Exclusive Labeling of the Extracytoplasmic Surface of Sodium Ion and Potassium Ion Activated Adenosinetriphosphatase and a Determination of the Distribution of Surface Area across the Bilayer†

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ABSTRACT: The relative distribution of the hydrophilic surface area of the α subunit of sodium ion and potassium ion activated adenosinetriphosphatase [$(Na^+ + K^+)$-ATPase] between the two sides of the lipid bilayer was determined with the impermeant protein modifying reagent diazotized *p*-[^{35}S]sulfanilic acid and sealed vesicles. Two types of vesicles, $(Na^+ + K^+)$-ATPase reconstituted with phosphatidylcholine and spontaneously sealed, crude membrane vesicles, were used. Both were shown to be sealed to small molecules by measurements of enzymatic latency and by flotation on density gradients. In the case of the reconstituted vesicles, selective proteolytic digestion of any α subunit present with an inside-out orientation allowed the extent of labeling of only the extracytoplasmic portion of the α subunit to be measured since α subunits with the opposite orientation were unaffected by the proteolytic treatment. On the other hand, the spontaneously sealed vesicles formed during homogenization of canine kidney medulla were shown to contain $(Na^+ + K^+)$-ATPase exclusively in a right-side-out orientation by virtue of their aggregation with ricin agglutinin and their resistance to digestion by trypsin. The specific radioactivity of the α subunit labeled in these vesicles therefore also represents that portion of the α subunit that is extracytoplasmic. Comparison of the specific radioactivities of the two α subunits labeled extracytoplasmically with the specific radioactivity of α subunits labeled in either disrupted vesicles or open membrane fragments yields a ratio for the distribution of the hydrophilic surface of the α subunit between the two sides of the lipid bilayer. Both the reconstituted preparation and the spontaneously sealed preparation gave the same ratio, three to one, cytoplasmic to extracytoplasmic surface area, demonstrating that this protein is split unequally between the two sides of the bilayer.

Sodium ion and potassium ion activated adenosinetriphosphatase [$(Na^+ + K^+)$-ATPase][1] is a member of a select group of proteins that span the membrane and transport cations actively (Kyte, 1981). A great deal of information about the kinetics of enzymatic catalysis and the interconversion of the conformational states of the intermediates in the overall reaction is known (Jørgensen et al., 1978; Moczydlowski & Fortes, 1980; Winslow, 1981). Structural information about $(Na^+ + K^+)$-ATPase, however, is sparse, and the salient features of the reported structural studies can be listed easily. The molecular weight of the α subunit of canine $(Na^+ + K^+)$-ATPase is 120 000; and that of the β subunit, 55 000 (Craig & Kyte, 1980). The α subunit spans the membrane (Kyte, 1974) and contains the site of phosphorylation (Uesugi et al., 1971; Kyte, 1971a). Because of these properties and the fact that calcium ion activated adenosinetriphosphatase (Ca^{2+}-ATPase)[1] does not require a β subunit in order to function (MacLennan, 1970), the α subunit should form the channel for cations. Finally, the location of the primary site of tryptic cleavage in the α subunit of the native enzyme is on its cytoplasmic portion (Giotta, 1975). Obviously, a great deal more information about the molecular architecture of the α subunit of $(Na^+ + K^+)$-ATPase must be ascertained before any understanding of the molecular specifics of the mechanism of active transport can be achieved.

The experiments described here address the specific question of the distribution of the surface area of the α subunit between extracytoplasmic and cytoplasmic domains. An earlier examination of this question, using a canine kidney cell line, suggested that there was an asymmetric partition of this protein across the lipid bilayer (Sharkey, 1980), favoring the cytoplasmic surface by about 4-fold. In the present study, $(Na^+ + K^+)$-ATPase was reconstituted into vesicles of phosphatidylcholine (Goldin, 1977) and labeled (Berg, 1969) with the impermeant modifying reagent, diazotized *p*-[^{35}S]-sulfanilic acid ([^{35}S]DABS).[1] After proteolytic digestion, which cleaves only those reconstituted $(Na^+ + K^+)$-ATPase molecules in the inside-out orientation, the specific radioactivity of the uncleaved, right-side-out α subunit was measured. This was compared to the specific radioactivity of the α subunit labeled from both surfaces in open membranes. From these studies it was possible to determine the ratio of the extracytoplasmic to cytoplasmic surface area of the α subunit in native $(Na^+ + K^+)$-ATPase. This approach was initially designed by Fung & Hubbell (1978) in structural studies on bovine rhodopsin and has since been used to establish the orientation of other transmembrane proteins in reconstituted systems (Wheeler & Hinkle, 1981; Lind et al., 1981).

In addition, membranes from the kidney, sealed entirely in a right-side-out orientation, were prepared by the procedure of Forbush (1982; B. Forbush III, unpublished results) on density gradients made from 1-deoxy-1-(methylamino)-D-glucitol 3,5-diacetamido-2,4,6-triiodobenzoate (Hypaque).[1] These vesicles were also labeled, and the ratio of the extra-

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[1] Abbreviations: $(Na^+ + K^+)$-ATPase, sodium ion and potassium ion activated adenosinetriphosphatase; DABS, diazotized *p*-sulfanilic acid; Hypaque, 1-deoxy-1-(methylamino)-D-glucitol 3,5-diacetamido-2,4,6-triiodobenzoate; $NaDodSO_4$, sodium *n*-dodecyl sulfate; Ca^{2+}-ATPase, calcium ion activated adenosinetriphosphatase; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; EDTA, ethylenediaminetetraacetic acid.

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cytoplasmic to cytoplasmic surface area of the α subunit was determined. The same ratio of incorporation relative to the controls was observed in the α subunit from both the reconstituted vesicles and the Hypaque vesicles.

Experimental Procedures

Materials. Phosphatidylcholine was purified from egg yolks by the method of Litman (1973). Cholic acid was recrystallized from 95% ethanol, and all concentrations of cholate are expressed in milligrams of the free acid. Aniline was distilled under vacuum on the day of use. Sodium dodecyl sulfate ($NaDodSO_4$)[1] was recrystallized according to Burgess (1969). Sulfanilic acid was recrystallized from boiling water and dried at 110 °C. Trypsin inhibitor (soybean), strophanthidin, Na_2ATP, and dextran (M_r 10000) were purchased from Sigma; TPCK-trypsin was from Millipore; Liquifluor, Protosol, and $H_2{}^{32}SO_4$ were from New England Nuclear; and Hypaque-M, 75% (w/v), was from Winthrop Laboratories. Ricin agglutinin was a gift from Dr. Nathan Kaplan, Department of Chemistry, University of California, San Diego.

