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# Purification of the Membrane-Spanning Tryptic Peptides of the $\alpha$ Polypeptide from Sodium and Potassium Ion Activated Adenosinetriphosphatase Labeled with 1-Tritiospiro[adamantane-4,3′-diazirine]†

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ABSTRACT: Five long, membrane-spanning tryptic peptides from the $\alpha$ polypeptide of sodium and potassium ion activated adenosinetriphosphatase [$(Na^+ + K^+)$-ATPase] have been purified. $(Na^+ + K^+)$-ATPase, isolated from canine kidney, was exposed to ultraviolet light in the presence of a high concentration of 1-tritiospiro[adamantane-4,3′-diazirine], a carbene precursor that partitions into the bilayer of the membrane. The $\alpha$ polypeptide, modified with 1.2 mol of [$^3$H]adamantylidene (mol of polypeptide)$^{-1}$, was isolated and digested with trypsin. Digestion with trypsin ensures that membrane-spanning sequences remain intact during the digestion, since lysine and arginine, being extremely hydrophilic, rarely appear in the membrane-embedded regions of membrane proteins. This digestion produced radioactive tryptic peptides greater than 25 residues in length. The tryptic digest of the labeled $\alpha$ polypeptide was chromatographed on Sephadex LH-60 in ethanol-formic acid, 4:1. The majority of the radioactivity (87%) eluted with distribution coefficients corresponding to peptides longer than melittin (26 residues), whereas 73% of the protein traveled with distribution coefficients corresponding to peptides less than 30 residues in length. Five radioactive peptides were further purified by high-pressure liquid chromatography, and each peptide displayed a unique, hydrophobic amino-terminal sequence. No other candidates could be found when a search for additional membrane-spanning peptides was conducted. Gel filtration of the tryptic peptides from the $\alpha$ polypeptide of $(Na^+ + K^+)$-ATPase labeled with 5-[$^{125}$I]iodo-1-naphthyl azide, a lipophilic nitrene precursor, produced no additional radioactive components. Amino-terminal sequences and amino acid compositions of the five purified peptides are presented.

Sodium and potassium ion activated adenosinetriphosphatase [$(Na^+ + K^+)$-ATPase][1] catalyzes the transport of three sodium ions out of the cell and two potassium ions into the cell, with the concomitant hydrolysis of MgATP (Skou, 1964). Purified preparations contain two components: a catalytic polypeptide of $M_r$ 110000 ± 15000 designated $\alpha$ and a sialoglycoprotein of unknown function with a molecular weight of 55000 ± 10000 designated $\beta$ (Craig & Kyte, 1980; Peterson & Hokin, 1981). The $\alpha$ polypeptide is known to span the membrane (Kyte, 1974) and contains the site of phosphorylation (Uesugi et al., 1971; Kyte, 1971a) and at least part of the binding site for cardiac glycosides (Ruoho & Kyte, 1974).

Since the homologous enzyme, calcium ion activated adenosinetriphosphatase ($Ca^{2+}$-ATPase), does not require a $\beta$ polypeptide in order to function (MacLennan, 1970), the $\alpha$

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[1] Abbreviations: $(Na^+ + K^+)$-ATPase, sodium and potassium ion activated adenosinetriphosphatase; $Ca^{2+}$-ATPase, calcium ion activated adenosinetriphosphatase; [$^3$H]adamantanediazirine, 1-tritiospiro-[adamantane-4,3′-diazirine]; HPLC, high-pressure liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; TPCK-trypsin, trypsin treated by the manufacturer with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; CM-Cys, (carboxymethyl)cysteine; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid.

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polypeptide alone must form the pathway through the membrane for the cations, and any knowledge of the molecular structure of the transmembrane portion of this subunit would be very important for an understanding of the mechanism of active transport. Recent evidence strongly indicates that the monomeric complex containing only one $\alpha$ and one $\beta$ polypeptide exhibits full enzymatic activity (Craig, 1982). For this to be so, the sodium and potassium ions must pass across the membrane through a passage formed by only one $\alpha$ polypeptide. How this is accomplished could be more clearly understood if the sequences of all of the membrane-spanning portions of this protein were known.

To identify such peptides, 1-tritiospiro[adamantane-4,3′-diazirine] ([$^3$H]adamantanediazirine), a radioactive carbene precursor that partitions into the lipid bilayer, has been employed (Bayley & Knowles, 1980). Upon exposure to light, this reagent labels portions of the $\alpha$ polypeptide of ($Na^+$ + $K^+$)-ATPase that are embedded in the membrane (Farley et al., 1980). In the present studies, labeled $\alpha$ polypeptide was isolated and a complete tryptic digestion was performed.

Although tryptic digestion of this protein produces over 100 fragments, the reason for choosing trypsin is straightforward. Lysine and arginine, being the most hydrophilic amino acid residues known (Chothia, 1976; Kyte & Doolittle, 1982), rarely appear in the membrane-embedded regions of membrane-spanning proteins (Khorana et al., 1979; Claudio et al., 1983; Noda et al., 1983; Anderson et al., 1982). Therefore, a peptide that spans the membrane should remain intact during the digestion. If a peptide transverses the membrane, the thickness of whose lipid bilayer is 40 Å (Levine & Wilkins, 1971), as an $\alpha$ helix (Henderson & Unwin, 1975), whose rise is 1.5 Å (amino acid residue)$^{-1}$ (Pauling & Corey, 1955), it would require approximately 26 amino acids to span the membrane. The membrane-spanning peptides produced by tryptic digestion of the $\alpha$ polypeptide should therefore be at least 26 residues in length, significantly larger than the mean length of 10 residues (tryptic peptide)$^{-1}$ (Kyte, 1972). These larger peptides could then be separated from the large number of smaller tryptic peptides produced from the cytoplasmic and extracytoplasmic regions of the protein.

Another aspect of this work was a comparison between the labeling achieved with [$^3$H]adamantanediazirine and that obtained with the lipophilic nitrene precursor, 5-[$^{125}$I]iodo-1-naphthyl azide (Bercovici & Gitler, 1978). There has been much debate concerning the advantages in selectivity or lifetime of carbenes or nitrenes in labeling proteins from within the bilayer of the membrane (Bayley & Knowles, 1978a,b; Farley et al., 1980; Jørgensen et al., 1982). In none of these studies, however, was the same protein labeled separately by the two reagents under similar circumstances and then submitted to digestion and peptide mapping. This would be a method by which the selectivity of these two reagents could be unequivocally compared.

The present report details the isolation of five membrane-spanning peptides from the $\alpha$ polypeptide of ($Na^+$ + $K^+$)-ATPase that had been labeled with [$^3$H]adamantanediazirine while in its native conformation. The five peptides were purified selectively from the majority of the peptides generated by trypsin by using gel filtration and high-pressure liquid chromatography (HPLC), and several residues of their amino-terminal sequences were determined. All five peptides exhibited unique amino-terminal sequences containing a very high frequency of hydrophobic amino acids. Analysis of the tryptic digest of the $\alpha$ polypeptide labeled with 5-[$^{125}$I]iodo-1-naphthyl azide showed that the same set of peptides was labeled by this reagent as well. A comparison of the labeling pattern observed with both reagents is presented.

## Experimental Procedures

*Materials.* Sodium dodecyl sulfate ($NaDodSO_4$) was recrystallized from 95% ethanol. Iodoacetamide was recrystallized from 90% ethanol. Trifluoroacetic acid and formic acid were redistilled before use. HPLC grade 2-propanol was from Burdick & Jackson. Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK–trypsin) was purchased from Worthington Biochemical Corp.

*Synthesis of [$^3$H]Adamantanediazirine.* The synthesis of [$^3$H]adamantanediazirine was exactly as described by Bayley & Knowles (1980). After purification on silica gel, it contained no detectable radioactive impurities on thin-layer chromatography with systems B and C of Bayley & Knowles (1980) and had an identical ultraviolet spectrum to that of unlabeled material. It was stored at −20 °C in ethanol (1 mCi mL$^{-1}$), and the final specific radioactivity of undiluted product was $4 \times 10^8$ cpm $\mu$mol$^{-1}$.

*Synthesis of 5-[$^{125}$I]Iodo-1-naphthyl Azide.* 5-Nitro-1-naphthylamine was converted to 5-nitro-1-naphthyl azide by the method of Foster & Fierz (1907). Treatment of the product with 0.3 M $Na_2S_2O_4$ in 80% ethanol at 60 °C for 15 min produced 5-amino-1-naphthyl azide in 20% yield. The 5-amino-1-naphthyl azide (0.5 $\mu$mol) was converted to 5-[$^{125}$I]iodo-1-naphthyl azide by the method of Bercovici & Gitler (1978) using 10 $\mu$L of a 9 mg mL$^{-1}$ NaI solution containing 1 mCi of carrier-free Na$^{125}$I. The product, after purification on silica gel, showed no detectable radioactive impurities on thin-layer chromatography and was stored in ethanol at −20 °C in the dark. The final specific radioactivity of undiluted product was $1.8 \times 10^9$ cpm $\mu$mol$^{-1}$.

