. The recovery of transport stoichiometry in our system implies that no drastic denaturation of the protein has taken place.

Our explanation is that small rearrangements of the α polypeptide have been effected during this reconstitution procedure. These movements expose and protect protease-sensitive sites of the large subunit. This is proposed as the reason for the decrease in specific activity of the reconstituted (Na^+, K^+)-ATPase and the loss of the K^+ -induced fluorescence change. Nevertheless, the protein is still capable of properly coupling the transport of ions to the hydrolysis of ATP and is still asymmetrically inserted in the bilayer. It is possible that the exposure to cholate or the residual detergent (less than 1% of the starting concentration) is the cause of this structural change. Unfortunately, in our hands, this cholate dialysis procedure was the only reconstitution protocol that produced a large proportion of sealed vesicles.

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REFERENCES

1. Cantley, L. C. (1981) *Curr. Top. Bioenerg.* **11**, 201–237
2. Jorgensen, P. L. (1982) *Biochim. Biophys. Acta* **694**, 27–68
3. Castro, J., and Farley, R. A. (1979) *J. Biol. Chem.* **254**, 2221–2228
4. Perrone, J. R., and Blostein, R. (1973) *Biochim. Biophys. Acta* **291**, 680–689
5. Forbush, B., Kaplan, J. H., and Hoffman, J. F. (1978) *Biochemistry* **17**, 3667–3676
6. Sen, A. K., and Post, R. L. (1964) *J. Biol. Chem.* **239**, 345–352
7. Goldin, S. M. (1977) *J. Biol. Chem.* **252**, 5630–5642
8. Chin, G., and Forgac, M. (1983) *Biochemistry* **22**, 3405–3410
9. Drickamer, L. K. (1975) *J. Biol. Chem.* **250**, 1952–1954
10. Forbush, B., III (1982) *J. Biol. Chem.* **257**, 12678–12684
11. Jorgensen, P. L. (1974) *Biochim. Biophys. Acta* **356**, 36–52
12. Forgac, M., and Chin, G. (1981) *J. Biol. Chem.* **256**, 3645–3646
13. Kagawa, Y., and Racker, E. (1971) *J. Biol. Chem.* **246**, 5477–5487
14. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
15. Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–117
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
17. Laemmli, U. K. (1970) *Nature (Lond.)* **227**, 680–685
18. Oakley, B. R., Kirsch, D. R., and Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363
19. O'Connell, M. A. (1982) *Biochemistry* **21**, 5984–5991
20. Racker, E., Violand, B., O'Neal, S., Alfonzo, M., and Telford, J. (1979) *Arch. Biochem. Biophys.* **198**, 470–477
21. Dixon, J. F., and Hokin, L. E. (1980) *J. Biol. Chem.* **255**, 10681–10686
22. Goldin, S. M., and Rhoden, V. (1978) *J. Biol. Chem.* **253**, 2575–2583
23. Papazian, D. M., Rahamimoff, H., and Goldin, S. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 3708–3712
24. Goldin, S. M., Rhoden, V., and Hess, E. J. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 6884–6888
25. Skou, J. C. (1960) *Biochim. Biophys. Acta* **42**, 6–23
26. Giotto, G. J. (1975) *J. Biol. Chem.* **250**, 5159–5164
27. Jorgensen, P. L. (1975) *Biochim. Biophys. Acta* **401**, 399–415
28. Jorgensen, P. L. (1977) *Biochim. Biophys. Acta* **466**, 97–108
29. Kyte, J. (1972) *J. Biol. Chem.* **247**, 7642–7649
30. Craig, W. S., and Kyte, J. (1980) *J. Biol. Chem.* **255**, 6262–6269
31. Forgac, M., and Chin, G. (1982) *J. Biol. Chem.* **257**, 5652–5655
32. Deguchi, N., Jorgensen, P. L., and Maunsbach, A. B. (1977) *J. Cell Biol.* **75**, 619–634
33. Small, D. M. (1967) *J. Lipid Res.* **8**, 551–560
34. Karlsh, S. J. D., and Pick, U. (1981) *J. Physiol. (Lond.)* **312**, 505–529
35. Karlsh, S. J. D., and Yates, D. W. (1978) *Biochim. Biophys. Acta* **527**, 115–130

Purification and proteolysis of vesicles containing inside-out and right-side-out oriented reconstituted (Na⁺, K⁺)-ATPase.

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