Synthesis of [^{35}S]Sulfanilic Acid. [^{35}S]Sulfuric acid (2.5 mCi at 400 Ci mmol^{-1}), dissolved in 0.5 mL of water, is added to a glass hydrolysis tube (0.8 × 16 cm). A dilute solution of nonradioactive sulfuric acid (0.5 μmol) and a dilute solution of aniline (0.5 μmol) are added, and the mixture (final volume of 0.6 mL) is chilled and taken to dryness in an evacuated dessicator over NaOH pellets and P_2O_5. The same solutions are added again to the same tube, and the evaporation is repeated to yield 1 μmol of solid anilinium[^{35}S]sulfate at a calculated specific radioactivity of 5 Ci mmol^{-1}. The hydrolysis tube is sealed under vacuum and baked for 8 h at 180 °C. After being cooled, the tube is broken open, and the contents are dissolved in a small volume of water and streaked on a thin-layer plate (5 × 20 cm) of silica gel with a fluorescent indicator. The chromatogram is developed with 1-butanol–acetic acid–water (120:30:50). The location of the products is determined by their absorption of ultraviolet light. The region of the plate that contains the [^{35}S]sulfanilic acid is scraped, and the silica is washed with water (2 × 2 mL). The silica is spun out, the supernates are pooled, and an absorption spectrum is measured and compared to that of a standard. Recrystallized sulfanilic acid has a λ_{max} of 248 nm in water and a molar extinction coefficient of 15 100. The specific synthesis described here yielded 0.15 μmol of product with the same spectrum as that of the standard.

Synthesis of [^{35}S]DABS. The diazotization of sulfanilic acid is conducted by a significant modification of the method of Tinberg et al. (1974). [^{35}S]Sulfanilic acid (90 μL of a 60 μM solution) is mixed with nonradioactive sulfanilic acid (1 μL of a 35 mM solution) to yield a 10-fold dilution in specific radioactivity. The solution, in a 6 × 50 mm test tube, is dried under a stream of N_2. The residue is dissolved with 20–50 μL of ice-cold 0.5 M HONO made by mixing, on ice, 0.9 mL of prechilled 0.556 M $NaNO_2$ in 2.5% Na_2CO_3 with 0.1 mL of prechilled concentrated HCl. The reaction proceeds in ice for 15 min and is halted by drying under a stream of N_2. The residue is resuspended in 400 μL of water and titrated to pH 7 with 1 M Na_2HPO_4. This solution is diluted 10-fold into the preparation of enzyme to be labeled. Nonradioactive DABS was synthesized as described by Tinberg et al. (1974) and was used as a standard to evaluate the products of the radioactive syntheses.

Enzyme Preparation and $(Na^+ + K^+)$-ATPase Assay. $(Na^+ + K^+)$-ATPase was purified from canine kidney medullas by the method of Jørgensen (1974), as modified by Munson (1981). Strophanthidin-sensitive $(Na^+ + K^+)$-ATPase activity was measured as described by Kyte (1971b). The enzyme routinely had specific activities of 750–900 μmol of P_i (mg of protein)$^{-1}$ h^{-1}. These completely unsealed, membranous fragments of pure enzyme will be referred to as unreconstituted $(Na^+ + K^+)$-ATPase. The concentration of protein was determined by the Lowry method (Kyte, 1971b).

Preparation of Reconstituted Vesicles. The procedure of Goldin (1977) was modified for the present requirements. A solution of phosphatidylcholine from egg yolk in 19:1 chloroform:methanol (0.7 mL of 75 mg mL^{-1}) is evaporated onto the wall of a test tube (13 × 100 mm) on a lyophilizer. The lipid film is suspended in 1.5 mL of 18 mg mL^{-1} cholate dissolved in 60 mM *N*-ethylmorpholine, pH 7.0, 0.5 M sucrose, 2 mM EDTA, 60 mM NaCl, 40 mM KCl, and 15 mM $MgCl_2$ (2× N-EM buffer). After addition of the solution of detergent, the tube is capped under N_2 and mixed vigorously until the film of lipid is completely dispersed. $(Na^+ + K^+)$-ATPase (1.5 mL at 1.8 mg of protein mL^{-1}) is added to the swirling mixture of lipid and cholate. The sample is kept at room temperature for 2 min and then on ice for 25 min, transferred to a centrifuge tube, and spun for 20 min at 28K rpm in a Beckman Ti 50 rotor (70000*g*). The supernate is collected and dialyzed at 4 °C for 24 h against 2 L of 30 mM *N*-ethylmorpholine, pH 7.0, 0.25 M sucrose, 1 mM EDTA, 30 mM NaCl, 20 mM KCl, and 7.5 mM $MgCl_2$ (N-EM buffer). The sample is loaded into the bag for dialysis so that it is about 1 mm in width. The dialyzate is collected and loaded as fractions of 0.5 mL onto linear (0–10%) density gradients of dextran (M_r 10000) made from N-EM buffer by mixing 2 mL of N-EM buffer with 2 mL of 10% dextran in N-EM buffer, and these are spun at 5 °C for 18 h at 50K rpm in a Beckman SW 60 rotor (380000*g*). After centrifugation, the turbid material that has remained floating at the top of the gradient is collected. This fraction will be referred to as reconstituted vesicles. The overall yield of reconstituted $(Na^+ + K^+)$-ATPase is usually 7% of the starting material with respect to protein. The cholate-activated, reconstituted enzyme usually displays 7% of the initial specific activity, 50 μmol of P_i (mg of protein)$^{-1}$ h^{-1}.

Preparation of Hypaque Vesicles. Sealed, right-side-out vesicles are isolated on Hypaque density gradients as described by Forbush (1982; B. Forbush III, unpublished results). Crude membranes are prepared from medullas of canine kidney by the method of Kyte (1971a). Crude membranes (78 mg of protein in 5 mL) are layered on top of a linear (10–22%) density gradient of Hypaque prepared from dilutions with water of the 75% stock solution of Hypaque supplied by the manufacturer to yield the 10% and 22% solutions for the gradient. The gradient (37 mL) is spun at 5 °C for 18 h at 27K rpm in a Beckman SW27 rotor (140000*g*). After centrifugation, the membrane population that has floated at the top of the gradient is collected. These preparations are referred to as Hypaque vesicles.