*Preparation of ($Na^+$ + $K^+$)-ATPase and $\alpha$ Polypeptide.* ($Na^+$ + $K^+$)-ATPase was purified from canine kidney by the method of Jørgensen (1974), as modified by Munson (1981). The specific enzymatic activity of these preparations was between 800 and 1000 $\mu$mol of $P_i$ mg$^{-1}$ h$^{-1}$. Determination of protein was by the method of Lowry et al. (1951) as described by Kyte (1971b). The $\alpha$ polypeptide was isolated on a column of Sepharose 4B equilibrated with 0.04 M Tris–$SO_4$, pH 8.0, and 0.1% $NaDodSO_4$ (Kyte, 1972) and was identified by electrophoresis on 5% polyacrylamide–$NaDodSO_4$ gels (Weber & Osborn, 1969). The concentration of $\alpha$ polypeptide was determined by quantitative amino acid analysis (Kyte, 1971b).

*Labeling of ($Na^+$ + $K^+$)-ATPase with [$^3$H]Adamantanediazirine and Isolation of $\alpha$ Polypeptide.* [$^3$H]-Adamantanediazirine ($1.95 \times 10^8$ cpm $\mu$mol$^{-1}$), dissolved in absolute alcohol at 27 mM, was mixed with a solution of ($Na^+$ + $K^+$)-ATPase on ice and in the dark so that the final concentrations would be 9.2 mg of protein mL$^{-1}$, 10 mM endogenous phospholipid, 0.92 mM [$^3$H]adamantanediazirine, 30 mM histidinium chloride, pH 7.1, 0.25 M sucrose, 1 mM EDTA, 1% 2-mercaptoethanol, and 3.3% ethanol. After 30 min, the sample was irradiated for 3.5 min at 4 °C with the light from a 400-W, high-pressure mercury lamp (General Electric HR400 DX33-1) as described by Munson (1981). After irradiation, the solution was made 4 g (g of protein)$^{-1}$ in $NaDodSO_4$ and heated at 100 °C for 1 min. It was applied to a column of Bio-Gel A5-m (2.6 cm × 90 cm) equilibrated with 0.04 M Tris–$SO_4$, pH 8.0, and 0.1% $NaDodSO_4$, and the $\alpha$ polypeptide labeled by [$^3$H]adamantylidene was eluted with the same buffer (Kyte, 1972). It was identified as the $\alpha$ polypeptide by electrophoresis on polyacrylamide gels. The bulk of the material was stored at 2.7 mg mL$^{-1}$ in 1.8% Na-

$DodSO_4$ at −70 °C and used for several successive experiments.

*Labeling of $(Na^+ + K^+)$-ATPase with 5-[$^{125}I$]Iodo-1-naphthyl Azide and Isolation of the α Polypeptide.* 5-[$^{125}$I]Iodo-1-naphthyl azide ($7.4 \times 10^8$ cpm μmol$^{-1}$), dissolved in ethanol at 0.15 mM, was mixed with a solution of $(Na^+ + K^+)$-ATPase on ice so that the final concentrations would be 3.2 mg of protein mL$^{-1}$, 3.5 mM endogenous phospholipid, 7.5 μM 5-[$^{125}$I]iodo-1-naphthyl azide, 30 mM histidinium chloride, pH 7.1, 0.25 M sucrose, 1 mM EDTA, 0.3% 2-mercaptoethanol, and 4.7% ethanol. After 1 h, the sample was irradiated with the same light source for 22 s at 4 °C, and the solution was then made 6 g (g of protein)$^{-1}$ in $NaDodSO_4$ and 1% in 2-mercaptoethanol, and heated at 50 °C for 5 min. The α polypeptide, labeled by the 5-[$^{125}$I]iodo-1-naphthylnitrene, was isolated by gel filtration. The fractions containing the α polypeptide were pooled, a portion ($2.2 \times 10^5$ cpm, 0.5 mg) was mixed with $4 \times 10^5$ cpm of α polypeptide labeled with [$^3$H]adamantanediazirine and 3.5 mg of unlabeled α polypeptide, and the mixture was lyophilized.

*Carboxamidomethylation.* In a typical experiment, a portion ($2.5 \times 10^6$ cpm $^3$H, 1.6 mg of protein) of labeled α polypeptide was mixed with 22 mg of unlabeled α polypeptide, and the mixture was dialyzed overnight against 0.2 M sodium borate, pH 9.5, and 10 mM dithiothreitol under Ar. The dialysate was made 8 M in urea and 5% in 2-mercaptoethanol, and the solution was incubated at 37 °C for 2 h under $N_2$. Following this incubation, the solution was dialyzed against 0.2 M sodium borate, pH 9.5, 8 M urea, and 10 mM dithiothreitol under Ar for 2 h. Solid iodoacetamide was added to the solution to a final concentration of 50 mM, and the pH was carefully monitored with indicator paper and maintained at 9.5. After 40 min at room temperature, additional iodoacetamide was added to make the solution 70 mM. The presence of free thiol groups was monitored throughout the reaction with 5,5′-dithiobis(2-nitrobenzoate). After all of the free thiol groups had reacted, the mixture was allowed to sit an additional 20 min, and it was dialyzed against 0.01 M Tris–acetate, pH 7.4, and lyophilized.

*Removal of $NaDodSO_4$.* The $NaDodSO_4$ was removed from the protein by the methods described by Sharkey (1983) and Weber & Kuter (1971). The lyophilized protein was dissolved in a minimum (1.7 mL) of $H_2O$, and recrystallized urea was added to 8 M. An aliquot (1 μL) was removed and assayed for $NaDodSO_4$ by the method of Mukerjee (1956), as modified by Sharkey (1983). Beaded XAD-4 resin [1.0 mL of packed, damp beads (100 mg of $NaDodSO_4$)$^{-1}$], prepared by extensive washing in methanol, $H_2O$, and then 0.1 M Tris–acetate, pH 8.0, was added to the solution of protein and $NaDodSO_4$ (Sharkey, 1983). The mixture was stirred at room temperature, and the removal of $NaDodSO_4$ by the XAD-4 beads was monitored by the assay for $NaDodSO_4$. When the concentration of $NaDodSO_4$ fell below 0.1%, the solution was removed from the beads. The beads were washed twice with 0.2 mL of 50 mM Tris–acetate, pH 7.8, and 8 M urea, and the washes were combined with the original solution. Dowex AG 1-X2 was prepared as described by Weber & Kuter (1971), and a small amount [10 μL of packed resin (mg of $NaDodSO_4$)$^{-1}$] was added to the solution. The mixture was stirred for 1 h, after which it was filtered through glass wool. The beads were washed twice with 0.2 mL of 50 mM Tris–acetate, pH 8.0, and 8 M urea, and the stripped protein was dialyzed at 4 °C against 50 mM $NH_4HCO_3$, pH 8.0. The final volume of the solution of protein was 3.8 mL. The α polypeptide, uncomplexed by detergent, is fully soluble in dilute aqueous buffer (Kyte, 1972). The yield of stripped, carboxamidomethylated α polypeptide to this point, based on the amount of protein present before carboxamidomethylation, was always 80–90%.

*Tryptic Digestion and Gel Filtration.* TPCK–trypsin was dissolved in 50 mM $NH_4HCO_3$, pH 8.0, at a concentration of 1 mg mL$^{-1}$ and used immediately. To the dialyzed solution of stripped α polypeptide was added 15 μg of TPCK–trypsin (mg of protein)$^{-1}$. The mixture was brought to 37 °C, after 6 h another aliquot of TPCK–trypsin was added, and then the solution was incubated overnight. The digest was lyophilized, and the protein (~20 mg) was dissolved in 1.0 mL of 88% formic acid. Ethanol (4.0 mL) and 2-mercaptoethanol (40 μL) were added, and the clear solution that resulted was applied to a column of Sephadex LH-60 (4 cm × 96 cm) equilibrated with 95% ethanol–88% formic acid, 4:1 (v/v). The tryptic peptides were eluted with the same solvent at 10 mL h$^{-1}$. The distribution of $^3$H was determined by liquid scintillation, and as necessary, the distribution of $^{125}$I was determined by counting γ radiation.

*Purification of Peptides by HPLC.* The pools from gel filtration were lyophilized, dissolved in 1.5 mL of 88% formic acid, and relyophilized. The protein was dissolved in 88% formic acid, and a portion was chromatographed on a high-pressure liquid chromatographic system consisting of the following components from Waters Associates: two M6000A pumps, a UK6 injector, a 680 automated gradient controller, and a 440 UV detector equipped with an extended wavelength module operating at 229 nm. One of the pumps delivered 0.1% (v/v) aqueous trifluoroacetic acid (solvent A) (Mahoney & Hermodson, 1980), and the other pump delivered 2-propanol (solvent B). Samples were injected onto either a Waters μBondapak CN column (0.39 cm × 30 cm, pools II–IV) or a Vydac 300-Å pore size C4 column (0.46 cm × 25 cm, pool V) equilibrated with various concentratioins of solvent B in solvent A. The peptides were eluted with a linear increase in the concentration of solvent B in solvent A at a flow rate of 1 mL min$^{-1}$.