Preincubation with Cholate and Assay of Enzyme. For assays of latency on reconstituted vesicles, 40 μL of vesicles at 0.1 mg of protein mL^{-1} was mixed with 20 μL of N-EM buffer containing cholate at an appropriate concentration. The samples were incubated at room temperature for 10 min, and then 10-μL samples were transferred to tubes containing 10 μL of 12 mM Na_2ATP dissolved in N-EM buffer, and 0.2 μM strophanthidin where required, and incubated for 10 min at 37 °C. The reaction was quenched by the addition of 200 μL of 5% $NaDodSO_4$, followed by 20 μL of 2.5% ammonium molybdate in 2 M HCl and 10 μL of 25 mg mL^{-1} powdered Fiske–SubbaRow reducing agent (Leloir & Cardini, 1957).

The absorbance at 700 nm was measured after 30 min.

Labeling with [^{35}S]DABS. Unreconstituted (Na^+ + K^+)-ATPase or reconstituted vesicles (each 0.5 mL at 0.1 mg of protein mL^{-1}) were preincubated for 10 min at room temperature at the indicated cholate concentrations where required, [^{35}S]DABS (50 μL of a 150 μM solution) was added to the samples, and the labeling reaction was conducted for 10 min at room temperature. The reaction was quenched by the addition of 60 μL of 100 mM histidine (unbuffered, pH 7), and the samples were transferred to bags for dialysis at 4 °C for 2 h against 25 mM imidazolium chloride, pH 7.0–1 mM EDTA.

Digestion of Membranes with Trypsin. Vesicles or unreconstituted (Na^+ + K^+)-ATPase was incubated at 37 °C for 20 min with 8–10 μg mL^{-1} TPCK-trypsin, and the reaction was quenched by the addition of 3-fold excess by weight of trypsin inhibitor over trypsin. When conducted during a labeling experiment, the digestion was run after the addition of the quenching solution of histidine but prior to the dialysis.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Cylindrical gels of polyacrylamide (7.5% in acrylamide) were formed in the $NaDodSO_4$–phosphate buffer system of Shapiro et al. (1967) as modified by Weber & Osborn (1969). Prior to electrophoresis, samples were made 2% in $NaDodSO_4$ and incubated at room temperature for 1 h. They were then layered on the gels, which were submitted to electrophoresis, stained with Coomassie Brilliant Blue, destained by diffusion, and scanned at 550 nm (Craig & Kyte, 1980). With radioactive samples, gels were run in parallel; one gel was scanned, and one gel was sliced and digested in 8 mL of Liquifluor, 4.5% in Protosol, for 24 h at 50 °C. Gel samples were not boiled since the azo linkage is heat labile (Howard & Wild, 1956). The samples were split again into pairs prior to electrophoresis so that one gel could be stained and the other sliced, digested, and submitted to liquid scintillation counting. This procedure was necessary because the azo linkage is also acid labile (Higgins & Harrington, 1959).

Results

Synthesis of [^{35}S]Sulfanilic Acid. An economical and simple method for the synthesis of [^{35}S]sulfanilic acid was devised because the [^{35}S]sulfanilic acid commercially available is of a low specific radioactivity and is synthesized on a limited schedule. The synthetic method based on baking of anhydrous anilinium sulfate was adopted (Morrison & Boyd, 1973). A photodensitometric scan of an autoradiogram made from a thin-layer chromatogram of the products from this synthesis is shown in Figure 1A. On the basis of the peak areas, a significant portion (40%) of the products that contain ^{35}S is [^{35}]sulfanilic acid. The overall preparative yields, after elution of the sulfanilic acid from the silica gel plate, have been 10–20%. This calculation is based on the ultraviolet absorption of the eluted product and the amount of anilinium sulfate (1 μmol) in the starting material. This [^{35}S]sulfanilic acid has a calculated specific radioactivity of 5 Ci $mmol^{-1}$. This rapid and simple method of synthesis utilizes readily available reagents and provides a radioisotope at 60 times the specific radioactivity of the commercially available compound for one-tenth the cost.

Synthesis of [^{35}S]DABS. The published syntheses of [^{35}S]DABS were found to produce product in extremely low yield, and consequently, they were modified considerably. For a standard, nonradioactive DABS was synthesized as described by Tinberg et al. (1974), and the physical appearance of the product was precisely that described by these authors. Silica gel thin-layer chromatography of this nonradioactive DABS yielded only one spot that absorbed ultraviolet light, and the relative mobility of this spot was used as a reference for the chromatograms of the radioactive products of the small-scale syntheses.

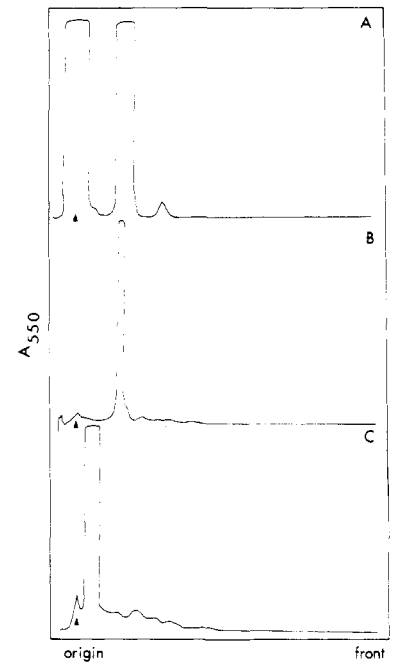

FIGURE 1: Scans of autoradiograms of silica gel thin-layer chromatograms of [^{35}S]sulfanilic acid reaction mixture, [^{35}S]sulfanilic acid, and [^{35}S]DABS reaction mixture. Photodensitometric scans of autoradiograms of silica gel thin-layer chromatograms of (A) anhydrous anilinium [^{35}S]sulfate after baking at 180 °C for 8 h, (B) [^{35}S]sulfanilic acid (2 μL of 43 μM at 3.2 Ci $mmol^{-1}$), and (C) [^{35}S]DABS (1 μL of 0.95 mM at 0.3 Ci $mmol^{-1}$). The peaks appear flattened in these scans because the X-ray film was slightly overexposed.

The modifications employed in the synthesis of [^{35}S]DABS had to be designed to accommodate the requirement that only nanomoles of product are required from each batch. This is due to the fact that DABS itself is unstable, and therefore, only the required amount of the radioactive reagent should be synthesized immediately prior to its employment in a labeling experiment. Thin-layer chromatograms were run routinely after each synthesis to verify the formation of the diazonium salt. Photodensitometric scans of autoradiograms made from chromatograms of the starting material, [^{35}S]-sulfanilic acid (Figure 1B), and the product, [^{35}S]DABS (Figure 1C), indicate that the conversion of [^{35}S]sulfanilic acid into [^{35}S]DABS is virtually complete when this protocol is followed.

Labeling of (Na^+ + K^+)-ATPase. [^{35}S]DABS reacted covalently to label the α subunit of unreconstituted (Na^+ + K^+)-ATPase (Figure 2). In several preliminary experiments the β subunit of (Na^+ + K^+)-ATPase, however, was never observed to react detectably with this reagent even when the labeling was conducted in the presence of millimolar concentrations of [^{35}S]DABS (data not shown). For this reason all of the gels to be described here were sliced only through the region containing the α subunit. The extent of labeling of the α subunit of (Na^+ + K^+)-ATPase was reduced markedly if 2-mercaptoethanol, histidine, or imidazole were present in the enzyme preparation. Obviously, these compounds can react with DABS (Howard & Wild, 1956), and they were removed by dialysis from enzyme samples to be used in labeling experiments.

Reconstitution of (Na^+ + K^+)-ATPase. The successful interpretation of the labeling patterns observed with the re-

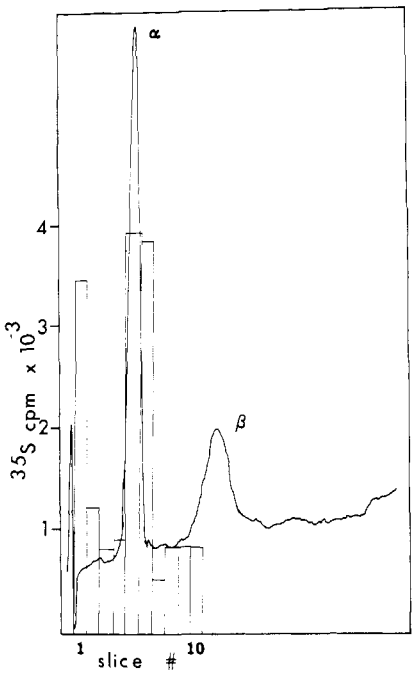

FIGURE 2: Scan of a $NaDodSO_4$–polyacrylamide gel of [^{35}S]DABS labeled, unreconstituted $(Na^+ + K^+)$-ATPase. Unreconstituted $(Na^+ + K^+)$-ATPase (0.06 mg of protein in 250 μL) was labeled with [^{35}S]DABS [5 μL of 7.6 × 10^{-4} M at 2.4 × 10^{14} cpm (mol of DABS)$^{-1}$]. After 10 min at room temperature, the reaction was stopped by the addition of 25 μL of 100 mM histidine. The sample was dialyzed against 25 mM imidazolium chloride, pH 7.0–1 mM EDTA and prepared for electrophoresis. It was split, and equal volumes were loaded on two 7.5% polyacrylamide gels. Following electrophoresis, one gel was stained and scanned at 550 nm (solid line), and the other was sliced, digested, and counted (bars). The position of the α subunit is noted. Electrophoresis was from left to right.

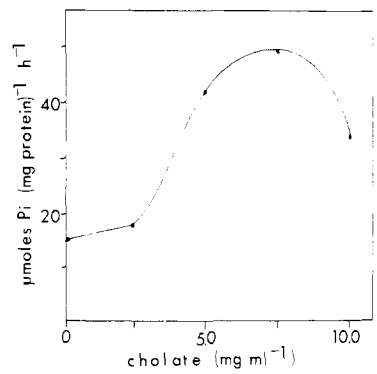

FIGURE 3: Effect of cholate preincubation on $(Na^+ + K^+)$-ATPase activity in reconstituted vesicles. Vesicles (4 μg of protein in 40 μL) were mixed with 20 μL of various cholate solutions to yield the indicated final concentrations. After 10 min at room temperature, samples (10 μL) of the mixtures were assayed for $(Na^+ + K^+)$-ATPase activity.

constituted vesicles required that all of those containing $(Na^+ + K^+)$-ATPase be sealed to the labeling reagent. Vesicle preparations were considered to be sealed to small molecules if the $(Na^+ + K^+)$-ATPase activity was latent, that is, if detergent treatment produced an increase in specific enzymatic activity over that of untreated controls. A random orientation of the enzyme was anticipated in the reconstituted vesicles (Goldin, 1977). If vesicles are sealed to small molecules such as MgATP, then full expression of enzymatic activity would require the addition of detergent to disrupt their integrity. $(Na^+ + K^+)$-ATPase activity was measured in reconstituted vesicles that had been preincubated at several concentrations of cholate (Figure 3). The $(Na^+ + K^+)$-ATPase activity of reconstituted vesicles was increased 4-fold by the addition of cholate. By comparison, the latency of the turbid material collected after the dialysis step, but prior to the dextran gradient step, was only 1.3-fold.

Digestion with Trypsin. The sites in the α subunit of $(Na^+ + K^+)$-ATPase that can be cleaved by trypsin when it is in the native enzyme are located on the cytoplasmic portion of the protein (Giotta, 1975). Therefore, only those α subunits with an inside-out orientation in a sealed, reconstituted vesicle will be cleaved by trypsin. Any α subunit present in a sealed vesicle in a right-side-out orientation, the one of most interest, should be unaffected by the proteolytic enzyme and be found on a $NaDodSO_4$ gel at the normal position. As a control, all of the α subunit present should be digested by trypsin if the vesicles are broken open with cholate.

For demonstration that the reconstituted vesicles were sealed to trypsin, the following experiment was performed. Reconstituted vesicles were incubated as paired samples at 0, 6, and 10 mg of cholate mL^{-1} for 10 min at room temperature, and then one set was digested with trypsin. All the samples were submitted to electrophoresis, and the gels were stained and scanned. The areas of the peaks of the α subunits were calculated, and percent cleavage was determined from them. In the absence of cholate, 25% of the α subunit was cleaved; at 6 mg of cholate mL^{-1}, 58%; and at 10 mg of cholate mL^{-1}, 82%. It can be seen that the availability of the α subunit to trypsin increases in parallel, although displaced to higher concentrations of cholate, with the enzymatic activity (Figure 3). In contrast, under the same digestion conditions in coincident incubations, 90% of the α subunit present in unreconstituted $(Na^+ + K^+)$-ATPase was digested by trypsin in the absence of cholate. The results indicate that a significant percentage of the enzyme in the reconstituted vesicles is unavailable to trypsin, just as the active sites are unavailable to MgATP, until the integrity of the bilayer is destroyed by the detergent. Since the enzyme in the open fragments of membrane is completely cleaved under the same conditions, it can be concluded that the trypsin-resistant enzyme in the reconstituted vesicles is protected from digestion because it has a right-side-out orientation in vesicles sealed to trypsin.