*Sequencing.* Sodium dodecyl sulfate (10–30 μL of a 20% solution) was added to the pools from the HPLC, and the samples were lyophilized. The peptides were dissolved in 1.5 mL of $H_2O$ and lyophilized again. A suitable portion of each pool (1–10 nmol) was dissolved in 0.5 M $NaHCO_3$, pH 9.8, and the peptides were sequenced by using the dansyl–Edman procedure outlined by Weiner et al. (1972) with the following modifications. After the incubation of the coupled peptide in trifluoroacetic acid at 50 °C for 5 min, 10 μL of 100 mg mL$^{-1}$ $NaDodSO_4$ in trifluoroacetic acid was added. An appropriate amount of the above solution was transferred to a small tube, the solvent was removed under a gentle stream of $N_2$, and dansylation of the resulting residue was performed after drying in vacuo for 30 min at 60 °C. Succinylated lysozyme was then added, and the protein was precipitated with 9 volumes of acetone rather than 20% trichloroacetic acid.

*Amino Acid Analysis.* Sodium dodecyl sulfate (20 μL of a 20% solution) was added to the pooled peptides from HPLC, which were then lyophilized and redissolved in $H_2O$. The samples were split into two equal aliquots and lyophilized in hydrolysis tubes. Norleucine, an internal standard, was included. One aliquot was hydrolyzed in 6 M HCl at 110 °C for 24 h, and the other aliquot was hydrolyzed in concentrated HCl–trifluoroacetic acid, 2:1, for 50 min at 157 °C (Tsugita & Scheffler, 1982). The latter conditions were chosen in order to obtain a maximum yield of the hydrophobic amino acids (valine, isoleucine, leucine, and phenylalanine) for each peptide. This procedure was tested by hydrolyzing poly(L-valine) for

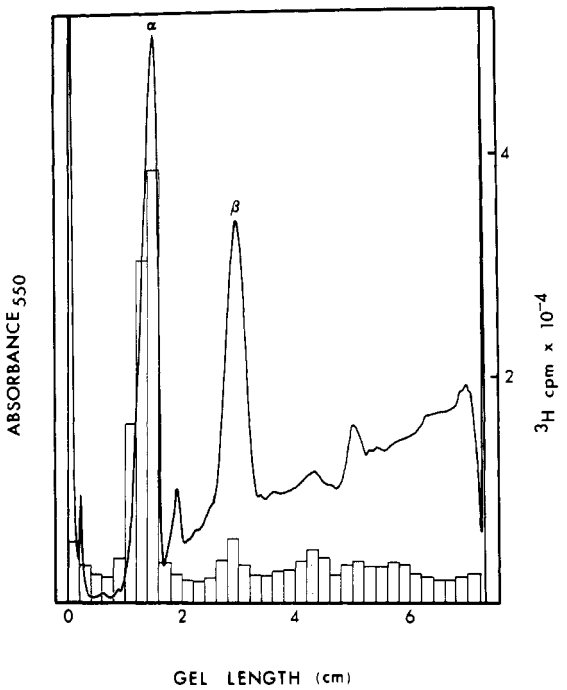


FIGURE 1: Incorporation of [$^3$H]adamantanediazirine into native ($Na^+$ + $K^+$)-ATPase. ($Na^+$ + $K^+$)-ATPase (3.8 mg $mL^{-1}$), as a suspension of membrane fragments, was incubated with 0.5 mM [$^3$H]-adamantanediazirine (4.2 × $10^8$ cpm $mol^{-1}$) in 30 mM histidinium chloride, pH 7.1, 0.25 M sucrose, 1 mM EDTA, 1% 2-mercaptoethanol, and 10% ethanol in the dark on ice for 45 min. The solution was irradiated with a continuous ultraviolet light source at 4 °C for 105 s. After photolysis, the sample was made 4% in $NaDodSO_4$ and heated at 100 °C for 1 min. A portion (50 μg) was subjected to electrophoresis on a 5% polyacrylamide–$NaDodSO_4$ gel. Following electrophoresis, the gel was stained and scanned at 550 nm to locate the protein bands. The gel was then sliced into 2-mm slices, and the radioactivity of each slice was determined after incubation in 5 mL of 4.6% Protosol and 4.6% Liquifluor in toluene at 50 °C for 24 h (bar graph). The electrophoresis was from left to right and the large (α) and small (β) polypeptides are indicated.

various times and measuring the amount of valine produced. The yield of valine was 67% after 250 min and reached 100% at 750 min. Of several methods attempted, this hydrolysis procedure gave 100% yield of valine from poly(L-valine) in the shortest amount of time. When the α polypeptide was hydrolyzed in concentrated HCl–trifluoroacetic acid, 2:1, at 157 °C, a maximum yield of the hydrophobic amino acids was obtained at 50 min, but the yields of methionine, serine, threonine, and tyrosine were low (yield of serine was decreased by 35% and threonine by 20%). Consequently, values for these amino acids, as well as (carboxymethyl)cysteine (CM-Cys), were obtained from the hydrolysis of the peptides in 6 M HCl for 24 h at 110 °C. The amount of tryptophan was estimated from the ratio of the absorbance at 229 nm to the absorbance at 280 nm obtained during HPLC, corrected for the approximate number of peptide bonds. This was calibrated by the ratio of the absorbance at 229 nm to the absorbance at 280 nm obtained for melittin [1 tryptophan (25 peptide bonds)$^{-1}$] upon HPLC.

## Results

*Incorporation of [$^3$H]Adamantylidene into Native ($Na^+$ + $K^+$)-ATPase.* Preliminary experiments revealed that, at the fluxes of light employed, the incorporation of [$^3$H]-adamantylidene into the α polypeptide of ($Na^+$ + $K^+$)-ATPase reached a maximum at 1 min and showed no further increase for 30 min. A high concentration of protein (9.2 mg $mL^{-1}$) was used to ensure maximum noncovalent incorporation of

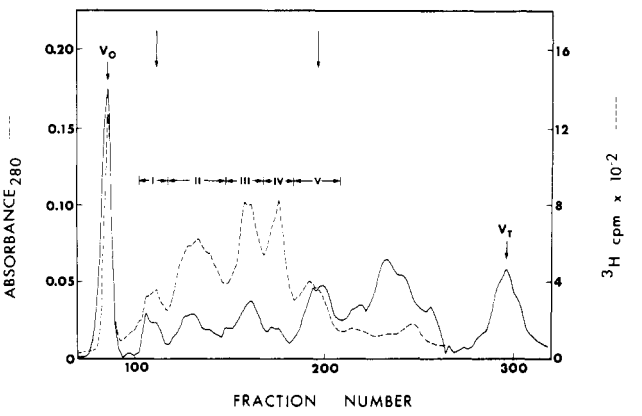


FIGURE 2: Gel filtration on Sephadex LH-60 of a tryptic digest of α polypeptide from ($Na^+$ + $K^+$)-ATPase labeled with [$^3$H]-adamantylidene. Purified α polypeptide (1.6 mg, 2.5 × $10^6$ cpm of $^3$H), labeled with [$^3$H]adamantylidene, was mixed with 22 mg of unlabeled α polypeptide, and the mixture was carboxamidomethylated, stripped of $NaDodSO_4$, and digested with trypsin. The digest was lyophilized and then dissolved in 1.0 mL of 88% formic acid. Ethanol (95%, 4.0 mL) and 2-mercaptoethanol (40 μL) were added, and the solution was applied to a Sephadex LH-60 column (4 cm × 96 cm) equilibrated with ethanol–formic acid, 4:1. The tryptic peptides were eluted with the same solvent at a flow rate of 10 mL $h^{-1}$. Fractions of 3.3 mL were collected, the absorbance at 280 nm was determined (solid line), and 40-μL aliquots were removed for measurement of $^3$H cpm (dashed line). The five radioactive pools (I–V) described in the text are indicated, as well as the void ($V_0$) and total volume ($V_T$) of the column. The arrows mark the elution positions of cytochrome *c* and melittin.

[$^3$H]adamantanediazirine into the lipid bilayer prior to photolysis (Bayley & Knowles, 1980). The high stoichiometries used in these experiments [0.1 mol of diazirine (mol of phospholipid)$^{-1}$] were chosen to guarantee that all of the sequences of ($Na^+$ + $K^+$)-ATPase which are embedded within the membrane would be labeled.

The distributions of radioactivity and staining that resulted when ($Na^+$ + $K^+$)-ATPase that had been labeled by [$^3$H]-adamantylidene was submitted to electrophoresis on a $NaDodSO_4$–polyacrylamide gel are displayed in Figure 1. A large majority of the radioactivity traveled with the α polypeptide, but minor incorporation into the β polypeptide was also observed.

*Tryptic Peptides from the α Polypeptide Labeled with [$^3$H]Adamantylidene.* The α polypeptide, from ($Na^+$ + $K^+$)-ATPase labeled with [$^3$H]adamantylidene, was isolated by gel filtration in a buffer containing 0.1% $NaDodSO_4$ (Kyte, 1972) and mixed with previously purified, unlabeled α polypeptide. This mixture was carboxamidomethylated, stripped of the $NaDodSO_4$, and digested with trypsin. The digest was submitted to gel filtration on Sephadex LH-60 equilibrated with 95% ethanol–88% formic acid, 4:1 (Khorana et al., 1979; Takagaki et al., 1980). The distribution of radioactivity and absorbance at 280 nm over the elution profile is shown in Figure 2.