Labeling of Vesicles with [^{35}S]DABS. Reconstituted vesicles and unreconstituted $(Na^+ + K^+)$-ATPase were preincubated with or without cholate. These samples were then labeled with 15 μM [^{35}S]DABS. After the reaction was quenched, half of each sample was digested with trypsin. All the samples were dialyzed and then submitted to electrophoresis. The radioactivity associated with the α subunit on these gels was corrected for background, and specific radioactivities were calculated from this corrected value and the peak areas of the α subunits on the scans of the corresponding gel that had been stained. The results of these measurements are gathered in Table I.

The treatment with cholate had no significant effect on the labeling of the α subunit in the open fragments of membrane containing unreconstituted $(Na^+ + K^+)$-ATPase. The specific radioactivity of the α subunit in reconstituted vesicles that were preincubated with cholate, however, was approximately twice (1.8 ± 0.2; $n = 3$) that of the α subunit from vesicles labeled in the absence of cholate. Furthermore, the α subunit in unreconstituted $(Na^+ + K^+)$-ATPase could be digested almost quantitatively (95%) by trypsin while only 36% of the α subunit in the reconstituted vesicles could be. It can be concluded that the reconstituted vesicles are sealed to [^{35}S]DABS, that the enzyme is inserted randomly in the reconstituted vesicles, that most of the reconstituted vesicles are unilamellar,

Table I: Effect of Preincubation with Cholate and Treatment with Trypsin on the Specific Radioactivity of the α Subunit in Reconstituted Vesicles Labeled with [^{35}S]DABS[a]

sample and treatment	specific radioactivity of the α subunit[b] control	cholate added (6 mg mL^{-1})
reconstituted vesicles		
control	510	940
digested with trypsin (8 μg mL^{-1})	210 (36%)[c]	405 (75%)[c]
unreconstituted ($Na^+ + K^+$)-ATPase		
control	740	840
digested with trypsin (8 μg mL^{-1})	(95%)[c]	(93%)[c]

[a] Reconstituted vesicles (0.12 mg of protein in 0.4 mL) and unreconstituted ($Na^+ + K^+$)-ATPase (0.12 mg of protein in 0.4 mL) were mixed with 0.1 mL of either N-EM buffer or 30 mg mL^{-1} cholate in N-EM buffer and incubated for 10 min at room temperature. [^{35}S]DABS (10 μL of 7.6×10^{-4} M at 2.4×10^{14} cpm mol^{-1}) was added, and the reaction progressed for 10 min at room temperature. Labeling was quenched by the addition of 50 μL of 100 mM histidine. Paired samples (250 μL) from each mixture were placed into two test tubes, TPCK-trypsin (10 μL of 0.2 mg mL^{-1}) was added to one test tube, and both were incubated for 20 min at 37 °C. Digestion was stopped by the addition of trypsin inhibitor (10 μL of 0.6 mg mL^{-1}). Samples were dialyzed and prepared for electrophoresis. [b] Specific radioactivity of the α subunit is expressed as ^{35}S cpm corrected for background from the sliced and counted gel, normalized by the peak area (cm^2) of the scan of the α subunit in the stained gel (^{35}S cpm cm^{-2}). [c] Cleavage of the α subunit by trypsin (%).

Table II: Cytoplasmic to Extracytoplasmic Surface Area Ratios of the α Subunit

expt	final protein concn (mg mL^{-1})	final concn of [^{35}S]DABS (μM)	specific radioactivity of DABS (cpm mol^{-1})	surface area ratio (cytoplasmic/extracytoplasmic)[a]
1	0.12	15	2.4×10^{14}	3.5
2	0.10	13	1.4×10^{14}	1.9
3	0.09	7	0.5×10^{14}	3.0
4	0.20	20	2.5×10^{14}	3.4

[a] Average value of 3.0 ± 0.7.

and that the fragments of membrane containing ($Na^+ + K^+$)-ATPase purified by the procedure of Jørgensen (1974) are, for the most part (≥85%), open.

If all of these conclusions are correct, the specific radioactivity of the α subunit from trypsin-treated reconstituted vesicles (210 cpm cm^{-2}) measures the portion of the α subunit exposed extracytoplasmically, and the specific radioactivity of the α subunit from vesicles labeled in the presence of cholate (940 cpm cm^{-2}) measures the sum of both the cytoplasmic and extracytoplasmic portions. The difference between these two numbers divided by the former (3.5) is an estimate of the ratio of the surface areas (cytoplasmic to extracytoplasmic) of the α subunit on the two sides of the bilayer. The ratios of these surface areas for the α subunit were calculated with the labeling data obtained from four separate preparations of reconstituted vesicles. These values are listed in Table II. The average for these measurements is approximately 3. If DABS inserts at random into the protein, it follows that the surface area of the cytoplasmic face of the α subunit of ($Na^+ + K^+$)-ATPase in its native conformation is 3 times as great as the surface area of the extracytoplasmic face.[2]

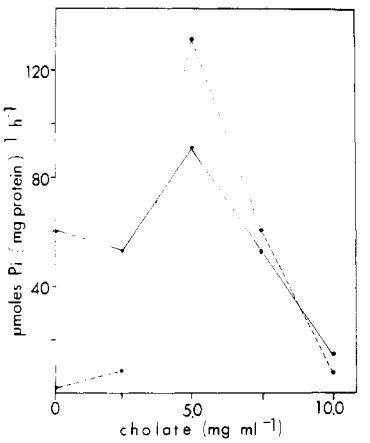

FIGURE 4: Effect of cholate preincubation on ($Na^+ + K^+$)-ATPase activity in Hypaque vesicles and crude membranes. Crude membranes or Hypaque vesicles (both 17 μg of protein in 100 μL) were mixed with 10 μL of various cholate solutions to yield the indicated final concentrations. After 10 min at room temperature, samples (20 μL) were assayed for ($Na^+ + K^+$)-ATPase activity. Solid line, crude membranes; broken line, Hypaque vesicles.