Five distinct peaks of radioactive material within the included volume were readily apparent. A calibration of the column with standards, whose elution positions are indicated in Figure 2, verified that the radioactive peptides were indeed large. The majority (87%) of the radioactivity traveled with distribution coefficients corresponding to peptides longer than melittin, a protein 26 residues in length. In a separate experiment, the distribution of total peptide was determined on an analytical column of Sephadex LH-60. As seen in Figure 3, the majority (73%) of the protein eluted with distribution coefficients corresponding to peptides less than 30 residues in length. These results demonstrated that tryptic peptides

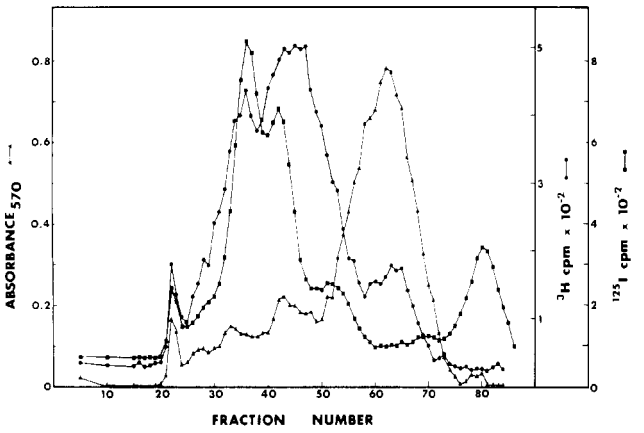


FIGURE 3: Distribution of tryptic peptides from a mixture of 5-[$^{125}$I]iodo-1-naphthylnitrene labeled, [$^{3}$H]adamantylidene labeled, and unlabeled $\alpha$ polypeptide submitted to chromatography on Sephadex LH-60. Purified $\alpha$ polypeptide, labeled with 5-[$^{125}$I]iodo-1-naphthylnitrene ($2.2 \times 10^5$ cpm), was mixed with $\alpha$ polypeptide labeled with [$^{3}$H]adamantylidene ($4 \times 10^5$ cpm) and 3.5 mg of unlabeled $\alpha$ polypeptide. The mixture was carboxamidomethylated, stripped of $NaDodSO_4$, and digested with trypsin. Following lyophilization, the digest was dissolved in 0.15 mL of 88% formic acid, and 0.4 mL of ethanol was added. The solution was applied to a column of Sephadex LH-60 (1.6 cm × 36 cm) equilibrated with ethanol–formic acid, 4:1, and the tryptic peptides were eluted in the same solvent at a flow rate of 7 mL h$^{-1}$. Fractions of 1.0 mL were collected and $^{125}$I and $^{125}$I + $^{3}$H determined by $\gamma$ and liquid scintillation counting, respectively. Net $^{3}$H radioactivity was calculated by correcting for the contribution due to $^{125}$I. The distribution of protein was determined by the alkaline ninhydrin method of Hirs et al. (1956). (●) $^{3}$H radioactivity; (■) $^{125}$I radioactivity; (▲) absorbance at 570 nm following alkaline ninhydrin reaction.

containing the membrane-spanning regions and identified by [$^{3}$H]adamantylidene could be separated on the basis of their length from the majority of the peptides generated by trypsin. The mean apparent lengths for the peptides in each pool, determined from the calibration of the column (Figure 2), were 110 residues, 70 residues, 50 residues, 35 residues, and 27 residues for pools I–V, respectively.

*Purification of Tryptic Peptides on HPLC.* Pools II–V (Figure 2) were submitted to further purification on HPLC. Distributions of the absorbance at 229 nm and $^{3}$H for each of the four pools are shown in Figure 4 and Figure 5. It was clear that upon HPLC the radioactivity no longer coeluted with the unlabeled peptides that formed the large majority of the mass of protein injected and that these chromatographic systems could detect the presence of the adamantyl group. Consequently, regions were pooled according to the absorbance at 229 nm for further analysis. A single radioactive peptide, designated T2, was isolated from pool II (Figure 4A), and it had the amino-terminal sequence Ile-Ala-Thr-Leu-. Pool III (Figure 4B) contained a major peptide, T3, with the amino-terminal sequence Val-Leu-Gly-Phe-. Purification of pool IV (Figure 4C) on HPLC, however, produced two major peptides: T4, which had an amino-terminal sequence Leu-Ile-X-Leu-Ala-, and T4a, which had an amino-terminal sequence Leu-Phe-Leu-Phe-. Both the profile of absorbance at 229 nm and the profile of $^{3}$H for pool V (Figure 5) displayed a broad, unresolved multiplet of peaks, and the envelope of the radioactivity appeared to mimic the multiplet of absorbance. These observations suggested that this broad, heterogeneous envelope contained either several peptides, each of which was labeled by [$^{3}$H]adamantylidene, or one peptide, labeled by [$^{3}$H]-adamantylidene, that had become microheterogeneous during its purification. The latter possibility was shown to be the correct one by pooling the indicated region in Figure 5 and submitting the protein to sequencing by the dansyl–Edman

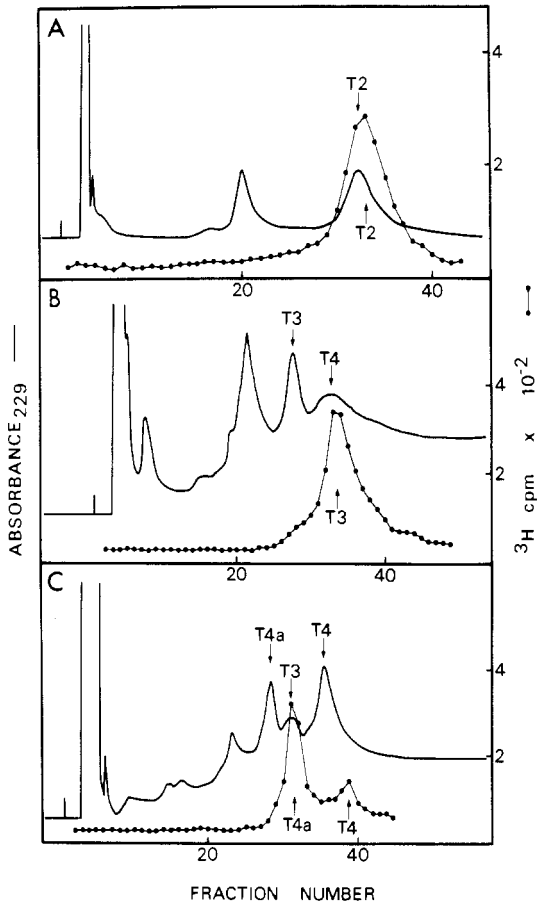


FIGURE 4: Purification of membrane-spanning peptides by HPLC upon a $\mu$Bondapak CN column (0.39 × 30 cm). Pools II–IV from Figure 2 were lyophilized and redissolved in 88% formic acid. (A) A sample (50 $\mu$L, $2 \times 10^4$ cpm) of pool II was injected onto the column equilibrated in 0.1% trifluoroacetic acid (solvent A) and eluted with a linear gradient of 100% solvent A to 100% 2-propanol (solvent B) over 1 h. (B) A sample (50 $\mu$L, $2.7 \times 10^4$ cpm) of pool III was injected onto the column and eluted with a linear gradient of 30% solvent B in A to 80% solvent B in A over 45 min. (C) A sample (50 $\mu$L, $1.9 \times 10^4$ cpm) of pool IV was injected onto the column and eluted with a linear gradient of 30% solvent B in A to 70% solvent B in A over 1 h. In all cases, fractions of 1 mL were collected, and 150-$\mu$L aliquots were removed to determine $^{3}$H cpm. Peptides producing the $A_{229}$ were identified by manual sequencing, and the identifications are indicated *above* the respective protein peak. The unidentified peaks contained no protein and are presumably organic impurities from ethanol–formic acid. The identity of the peptide to which each radioactive peak was assigned is indicated *underneath* the radioactive peak.

procedure. An unambiguous sequence of Met-Tyr-Leu-Pro- was obtained. The origin of this microheterogeneity has not been identified, but the partial oxidation of methionine or tryptophan and the partial hydrolysis of asparagine or glutamine are obvious possibilities. Each of these five peptides is identified in the respective figures. All five peptides, T2 through T5, were isolated and sequenced from two separate digests.

Since pool III and pool IV were directly adjacent to each other on the original gel filtration column (Figure 2), they were cross-contaminated. The cross-contaminants, T4 and T3, are labeled in parts B and C of Figure 4, respectively. Their identity was confirmed by amino-terminal sequencing.