[^{35}S]DABS Labeling of Hypaque Vesicles. Sealed vesicles were purified from a crude preparation of membranes derived from canine renal medulla. These vesicles were separated from open fragments of membrane by flotation on a linear Hypaque density gradient (Forbush, 1982; B. Forbush III, unpublished results). Only a small fraction (13%) of the protein applied to the gradient was recovered as sealed vesicles floating on the top. When these were submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis, it was found that they contained many other proteins in addition to ($Na^+ + K^+$)-ATPase. The α subunit of ($Na^+ + K^+$)-ATPase present in these Hypaque vesicles, however, was easily identified on the polyacrylamide gels despite the presence of the contaminating proteins.

Enzymatic latency was measured to determine if these vesicles were sealed to small molecules. The specific activity of ($Na^+ + K^+$)-ATPase was assessed in samples of Hypaque vesicles that had been preincubated at several cholate concentrations, and the results are plotted in Figure 4. For comparison, the effect of cholate on ($Na^+ + K^+$)-ATPase activity in the original crude preparation of membranes is also presented. Expression of any ($Na^+ + K^+$)-ATPase activity [≥2 μmol of P_i (mg of protein)$^{-1}$ h^{-1}] in the Hypaque vesicles required the presence of detergent. These results demonstrate that they are completely sealed to substrate, MgATP.

It can be assumed that, during homogenization of the tissue, all of the ($Na^+ + K^+$)-ATPase molecules in a given plasma membrane fragment remain oriented in the same direction and only purely inside-out or purely right-side-out vesicles can result. Therefore, if all of the sealed vesicles collected on the

[2] One entry in Table I requires further comment. The tryptic digestion of the reconstituted vesicles that have been treated with cholate was less than quantitative (75%), raising the possibility that the digestion of the untreated reconstituted vesicles (36%) was also incomplete. This would bring into question the value for the specific radioactivity of the α subunit labeled only from the extracytoplasmic face (210 cpm cm^{-2}). It has already been noted that higher concentrations of cholate are required to increase trypsin sensitivity of the α subunit in reconstituted vesicles than are required to expose latent enzymatic activity (Figure 3). For the experiment presented in Table I, a cholate concentration (6 mg mL^{-1}) that produces the maximum increase in specific enzymatic activity was chosen. When the cholate concentration was increased to 9 mg mL^{-1} in another experiment, all (>95%) of the α subunit in the reconstituted vesicles was cleaved by the trypsin.

Table III: Aggregation of $(Na^+ + K^+)$-ATPase Activity in Hypaque Vesicles by Ricin Agglutinin[a]

additions	activity[b] control	activity[b] cholate added (5 mg mL^{-1})
buffer	0.017	0.052
ricin	0.023	0.035
ricin + super	0.005	0.006

[a] Hypaque vesicles (0.03 mg of protein) were incubated for 30 min at 37 °C in 30 mM histidinium chloride, pH 7.1, 0.25 M sucrose, 1 mM EDTA, and, where indicated, ricin agglutinin (0.1 mg) in a final volume of 125 μL. After incubation, one sample of vesicles plus ricin agglutinin was spun in a clinical centrifuge for 5 min, and the supernate was collected. Samples (50 μL) from the described mixtures directly or supernates were mixed with 5 μL of either buffer or 55 mg mL^{-1} cholate in buffer and incubated for 10 min at room temperature. Samples (10 μL) from these incubations were assayed for $(Na^+ + K^+)$-ATPase. [b] μmol of P_i h^{-1}.

Table IV: Effect of Preincubation with Cholate and Treatment with Trypsin on the Specific Radioactivity of the α Subunit in Hypaque Vesicles Labeled with [^{35}S]DABS[a]

treatment of hypaque vesicles	specific radioactivity of α subunit[b] control	specific radioactivity of α subunit[b] cholate added (5 mg mL^{-1})
control	130	430
digested with trypsin	110 (6%)[c]	(>95%)[c]

[a] Hypaque vesicles (0.17 mg of protein in 0.2 mL) were mixed with 20 μL of either N-EM buffer or 55 mg mL^{-1} cholate in N-EM buffer and incubated for 10 min at room temperature. [^{35}S]-DABS (30 μL of 1.9×10^{-4} M at 3.8×10^{14} cpm mol^{-1}) was added, and after 10 min at room temperature the labeling was quenched by the addition of 50 μL of 100 mM histidine. Two samples (150 μL) from each were transferred to paired test tubes, TPCK-trypsin (5 μL of 0.35 mg mL^{-1}) was added to one test tube from each pair, and all of the samples were incubated for 20 min at 37 °C. Digestion was stopped by the addition of trypsin inhibitor (20 μL of 0.41 mg mL^{-1}). Samples were dialyzed and prepared for electrophoresis. [b] Specific radioactivity of the α subunit is expressed as ^{35}S cpm corrected for background from the sliced and counted gel, normalized by the peak area of the scan of the α subunit in the stained gel (^{35}S cpm cm^{-2}). [c] Cleavage of the α subunit by trypsin (%).

Hypaque gradient contain only $(Na^+ + K^+)$-ATPase molecules oriented in a right-side-out direction, then ricin agglutinin should aggregate all of the vesicles, since only the extracytoplasmic surface of the enzyme is glycosylated. As a test of this hypothesis, Hypaque vesicles were incubated with ricin agglutinin and submitted to centrifugation, and the supernate was assayed for $(Na^+ + K^+)$-ATPase activity in the presence and absence of cholate. Control incubations contained either no ricin or ricin but no centrifugation step. $(Na^+ + K^+)$-ATPase activities measured in these samples are listed in Table III. As expected, the level of measurable enzymatic activity in the control samples was increased by the addition of cholate. Ricin agglutinin aggregated 90% of the Hypaque vesicles that contained $(Na^+ + K^+)$-ATPase activity as judged by the level of enzymatic activity present in the supernate of the sample incubated with ricin agglutinin. Furthermore, this remaining enzymatic activity was unaffected by the addition of cholate, suggesting that the residual $(Na^+ + K^+)$-ATPase activity in this preparation was present in inside-out vesicles. Since most preparations of Hypaque vesicles contained much less $(Na^+ + K^+)$-ATPase activity in the absence of cholate (Figure 4) than the one used in this experiment, it is clear that the majority (>90%) of the vesicles that contain $(Na^+ + K^+)$-ATPase activity are sealed and contain the enzyme in a right-side-out orientation. Further evidence for a right-side-out orientation is that only 6% of the α subunit in the Hypaque vesicles was sensitive to trypsin, as judged by the areas of peaks on stained gels (Table IV). In addition, Forbush (1982; B. Forbush III, unpublished results) has presented exhaustive independent results that demonstrate that the vesicles in this preparation that contain $(Na^+ + K^+)$-ATPase are all sealed with a right-side-out orientation.