*Search for Additional Membrane-Spanning Peptides.* To determine whether there were any other membrane-spanning peptides that were present within the elution profile of the column of Sephadex LH-60 (Figure 2) but that had gone undetected during HPLC, amino-terminal analysis was performed directly on the pools shown in Figure 2. The identity

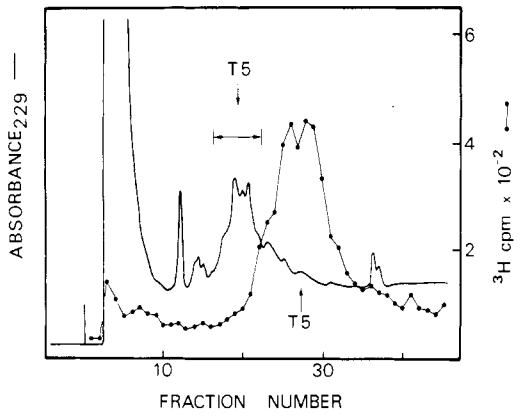


FIGURE 5: Purification of peptide T5 by HPLC upon a Vydac C-4 bonded column. Pool V from Figure 2 was lyophilized and redissolved in 1.0 mL of 88% formic acid. A sample (50 μL, 9.5 × $10^3$ cpm) was injected onto a Vydac 300-Å pore size C-4 bonded stationary phase column (0.46 cm × 25 cm) equilibrated in 7 parts 0.1% trifluoroacetic acid (solvent A)–3 parts 2-propanol (solvent B). The sample was eluted with a linear gradient of 30% solvent B in A to 60% solvent B in A over 1 h. In this one run, each entire fraction was used to determine $^3$H cpm. In subsequent runs, the region indicated was pooled and sequenced.

and approximate intensity of the amino acid residues obtained in each Edman cycle from pools I to V, as well as the void volume, are shown in Table I. Only a few amino acids appeared in each cycle from a given pool. This suggests that the majority of the purification had already occurred on gel filtration. The major amino-terminal residue present in pools II–V was identical with the amino-terminal residue of the major radioactive peptide purified from that pool. Further sequencing of pools II–IV through four cycles provided additional confirmation of the sequences obtained with the purified peptides. Few additional amino acids, beyond those present at a given position in the four major peptides, were detected in significant amounts in any of the cycles. The sequence of T4a (Leu-Phe-Leu-Phe-) was not detected when pool IV was sequenced. Analysis by HPLC showed that the yield of T4a in this particular digest was very low, and thus its sequence could not be observed due to the large amounts of T3 and T4 in pool IV. A subsequent digest contained a much larger amount of T4a than did the previous one, and the HPLC profile in Figure 4C was obtained from the latter digest. Even though the major amino acid was methionine, several other amino acids were also detected when pool V was submitted to amino-terminal analysis. This is consistent with its coelution with the leading edge of the large number of small tryptic peptides (Figure 2).

The amplitude of the void volume from the column of Sephadex LH-60 was found to vary significantly (compare Figures 2 and 3). When the void volume from the run presented in Figure 2 was digested with trypsin and resubmitted to chromatography on Sephadex LH-60, all of the peaks previously seen (I–V) were produced, and each peak had an identical distribution coefficient to its counterpart in Figure 2. More important, however, was that no new radioactive peaks could be found. It was therefore concluded that the original void volume contained only products of partial digestion or aggregates of the five membrane-spanning peptides previously isolated. This conclusion is consistent with the distribution of amino acid residues observed upon amino-terminal analysis of these fractions (Table I).

Pool I was submitted to gradient elution on HPLC using a CN-bonded stationary phase, and two radioactive peptides, both obtained in low yield, were isolated. The amino-terminal

Table I: Sequencing of the Pools from Figure 2[a]

| | residue[b] | | | | |
|---|---|---|---|---|---|
| pool | $n_0$ | $n_{-1}$ | $n_{-2}$ | $n_{-3}$ | $n_{-4}$ |
| $V_o$ | Leu (4)[c] | | | | |
| | Ile (3) | | | | |
| | Lys (3) | | | | |
| | Gly (2) | | | | |
| | Ala (2) | | | | |
| I | Leu (3) | Ile (3) | Ile (2) | Leu (3) | |
| | Lys (2) | Leu (3) | Ala (2) | Phe (3) | |
| | Ile (1) | Ala (1.5) | | | |
| IIa | Ile (4) | Ala (4) | Thr (3.5) | Leu (5) | |
| | Leu (2.5) | Ile (2) | Ala (2) | Phe (3) | |
| IIb | Leu (4) | Ile (3) | Val (3) | Phe (5) | Ala (3.5) |
| | Ile (2) | Ala (2.5) | Thr (3) | Leu (3) | Phe (3) |
| | Ala (3) | Leu (4) | Gly (2) | | Leu (2.5) |
| III | Val (4) | Leu (5) | Gly (4) | Phe (3) | Phe (3) |
| | V-L[d] (3) | Ile (3) | Ala (2) | Leu (2) | Leu (3) |
| | Leu (2) | Val (1) | Leu (1) | Gly (3) | Gly (3) |
| | | | | | Ala (2) |
| IV | Leu (4) | Ile (5) | Leu (3) | Leu (3) | Ala (4) |
| | Val (2) | Leu (3) | Gly (4) | Phe (4) | Phe (3) |
| | V-L[d] (2) | | Ala (2) | Gly (2.5) | Gly (2.5) |
| V | Met (4) | | | | |
| | $MetSO_2$[e] (2) | | | | |
| | Ile (2) | | | | |
| | Leu (2) | | | | |
| | Gly (2) | | | | |
| | Ala (2) | | | | |
| | Lys (2) | | | | |

[a] Samples (~10 nmol) from the pools shown in Figure 2 were lyophilized after adding $NaDodSO_4$ and then sequenced directly by using the dansyl–Edman procedure of Weiner et al. (1972). [b] The products of each Edman cycle are presented, starting with the amino terminus ($n_0$) and followed by those of each successive step ($n_{-i}$). [c] The approximate relative intensity of each dansyl amino acid on the plate from each cycle is indicated in parentheses. [d] Valine-leucine dipeptide. [e] Methionine sulfone.

sequences of these two peptides confirmed that they were also products of partial digestion because the sequences were identical with those of peptides T2 and T4, respectively. This pool, therefore, also did not contain any new membrane-spanning peptides.

From the amino-terminal sequence analysis of pool IIb (Table I), it was clear that, along with the major peptide T2, a product arising from the partial digestion of peptide T4 was also present. Although the major sequence in pool II was that of peptide T2 (Table I), the amino-terminal sequence of T4 (Leu-Ile-X-Leu-Ala-) could be readily followed.

A peptide with alanine as its amino terminus was also seen in the analysis of pool II, but no radioactive peptide bearing an alanine amino-terminal residue could be isolated from this pool by HPLC (Figure 4A). If it existed, either the alanine peptide did not elute from the high-pressure liquid chromatographic column under the conditions used or it was not labeled by [$^3$H]adamantylidene.

*Tryptic Peptides from the α Polypeptide Labeled with 5-[$^{125}$I]Iodo-1-naphthyl Azide.* Another reagent that has been used to label membrane-spanning proteins from within the lipid bilayer is the lipophilic nitrene precursor, 5-[$^{125}$I]iodo-1-naphthyl azide (Bercovici & Gitler, 1978). This reagent was used to label the α polypeptide of ($Na^+ + K^+$)-ATPase in the hope that it might identify membrane-spanning sequences that were not detected with [$^3$H]adamantanediazirine. It has been stated that these two reagents label distinctly different regions of this protein (Jørgensen et al., 1982). This experiment also allowed a direct comparison of the tryptic peptides labeled with 5-[$^{125}$I]iodo-1-naphthylnitrene and those labeled with [$^3$H]-adamantylidene.

The $\alpha$ polypeptide, from $(Na^+ + K^+)$-ATPase labeled with 5-[$^{125}$I]iodo-1-naphthylnitrene and isolated by gel filtration in 0.1% $NaDodSO_4$, was mixed with purified $\alpha$ polypeptide labeled with [$^3$H]adamantylidene. Mixing the two labeled, intact $\alpha$ polypeptides while they were still in a solution of $NaDodSO_4$ ensured that both were treated identically in subsequent steps. The protein was carboxamidomethylated, stripped of $NaDodSO_4$, and digested with trypsin. The digest was chromatographed on an analytical column of Sephadex LH-60, and the distribution of $^{125}$I and $^3$H is shown in Figure 3. Approximately 85% of the $^{125}$I traveled in the regions containing the five peptides previously discussed, and no peptides of either smaller or larger molecular weight were labeled to any significant degree. Although the smaller analytical column was not able to separate the peptides in pools III and IV labeled with [$^3$H]adamantylidene, the distribution of $^3$H shown here was identical with the distribution obtained when a portion of the tryptic digest used in Figure 2 was chromatographed on the same analytical column. Because it had been demonstrated that the regions of the elution profile embracing the $^{125}$I contained only the five peptides previously identified, or the products of their partial digestion, it was concluded that the 5-[$^{125}$I]iodo-1-naphthylnitrene had not labeled any peptides not already labeled by [$^3$H]-adamantylidene.

While the work reported here was in progress, a report appeared (Jørgensen et al., 1982) that also described the labeling of the transmembrane portions of the $\alpha$ polypeptide of $(Na^+ + K^+)$-ATPase with 5-[$^{125}$I]iodo-1-naphthyl azide. In that study the authors concluded that several labeled peptides, all with a molecular weight of 12 000, were produced upon extensive tryptic digestion of the modified protein. This followed from the results of both electrophoresis on 7.5% polyacrylamide gels and chromatography on Sephadex G-50, each run in 0.1% $NaDodSO_4$. The results described in the earlier study appear to contradict those obtained in the present work because several tryptic peptides of very different molecular weight have been isolated. Experiments performed in the early stages of this work, however, can clear up this discrepancy. When a tryptic digest of the $\alpha$ polypeptide labeled with [$^3$H]adamantylidene was submitted to chromatography on Sephadex G-100 in buffer containing 0.2% $NaDodSO_4$ and 8 M urea, over 90% of the radioactivity eluted with a single distribution coefficient (Figure 6). This result is identical with that obtained by Jørgensen et al. (1982). When an identical digest was chromatographed on Sephadex LH-60 in ethanol–formic acid, 4:1, all of the radioactive peaks observed in Figures 2 and 3 resulted (Figure 6, inset). It is likely that the "12K peptides" discussed by Jørgensen et al. (1982) are a heterogeneous mixture of at least the peptides obtained in this work, all of which elute within micelles of $NaDodSO_4$, at the same position on gel filtration.

*Amino Acid Compositions.* The amino acid compositions of the five purified peptides are presented in Table II. The values for valine, isoleucine, leucine, and phenylalanine were obtained from the hydrolysis of the peptides in concentrated HCl–trifluoroacetic acid, 2:1, at 157 °C for 50 min (Tsugita & Scheffler, 1982), whereas the values for serine, threonine, methionine, and tyrosine were obtained from hydrolysis in 6 M HCl at 110 °C for 24 h.

## Discussion

If 110 000 ± 15 000 is the molecular weight of the $\alpha$ polypeptide (Craig & Kyte, 1980; Peterson & Hokin, 1981), then only 1.2 mol of [$^3$H]adamantylidene was incorporated per mol of polypeptide in even the most efficient reactions (Figure 1).

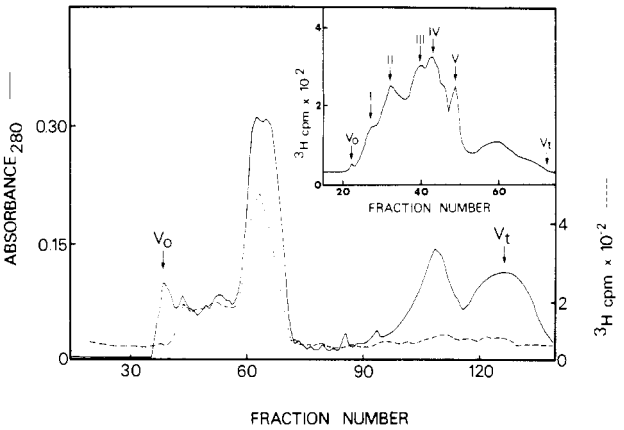


FIGURE 6: Gel filtration of a tryptic digest of the $\alpha$ polypeptide, labeled with [$^3$H]adamantylidene, on Sephadex G-100 in buffer containing $NaDodSO_4$ and urea. A sample (10 mg, $4.4 \times 10^5$ cpm) of $\alpha$ polypeptide, labeled with [$^3$H]adamantylidene, was carboxamidomethylated, stripped of $NaDodSO_4$, and digested with trypsin. The digest was made 25% in acetic acid and extracted 4 times with $^1/_4$ volume of 1-butanol. The butanol phase, which contained 35% of the protein and 70% of the $^3$H present before the extraction, was evaporated to dryness under a stream of $N_2$. The residue was dissolved in 0.4 mL of 40 mM Tris–acetate, pH 7.8, 8 M urea, and 4% $NaDodSO_4$ and applied to a column of Sephadex G-100 (2.1 cm × 38 cm) equilibrated with 40 mM Tris–acetate, pH 7.8, 8 M urea, and 0.1% $NaDodSO_4$. The peptides were eluted with the same buffer at 4.5 mL h$^{-1}$. Fractions of 0.8 mL were collected, the absorbance at 280 nm was determined (solid line), and 25-$\mu$L aliquots were removed for measurement of $^3$H cpm (dashed line). The inset shows the distribution of radioactivity obtained when an identical sample was chromatographed on a column of Sephadex LH-60 (1.6 cm × 36 cm) in ethanol–formic acid, 4:1. The five radioactive peaks identified in Figure 2 are indicated.

Table II: Amino Acid Compositions of the Purified Peptides[a]

| amino acid | peptide | | | | |
|---|---|---|---|---|---|
| | T2 | T3 | T4 | T4a | T5 |
| CM-Cys[b] | 0 (0) | 0 (0) | 0 (0) | 0.8 (1) | 0 (0) |
| Asp | 5.5 (6) | 9.0 (9) | 3.0 (3) | 5.0 (5) | 2.3 (2) |
| Thr[b] | 5.1 (5) | 1.6 (2) | 1.8 (2) | 1.1 (1) | 1.5 (2) |
| Ser[b] | 4.5 (5) | 1.8 (2) | 1.5 (2) | 1.4 (1) | 1.4 (1) |
| Glu | 5.9 (6) | 4.9 (5) | 2.2 (2) | 3.6 (4) | 2.1 (2) |
| Pro | 0 (0) | 4.5 (5) | 1.6 (2) | 2.0 (2) | 2.3 (2) |
| Gly | 10.0 (10) | 4.0 (4) | 4.2 (4) | 2.8 (3) | 1.6 (2) |
| Ala | 7.0 (7) | 1.1 (1) | 2.4 (2) | 1.8 (2) | 1.8 (2) |
| Val[c] | 4.9 (5) | 4.5 (5) | 2.1 (2) | 2.2 (2) | 2.3 (2) |
| Met[b] | 0 (0) | 1.4 (1) | 0 (0) | 1.3 (1) | 0.7 (1) |
| Ile[c] | 7.3 (7) | 3.2 (3) | 5.2 (5) | 1.8 (2) | 1.5 (2) |
| Leu[c] | 7.4 (7) | 5.2 (5) | 4.5 (5) | 3.8 (4) | 2.8 (3) |
| Tyr[b] | 1.2 (1) | 0.1 (0) | 0.5 (1) | 0.3 (0) | 1.5 (2) |
| Phe[c] | 3.2 (3) | 6.1 (6) | 2.6 (3) | 3.8 (4) | 2.2 (2) |
| His | 1.1 (1) | 1.0 (1) | 0.5 (1) | 0.4 (0) | 0.3 (0) |
| Lys | 2.9 (3) | 0.6 (1) | 0.8 (1) | 1.0 (1) | 1.6 (2) |
| Trp[d] | 2 (2) | 0 (0) | 1 (1) | 1 (1) | 1 (1) |
| Arg | 1.9 (2) | 1.1 (1) | 1.0 (1) | 0.8 (1) | 1.1 (1) |
| | (70) | (51) | (37) | (35) | (29) |

[a] Values are expressed as moles of amino acid per mole of peptide. The molecular weight of each peptide was estimated from its elution position on Sephadex LH-60 (Figure 2). Values rounded to the nearest whole number are indicated in parentheses. All values, except those indicated, were averages from hydrolysis in 6 M HCl at 110 °C for 24 h and hydrolysis in concentrated HCl–trifluoroacetic acid, 2:1, at 157 °C for 50 min. [b] Values only from hydrolysis in 6 M HCl at 110 °C for 24 h. [c] Values only from hydrolysis in concentrated HCl–trifluoroacetic acid, 2:1, at 157 °C for 50 min. [d] Content of Trp was estimated from the ratio of $A_{229}(A_{280})^{-1}$ obtained on HPLC (Figures 4 and 5).

[$^3$H]Adamantylidene is known to insert into the $\alpha$ polypeptide at more than one site (Farley et al., 1980), and as a carbene, it should be fairly promiscuous. It was clear at the outset that

it would be very difficult, if not impossible, to incorporate the reagent stoichiometrically at any specific site or set or sites within the membrane-spanning regions of $(Na^+ + K^+)$-ATPase. This consideration rendered impractical the purification of the labeled, radioactive peptides themselves; rather the radioactive peptides were used as tracers for the unlabeled peptides of the same sequence. Unlabeled protein, available in large quantities, was mixed prior to digestion with an appropriate amount of the highly radioactive protein, available only in limited quantities. For this strategy to operate successfully, however, the first separation procedure applied to the digest would have to be insensitive to the presence or absence of the adamantyl functionalities, which were attached only to the small amount of labeled material and which permitted the identification of the membrane-spanning peptides. Certainly, the longer the peptide that contained the tracer, the less important its presence would become. Furthermore, procedures that separate linear polymers as a function of their length would be unaffected by the addition of a large, bulky group to an internal position in a given polymer.

With the expectation that the membrane-spanning sequences would yield unusually large tryptic peptides, a system of gel filtration was sought that would separate these peptides from the multitude of smaller peptides produced by trypsin. Green et al. (1980) had reported severe aggregation of all of the hydrophobic regions of $Ca^{2+}$-ATPase when the carboxymethylated and succinylated protein was digested with trypsin and submitted to gel filtration in aqueous buffer [see also Allen et al. (1980a,b)]. Initial attempts in this laboratory to separate the membrane-spanning peptides on Sephadex G-100 in $NaDodSO_4$ or $NaDodSO_4$ and urea buffers (Dorner, 1971) were also unsuccessful (Figure 6). The gel filtration system devised by Khorana and co-workers (Khorana et al., 1979; Takagaki et al., 1980), which uses a mixture of ethanol and formic acid as the solvent on Sephadex LH-60, proved to be successful.