Hypaque vesicles alone and Hypaque vesicles treated with cholate were labeled with [^{35}S]DABS. These samples were split, and one of each pair was digested with trypsin. The α subunit from each of these preparations was isolated on $NaDodSO_4$–polyacrylamide gels, and the specific radioactivities were determined in the usual manner from scintillation and area measurements (Table IV). Since the Hypaque vesicles are sealed and contain the enzyme in a right-side-out orientation, only the exposed extracytoplasmic amino acids of the α subunit were modified by [^{35}S]DABS. In the Hypaque vesicles treated with cholate, however, both the cytoplasmic and the extracytoplasmic surfaces were modified. Therefore, the ratio of the cytoplasmic to the extracytoplasmic surface area of the α subunit in the Hypaque vesicles can be calculated from the data presented in Table IV, and a value of approximately 3 (2.9) is obtained for this preparation of the ATPase.

Both the $NaDodSO_4$–polyacrylamide gels and the maximum specific activity of $(Na^+ + K^+)$-ATPase in the Hypaque vesicles (Figure 4) clearly indicate that the enzyme is impure at this stage. Therefore, the possibility that contaminating proteins, running under the α subunit of the gels, could be confusing the results was ruled out by purifying $(Na^+ + K^+)$-ATPase from a preparation of Hypaque vesicles that had been labeled with [^{35}S]DABS. As a control, $(Na^+ + K^+)$-ATPase was also purified from a crude membrane preparation that had been labeled with [^{35}S]DABS because it is clear from both the yield of Hypaque vesicles [13% of the total $(Na^+ + K^+)$-ATPase activity] and the weak latency of the crude membranes themselves (Figure 4) that they are, for the most part, unsealed. $(Na^+ + K^+)$-ATPase was purified from both samples with the standard purification procedure applied on a smaller scale. Enzyme purified from labeled Hypaque vesicles had a specific activity of 820 μmol of P_i (mg of protein)$^{-1}$ h^{-1}, while enzyme purified from the labeled crude membrane preparation had a specific activity of 550 μmol of P_2 (mg of protein)$^{-1}$ h^{-1}. The radioactivity in each of these purified, labeled $(Na^+ + K^+)$-ATPase preparations was assessed, and the amount of protein present was determined by the Lowry method (Kyte, 1971b). The specific radioactivity of $(Na^+ + K^+)$-ATPase purified from labeled Hypaque vesicles was 130 cpm (μg of protein)$^{-1}$, and that purified from crude membranes was 540 cpm (μg of protein)$^{-1}$. Once again, a comparison of these specific radioactivities yields a ratio of surface areas of approximately 3 (3.2). Because only the α subunit of the enzyme is modified by [^{35}S]DABS, these results corroborate the measurements described above made on labeled α subunit isolated from unpurified $(Na^+ + K^+)$-ATPase in the Hypaque vesicles.

From a consideration of all of these results, it can be concluded that the α subunit in $(Na^+ + K^+)$-ATPase purified from Hypaque vesicles labeled with [^{35}S]DABS bears the radioactive modification only on amino acid residues that are located extracytoplasmically in the native enzyme and that this protein can be used to identify those sequences that constitute this surface.

Discussion

Since its development by Kagawa & Racker (1971), the method of reconstitution has been used in many instances to study various aspects of the structure and function of membrane-spanning proteins. When such preparations are used, the issue of their composition must always be considered carefully. Theoretically, in these turbid suspensions there could be pure phospholipid vesicles and multibilayers, unilamellar vesicles of a wide array of sizes containing the protein of interest, as well as multibilayers and fragments of membrane also containing the protein. The permeabilities of the vesicles could encompass a range of values from those sealed to molecules of all sizes to those sealed to large molecules but not small molecules to those leaky to all sizes of molecules. Additionally, the conformation of the protein that has been incorporated could vary; native and fully active protein, inactive but native protein, and inactive and denatured protein could all be present in random orientations and distributed equally through all the lamellar, fragmented, and vesicular forms. In addressing these problems, what can be said in favor of the present conclusions?

Open fragments of membrane and denatured, soluble enzyme should have been removed from the reconstituted preparation during the flotation on the gradient of dextran because it was a long centrifugation at high centrifugal force. A similar procedure was used by Wheeler & Hinkle (1981) to eliminate open fragments of membrane and unincorporated protein from a preparation of reconstituted glucose carrier, and a significant improvement in the transport activity, which in that case was completely dependent on the fraction of the protein incorporated into impermeable vesicles, was observed. In the present instance, the increase in enzymatic latency expressed by the vesicles collected after the centrifugation is also consistent with a depletion in the number of open vesicles. Certainly, the populations of vesicles used in these labeling studies were sealed to large molecules ($M_r \geq 10\,000$) because they floated on gradients of dextran. Any open fragments of membrane that might float on the dextran gradients because they had a very low buoyant density would still be susceptible to the treatment with trypsin. The α subunits in such fragments thus would be eliminated by the tryptic digestion and could not contribute to the final results.

Demonstrating that the vesicles are sealed to small molecules is not easy, but the information presented here allows for a maximum estimate of the percentage of the reconstituted vesicles that are sealed to charged, small molecules like MgATP. On the one hand, if one makes the stringent assumption that the enzyme activity expressed in the absence of treatment with detergent represents active enzyme present in vesicles unsealed to small molecules, then the extent of latency reflects the percentage of sealed vesicles in the population. A preparation of vesicles with a latency of 4-fold would then contain 75% of the enzyme in sealed vesicles. Because cholate can slowly inactivate the enzyme while it disrupts the phospholipid bilayer, thereby lowering the apparent degree of latency (Figure 3), this value may be too low and is thus a minimum estimate. On the other hand, if one is optimistic and assumes that all the vesicles are sealed to a charged molecule such as MgATP, then the enzymatic activity expressed in the absence of treatment with cholate arises from molecules of enzyme with an externally exposed hydrolytic site. If this is the case, then the extent of cleavage with trypsin should be greater than or equal to the level of enzymatic activity insensitive to cholate. A latency of 4-fold would suggest that 25% of the enzyme present has externally directed active sites, and about the same fraction (35%) of the α subunit was sensitive to trypsin cleavage (Table I). That the degree of cleavage with trypsin is greater than the percent of the enzyme that is not latent is consistent with the expectation that the potassium on the inside of small, thickly populated vesicles would be rapidly depleted, dropping activity below maximum velocity. It should be noted, however, that this interpretation requires that strophanthidin, the inhibitor used to define ($Na^+ + K^+$)-ATPase activity, be effectively permeant on the time scale of the assay (Mercer & Dunham, 1981).