The distribution of radioactivity that resulted when the tryptic digest of the labeled $\alpha$ polypeptide was submitted to gel filtration (Figure 2) confirmed that the membrane-spanning peptides, produced by trypsin and identified by the radioactivity, were large. The mean apparent lengths of the peptides in the five distinct, radioactive pools ranged from 110 amino acids for pool I to 27 amino acids for pool V. The distribution of total peptide obtained from an identical tryptic digest demonstrated that the large membrane-spanning peptides could be separated selectively from the multitude of smaller tryptic peptides produced from the cytoplasmic and extracytoplasmic regions of the $\alpha$ polypeptide. Over 70% of the total peptide traveled with distribution coefficients corresponding to peptides less than 30 residues in length. This resulted in a substantial purification of the membrane-spanning peptides during gel filtration.

When pools II–V were submitted to further purification on HPLC, a total of five peptides were isolated and their amino-terminal sequences determined. Upon chromatography on a CN-bonded stationary phase (Figure 4), pool II produced a major peptide, T2, with an amino-terminal sequence Ile-Ala-Thr-Leu-. Pool III also produced one major peptide, T3, with a sequence Val-Leu-Gly-Phe-. Pool IV, however, produced two peptides; T4 had an amino-terminal sequence Leu-Ile-X-Leu-Ala-, and T4a had a sequence Leu-Phe-Leu-Phe-. Pool V presented more difficult problems of resolution than the other pools. Multiple unresolved peaks were obtained when it was chromatographed on a CN-bonded stationary phase. Chromatgraphy on a $C_4$-bonded stationary phase of 300-Å pore size provided a better separation of the peptides, and the radioactivity was observed to follow and mimic a multiplet of peaks eluting near the middle of the gradient (Figure 5). The multiplet was shown to be one peptide, exhibiting heterogeneous behavior on HPLC, by sequencing. The sequence of this peptide was Met-Tyr-Leu-Pro-. Each of these five amino-terminal sequences are hydrophobic. In addition, it was found that no radioactivity could be eluted when a column containing a $C_{18}$-bonded stationary phase was used. Even the column containing the much less hydrophobic CN-bonded stationary phase required up to 70% 2-propanol to elute the radioactive peptides (Figure 4). These observations demonstrate the hydrophobicity of the labeled membrane-spanning peptides.

The radioactivity was observed to elute later in the gradient than the absorbance at 229 nm in all cases (Figures 4 and 5). The longer the peptide, however, the closer the peaks of radioactivity eluted to the peaks of the protein. This is to be expected because the covalent attachment of an adamantyl group would have more of an effect on the hydrophobicity of a smaller peptide. Because the radioactivity did not coelute with the protein, the problem of assigning the radioactive peaks to their respective peaks of protein arose.

Since the adjacent pools III and IV were not fully separated by the column of Sephadex LH-60 (Figure 2), there was cross-contamination between each pool. The cross-contaminating peptides were observed on HPLC and were identified unequivocally by their retention times and their amino-terminal sequences. The elution positions of these cross-contaminants are noted in Figure 4. The cross-contaminating peptides afforded a means by which the radioactivity could be matched with its corresponding peptide.

The concentration of peptide T3 in pool IV (Figure 4C) was much less than the concentration of peptide T3 in pool III (Figure 4B). The radioactivity that coeluted with peptide T3, however, was much greater in pool IV then in pool III. This inversion clearly demonstrates that the modified, radioactive and the unmodified, nonradioactive peptides were not coeluting. Therefore, the radioactivity coeluting with peptide T3 in Figure 4C cannot be attached to T3, but must be attached to peptide T4a. An identical argument can be used for peptide T4. Although the concentration of peptide T4 was much less in pool III than in pool IV, the radioactivity coeluting with T4 was much greater in pool III than in pool IV. The radioactivity coeluting with peptide T4 in pool III cannot be attached to T4 but must be attached to peptide T3. The radioactivity assigned to peptide T4 in pool IV was seen to elute nearly 4 min after the unlabeled peptide had eluted from the column (Figure 4C). The radioactivity due to the small amount of peptide T3 in pool IV was seen in between the two radioactive peaks corresponding to peptides T4a and T4, respectively. The radioactivity expected from peptide T4 in pool III was present as a shoulder to the right of the main peak. Finally, the striking similarities in both the width and fine structure between the peaks of absorbance for peptide T5 and the peaks of radioactivity within the same elution profile (Figure 5) strongly support the conclusion that the [$^3$H]-adamantyl groups are attached to the same peptide that produced the multiplet of absorbance. This supports the assignments of radioactivity to absorbance made in Figure 5. If these four assignments are accepted, the distances between each peptide and its adamantyl derivative are all very similar, and this adds credence to the assignments.

A systematic search for any additional peptides was conducted on each pool from the column of Sephadex LH-60. To be certain that there were no peptides that were contained in

these pools but had gone undetected on HPLC, the pools themselves were sequenced (Table I). There were relatively few amino acids detected at each step of the sequencing. In this particular experiment sequences of the known peptides were easily observed in the pools from which they could be purified, with the exception of peptide T4a in pool IV. Pool II was split in half to give pools IIa and IIb because its breadth suggested that it might contain more than one peptide. Along with the major peptide T2, both pools contained a peptide possessing a leucine amino terminus (Table I). The majority of this peptide resided in pool IIb. Along with the sequence of T2, the sequence of T4 was observed in pool IIb (Table I), and it was concluded that the peptide with the leucine amino terminus was a product of the partial digestion of peptide T4. Attempts to purify this peptide on HPLC were unsuccessful. The peptide possessing an alanine amino terminus (Table I) could not be isolated from pool IIb upon HPLC, but the low yield of this peptide suggested that it might also be a product of partial digestion. From the results of all of these experiments, it was concluded that there were no membrane-spanning peptides labeled with [$^3$H]adamantylidene other than the five peptides identified here.

One aspect of this work was to shed light on the controversy over the selectivity and reactivity of the two different lipophilic photoreagents, [$^3$H]adamantanediazirine and 5-[$^{125}$I]iodo-1-naphthyl azide. Although it has been stated that the labeling patterns observed when these two reagents were applied to ($Na^+ + K^+$)-ATPase were distinctly different from each other (Jørgensen et al., 1982), this was not observed in the present studies, which examined the products of these two reactions in much greater detail than was possible in the earlier experiments. By a comparison of the tryptic peptides that each compound labeled, a direct correlation between the two reagents was possible. In these experiments, $\alpha$ polypeptide labeled with 5-[$^{125}$I]iodo-1-naphthylnitrene was mixed with $\alpha$ polypeptide labeled with [$^3$H]adamantylidene to ensure identical treatment. Chromatography of the tryptic digest of the mixture on Sephadex LH-60 produced no additional radioactive components from the sample labeled with the naphthylnitrene (Figure 3). There was, however, a noticeable absence of $^{125}$I in the region of pool IV, and the majority of the radioactivity was located in pools II and III. Since it was shown previously that these pools contained only the two membrane-spanning peptides labeled by [$^3$H]adamantylidene, peptides T2 and T3, these two reagents do not label different parts of the $\alpha$ polypeptide.

It is important to discuss the experimental observations of Jørgensen et al. (1982) in light of the results presented in this paper. These authors stated that, upon extensive tryptic digestion of native ($Na^+ + K^+$)-ATPase, several peptides each of $M_r$ 12000 and each labeled by 5-[$^{125}$I]iodo-1-naphthylnitrene were produced. This conclusion was drawn from the distribution of $^{125}$I when the digest was submitted to electrophoresis on 7.5% polyacrylamide–$NaDodSO_4$ gels or chromatography on Sephadex G-50 eluted with a buffer containing 0.2% $NaDodSO_4$. The results from the chromatography on the column of Sephadex G-50 are identical with the results presented in this work (Figure 6). Since the tryptic digest run upon the latter column was identical with the digest chromatographed on the column of Sephadex LH-60 eluted with ethanol–formic acid, 4:1 (Figure 6, inset), it is clear that gel filtration in the presence of $NaDodSO_4$ cannot separate these peptides from each other. It is reasonable to assume that the 12K peptides discussed at length by Jørgensen et al. (1982) are a heterogeneous mixture of some or all of the peptides isolated in this study, as well as an unknown number of other peptides unrelated to the membrane-spanning portions of the $\alpha$ polypeptide. Presumably this mixture is not resolved by the column because each of these peptides is eluting within a micelle of $NaDodSO_4$, which necessarily causes all of the peptides to run at the same position. This phenomenon has been noted before by Fish et al. (1970). These authors stated that the log $R_s$ ceased to decrease as the log $M$ decreased below 4.2. As a result, proteins whose molecular weight was less than 15000 all eluted in the same position. It is well-known that separation of polypeptides on $NaDodSO_4$–polyacrylamide gels fails below a specific molecular weight (Neville, 1971; Williams & Gratzer, 1971). Again, all peptides below a molecular weight of 15000 travel with identical electrophoretic mobilities. It can be concluded from these considerations and the results presented here that tryptic digestion of the $\alpha$ polypeptide does not yield a set of membrane-spanning peptides all of the same length.

Recently, the sequences of several membrane-spanning proteins have been deduced from the sequences of the cDNAs produced from their mRNA. These proteins include all four subunits of acetylcholine receptor (Claudio et al., 1983; Noda et al., 1982, 1983) and subunits I and II of bovine cytochrome oxidase (Anderson et al., 1980). A significant difference exists in the mole fractions of the hydrophobic amino acids valine, isoleucine, leucine, and phenylalanine between the published amino acid compositions of the purified subunits (Vandlen et al., 1980; Verheul et al., 1979) and the actual amino acid compositions of each of these proteins. The values for the molar percentages of these four amino acids, obtained upon hydrolysis of the purified proteins in 6 M HCl at 110 °C for 72 h, were each as much as 3 mol % lower than the true values. This observation initiated a search for hydrolysis conditions that would give maximum yields for all of the hydrophobic amino acids in the membrane-spanning peptides. Hydrolysis of the model compound poly(L-valine) in concentrated HCl–trifluoroacetic acid, 2:1, at 157 °C (Tsugita & Scheffler, 1982) required 750 min to give 100% yield of valine, whereas hydrolysis of the $\alpha$ polypeptide showed no detectable increase in valine and isoleucine after 50 min. Therefore, 50 min was chosen as the hydrolysis time for the isolated peptides. Since this method caused significant decomposition of threonine, serine, methionine, and tyrosine, conventional hydrolysis in 6 M HCl at 110 °C for 24 h was used to obtain values for these amino acids.

The amino acid compositions of the five purified peptides are presented in Table II. The five peptides each displayed a significant increase in the sum of the molar percentages of valine, isoleucine, leucine, and phenylalanine when compared to their sum in the $\alpha$ polypeptide (Kyte, 1972). This result, as well as the amino-terminal sequences themselves and the labeling with [$^3$H]adamantanediazirine, indicates that each of these peptides contains a membrane-spanning sequence from the $\alpha$ polypeptide of ($Na^+ + K^+$)-ATPase. A careful search for additional peptides with these properties failed to discover any. Thus, these five peptides appear to be an exclusive set of the membrane-spanning segments of the protein and must contain the residues that form the pathway for the cations (Craig, 1982). Peptides T3 and T4a displayed a high molar percentage of aspartic and glutamic acid, an observation that suggests that these two peptides might be involved in transporting the cations across the lipid bilayer.

Another interesting feature of the amino acid compositions is the total number of tryptophans present in the five peptides. Allen and co-workers (Allen, 1980a,b; Allen et al., 1980a,b)

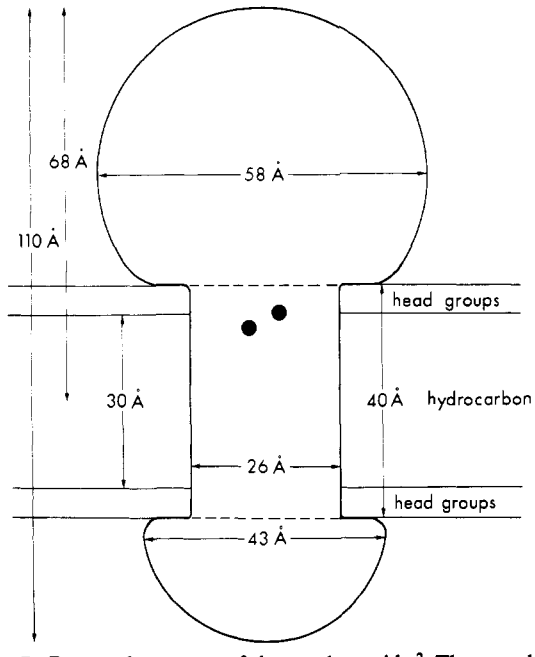


FIGURE 7: Proposed structure of the α polypeptide.[2] The cytoplasmic domain is above the membrane and contains 690 amino acids. The extracytoplasmic domain is below the membrane and contains 150 amino acids. The black circles are two potassium ions drawn to scale.

have sequenced over 70% of $Ca^{2+}$-ATPase, but only one tryptophan out of 18 or 19 present in the protein could be accounted for. It was presumed that the missing tryptophans were located in the membrane-spanning peptides, from which no sequence information could be obtained. Since the five membrane-spanning peptides isolated here constitute all of the membrane-spanning peptides present in $(Na^+ + K^+)$-ATPase, it had been assumed that these peptides would contain most of the missing tryptophan. Inspection of Table II, however, shows that only five tryptophans are present in the purified peptides. Possibly the rest of the tryptophans in the α polypeptide (Kyte, 1972) are located in the regions of the protein closely adjacent to the bilayer. Inspection of the membrane-spanning portions of the subunits of acetylcholine receptor (Claudio et al., 1983; Noda et al., 1982, 1983) and cytochrome oxidase (Anderson et al., 1980), as well as bacteriorhodopsin (Khorana et al., 1979; Kyte & Doolittle, 1982), shows that, on the average, only one tryptophan appears in the sequence of each membrane-spanning segment. This is also the frequency observed in these studies (Table II).

Since all of the membrane-spanning peptides have been accounted for, it is interesting to speculate on the number of membrane-spanning helices present in the α polypeptide of $(Na^+ + K^+)$-ATPase. Peptides T4, T4a, and T5 each could contain only one membrane-spanning helix, since their lengths (30–40 residues) would not allow for any more than one. Peptides T2 and T3 are long enough (~70 residues and ~50 residues, respectively) to transverse the bilayer twice. Since the length of peptide T3 is at the lower limit of the number of residues required, peptide T2 seems to be the best candidate for containing more than one membrane-spanning helix. Analysis of the four membrane-spanning helices in each subunit of acetylcholine receptor (Claudio et al., 1983; Noda et al., 1982, 1983), as well as the seven helices present in bacteriorhodopsin (Khorana et al., 1979; Kyte & Doolittle, 1982), indicates that membrane-spanning sequences contain a relatively constant number of the hydrophobic amino acids isoleucine, valine, leucine, phenylalanine, cysteine, and methionine. This value, when averaged over the 23 helices examined, was 14 ± 2.5 hydrophobic amino acids (membrane-spanning helix)$^{-1}$. The number of these amino acids in T2 is 22 (Table II), which falls within two standard deviations of the mean for two helices. Because the actual number of these hydrophobic amino acids in this peptide may be greater than 22, however, it is quite possible that T2 contains two membrane-spanning helices, which would bring the total to six.

A model of the α polypeptide of $(Na^+ + K^+)$-ATPase can be constructed by assembling all of the present knowledge of its structure. From the present work it can be concluded that there are probably six membrane-spanning helices present in the α polypeptide. Transversing the membrane 6 times would require 160 amino acids, which translates into a cylinder 26 Å in diameter and 40 Å in height with $\bar{v} = 0.74$ cm$^3$ g$^{-1}$. Since there are approximately 1000 amino acids in the α polypeptide (Craig & Kyte, 1980; Peterson & Hokin, 1981), this would leave 840 amino acids outside of the membrane. These 840 amino acids would have a volume of $1.2 \times 10^5$ Å$^3$ if 110 is the average molecular weight of an amino acid and 0.74 cm$^3$ g$^{-1}$ is the partial specific volume of the protein. O'Connell (1982) and Sharkey (1983) have estimated the ratio of the surface areas of the cytoplasmic to extracytoplasmic regions of the native α polypeptide to be 3. The model in Figure 7 was constructed[2] by using only these three observations: namely, six membrane-spanning helices, a surface area of the cytoplasmic region 3 times the surface area of the extracytoplasmic region, and a sum of the volumes of the cytoplasmic and extracytoplasmic regions equal to $1.2 \times 10^5$ Å$^3$. When this was done, the dimensions shown in Figure 7 were obtained.

Although this model is still conjecture, a pair of recent, completely independent measurements reinforce its accuracy. Paired membranes, in which two fragments of plasma membrane were associated through a coaxial interaction between the cytoplasmic domains of their α polypeptides, have been observed in electron micrographs of crystalline, native $(Na^+ + K^+)$-ATPase. The center-to-center distance between the two membrane bilayers, which was constant throughout the complex, was 150 Å.[3] This would mean that the distance from the center of the bilayer to the end of the cytoplasmic region of the α polypeptide is 75 Å, which is very close to the distance, 68 Å, obtained in the model. The other measurements that could be made from crystalline arrays of $(Na^+ + K^+)$-ATPase were the dimensions of the unit cell parallel to the bilayer. Within the hexagonal, monomeric arrays observed by Herbert et al. (1982) and Zampighi et al.,[3] the dimensions of the unit cell were found to be 53 Å × 69 Å and 55 Å × 65 Å, respectively, both of which are very close to the horizontal diameter, 58 Å, of the cytoplasmic sphere.

Included in Figure 7 are two circles whose diameters are those of two potassium ions. Somewhere among the six helices that span the plasma membrane must be the channel for these cations.

## Acknowledgments

I thank Jack Kyte, in whose laboratory this research was performed, for his advice, support, and guidance. I also thank Jim Gallagher for preparing the unlabeled α polypeptide.

**Registry No.** ATPase, 9000-83-3; [$^3$H]adamantanediazirine, 74280-01-6; 5-[$^{125}$I]iodo-1-naphthyl azide, 66640-74-2; 5-amino-1-naphthyl azide, 66640-76-4.

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