If one is pessimistic, however, and assumes that all of the vesicles are slowly leaky to MgATP and that the rate of enzymatic activity expressed in the absence of cholate is limited by the slow leak of MgATP into the vesicles, then the addition of detergent, rather than opening the vesicles, merely increases the extent of diffusion of MgATP to the active sites. If this were, however, the explanation for the latency displayed by the vesicles, one would expect the rate of diffusion of DABS into the vesicles to be much faster than that of MgATP since DABS is a smaller and less polar molecule. Therefore, DABS would equilibrate across the vesicles more rapidly than MgATP, and no differences in specific radioactivity should be observed between the sealed vesicles and the open fragments. This, however, is not the case (Table I). In fact, cholate treatment, as expected, increases the specific radioactivity by approximately a factor of 2 (1.8 ± 0.2). Furthermore, since the assumption that the vesicles are sealed is essential to the calculation of the surface area ratios (Table II) and since the same surface area ratio results from the experiments with the Hypaque vesicles (Table III), a completely different preparation, the latter result serves to confirm the former calculation and the assumption upon which it was based.

The cholate dialysis procedure used here for reconstituting ($Na^+ + K^+$)-ATPase was based on that used by Goldin (1977). It was demonstrated at that time, with great care, that this method yields unilamellar vesicles. Initially, this procedure was reproduced exactly as described. Unfortunately, the majority of the vesicles in these preparations were not sealed to MgATP, and minor modifications and improvements had to be made. As each modification was adopted, both the latency of the enzymatic activity and the sensitivity of the enzyme to trypsin were examined to make sure that no detrimental or inexplicable changes had occurred. Certainly, the specific values for the latency of the enzymatic activity (Figure 3), the susceptibility to trypsin (Table I), and the incorporation of [^{35}S]DABS (Table I) are consistent with the conclusion that the vesicles used here are also unilamellar.

The aspect of the reconstituted vesicle preparation that is not well-defined is the conformation of the enzyme in the sealed vesicles. Since the specific activity of ($Na^+ + K^+$)-ATPase in these preparations is so low, there is a serious possibility that a large percentage of the enzyme is inactive. If the structure of the protein is not significantly altered, then the low specific activity is not a major concern. Intuitively, an extensive amount of denaturation would have to occur before a certain length of the polypeptide would end up on the opposite side of the bilayer from its neighbors in the native structure. Therefore, very extensive unfolding has to occur before orientations are lost. Since the specific radioactivities of the α subunits in the modified ($Na^+ + K^+$)-ATPase from the purified fragments of membrane and that from the reconstituted vesicles treated with cholate (Table I) are so similar, however, it is unlikely that the inactive enzyme in the vesicle preparation has unfolded to reveal a significant number of new reactive amino acid residues. The strongest argument

in favor of the structural integrity of the inactive form of the reconstituted enzyme is again the close agreement between the surface area ratios obtained with the two types of vesicles. The specific enzymatic activity of $(Na^+ + K^+)$-ATPase purified from labeled Hypaque vesicles is at the level expected for fully active enzyme at this degree of purity. Since both active and inactive preparations of the enzyme give the same results, the alterations in structure that have inactivated the enzyme apparently did not significantly destroy its native conformation. Finally, Goldin (1977) arrived at the conclusion that the decreased specific activity of $(Na^+ + K^+)$-ATPase in his preparations of reconstituted $(Na^+ + K^+)$-ATPase was due to a decreased turnover number. He calculated that virtually all of the reconstituted enzyme was active, albeit at a much reduced level.

Finally, the levels of incorporation in all of the experiments described in this report are between 0.002 and 0.080 mol of DABS (mol of α subunit)$^{-1}$. Because of the experimental design, these values are only approximations. They make the point, however, that only a small fraction of the enzyme molecules even bear one site of modification; the majority are unlabeled. The necessity for such an approach has been discussed previously (Bretscher, 1971).

If one takes into consideration all of these observations, the experiments described here indicate that the α subunit is disproportionately distributed across the lipid bilayer. Three-fourths of its hydrophilic surface area is located on the cytoplasmic side and one-fourth on the extracytoplasmic side of the membrane. Inherent in this analysis is the assumption that the distribution of labeled amino acids accurately reflects the distribution of hydrophilic segments of the protein. The amino acids that form azo derivatives are histidine, lysine, and tyrosine (Howard & Wild, 1956). The reactive functionalities on these amino acids are only rarely buried in the tertiary structure of a protein (Chothia, 1976), and consequently, free access of the reagent to these reactive locations can be assumed. Furthermore, there are over 100 of these residues in each α polypeptide chain (Kyte, 1971b). Therefore, it is to be expected that distribution of these residues across the membrane would reflect the distribution of surface area in the native structure.

An examination of the structural information available for Ca^{2+}-ATPase will reinforce this conclusion. Ca^{2+}-ATPase resembles $(Na^+ + K^+)$-ATPase on such a number of functional and structural points that the two proteins are expected to be derived from a common, ancestral transport protein (Kyte, 1981). The primary sequence of over half of the Ca^{2+}-ATPase is available (Allen et al., 1980). The sequenced fragments are all from hydrophilic domains, and it has been proposed that they are located exclusively in the cytoplasmic portion of the protein (Green et al., 1980). About the same percentage (11% against 8%) of the amino acids is either lysine, histidine, or tyrosine in the sequenced fragments as in the total amino acid composition (MacLennan et al., 1971). It would appear then that the distribution of these three amino acids would provide an accurate indication of the mass distribution of the hydrophilic domains of the Ca^{2+}-ATPase. While this does not require that the same statement be true for $(Na^+ + K^+)$-ATPase, it is a reasonable assumption, given the very similar percentage of these amino acids in the total composition and the close similarity of the two transport enzymes.

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