Purification of Labeled Cyanogen Bromide Peptides of the α Polypeptide from Sodium Ion and Potassium Ion Activated Adenosinetriphosphatase Modified with *N*-[^{3}H]Ethylmaleimide†

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Received July 18, 1985; Revised Manuscript Received October 18, 1985

ABSTRACT: Sodium ion and potassium ion activated adenosinetriphosphatase, isolated from canine kidney, was reacted with *N*-[^{3}H]ethylmaleimide while it was poised in three different conformations, ostensibly E_2–P, E_2, and E_1, respectively. These assignments were made from a consideration of the particular concentrations of ligands in the respective alkylation mixtures. After a 30-min reaction, the remaining enzymatic activity was found to vary among these three different samples from 90 to 30% of that of unalkylated controls. In all cases, the α polypeptide was purified and subjected to digestion with cyanogen bromide, and in each digest the same two distinct radioactive peptides were identified and purified by gel filtration on a column of Sephadex LH-60. The incorporation of *N*-[^{3}H]ethylmaleimide into one of these two peptides correlated closely with enzymatic inactivation, while the incorporation into the other was most extensive when the portion of the active site to which ATP binds was unoccupied. Alkylation of the residue within the latter peptide, however, does not result in inactivation of the enzyme. Both peptides were further purified by high-pressure liquid chromatography, and their amino-terminal sequences were determined by manual dansyl Edman or solid-phase techniques. The peptide containing the sulfhydryl protected by ATP has, as its amino terminus, the lysine that reacts exclusively with fluoresceinyl 5′-isothiocyanate [Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1984) *J. Biol. Chem. 259*, 9532–9535].

Sodium ion and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase][1] is the enzyme responsible for the active transport of sodium and potassium across the plasma membranes of all animal cells (Kyte, 1981). During each turnover, the free energy from the hydrolysis of one molecule of MgATP is coupled to the movement of three sodium ions out of the cell and two potassium ions into the cell. Kinetic studies have indicated that the enzyme passes through at least four unique conformations during each turnover. The enzyme can be poised in any one of these conformations, which are designated E_1, E_1~P, E_2–P, and E_2, by choosing appropriate concentrations of various substrates or inhibitors (Winslow, 1981).

The α polypeptide of canine (Na^+ + K^+)-ATPase contains 20–25 cysteine residues (Kyte, 1972). The relationship between these sulfhydryl groups and enzymatic activity has been studied extensively (Gupte & Lane, 1979; Harris & Stahl, 1980; Schoot et al., 1978). One of the most commonly used sulfhydryl reagents is *N*-ethylmaleimide. This reagent has been shown to inactivate the enzyme (Fahn et al., 1968; Skou & Hilberg, 1965), and the inactivation can be prevented by the addition of ATP (Skou, 1974; Schoot et al., 1977). By determining the actual concentration and the specific radioactivity of *N*-[^{3}H]ethylmaleimide during its reaction with (Na^+ + K^+)-ATPase, Winslow (1981) has demonstrated that, under optimum conditions and with full inactivation, about 4 mol of *N*-[^{3}H]ethylmaleimide can be incorporated into a mole of the catalytic subunit, which is the folded α polypeptide, when the enzyme is in the native, enzymatically active complex. One mole of this incorporation should have been due to alkylation of the unique cysteine whose modification inactivates the enzyme and whose reactivity depends on the level of sodium or potassium present during the reaction (Skou, 1974). Another mole of the incorporation was shown to be into a sulfhydryl that seems to react with *N*-ethylmaleimide only when the binding site for ATP is not occupied, but alkylation of this residue does not affect enzymatic activity.

This paper describes experimental procedures for the isolation of the two cyanogen bromide peptides of the α polypeptide of (Na^+ + K^+)-ATPase that contain the two cysteine residues whose reaction with *N*-ethylmaleimide changes when the native enzyme is poised in different conformational states. A unique peptide, which contained a sulfhydryl residue whose incorporation of *N*-[^{3}H]ethylmaleimide correlated closely with the extent of enzymatic inactivation, was purified, and its partial amino-terminal sequence was determined. In addition, another fragment, containing a cysteine residue that reacts with *N*-ethylmaleimide most vigorously in the absence of ATP, was purified, and its amino-terminal sequence was also determined. This latter peptide contained, as its amino-terminal residue, the same lysine that reacts with the fluorescent thiocarbamylating agent fluoresceinyl 5′-isothiocyanate (Karlish, 1980; Farley et al., 1984).

Experimental Procedures

Materials. *N*-[^{3}H]Ethylmaleimide was purchased from New England Nuclear. Unradioactive *N*-ethylmaleimide was purchased from Eastman Kodak Co. Sodium dodecyl sulfate ($NaDodSO_4$) was recrystallized from 95% ethanol. Trifluoroacetic acid, ethanolamine, formic acid, *N*,*N*-dimethyl-

† This research was supported by Grants GM-33962 and AM-07233 from the National Institutes of Health, Grant DMB-8413772 from the National Science Foundation, and Grant-in-Aid AHA-81-1003 from the American Heart Association, all of which provide support to the laboratory of Jack Kyte. D.T.L. was also supported by Predoctoral Training Grants GM-07313-08 and GM-07313-09 from the National Institutes of Health.

[1] Abbreviations: (Na^+ + K^+)-ATPase, sodium ion and potassium ion activated adenosinetriphosphatase; Ca^{2+}-ATPase, calcium ion activated adenosinetriphosphatase; (H^+ + K^+)-ATPase, proton and potassium ion activated adenosinetriphosphatase; $NaDodSO_4$, sodium dodecyl sulfate; PTH, phenylthiohydantoin; CDTA, *trans*-1,2-diaminocyclohexanetetraacetic acid; HPLC, high-pressure liquid chromatography; DMBA, *N*,*N*-dimethylbenzylamine.

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benzylamine (DMBA), and phenyl isothiocyanate were redistilled before use. *p*-Phenylenyl diisothiocyanate (Eastman) was recrystallized from acetic acid and then hexane. For high-pressure liquid chromatography (HPLC), acetonitrile (HPLC grade) was purchased from Fisher Scientific, and 1-propanol and 2-propanol (HPLC grade) were from Burdick and Jackson. Phenylthiohydantoin (PTH) amino acid standards were purchased from Sigma, and [*N*-(β-aminoethyl)-γ-aminopropyl]trimethoxysilane was from Pierce.

Isothiocyanate glass beads (Laursen et al., 1972) were made by modification[2] of the method of Wachter et al. (1973) as it has been described by Mross and Doolittle (1977). [(Aminoethyl)amino]propyl glass beads (CPG 500-120) were made as described by Mross and Doolittle (1977) except that [*N*-(β-aminoethyl)-γ-aminopropyl]trimethoxysilane was substituted for the (aminopropyl)triethoxysilane. To 2 g of these beads, 1.5 g of *p*-phenylenyl diisothiocyanate, dissolved in 8 mL of dimethylformamide at 55 °C, was added. The beads were immersed in the solution by centrifugation, the tube was flushed with N_2, and the sample was incubated at 55 °C for 90 min. The modified beads were washed thoroughly with 1% pyridine in dimethylformamide and then with methanol and dried. When this procedure is followed, 40 μmol of phenyl isothiocyanate are available to react with lysine per gram of glass beads.

Preparation of $(Na^+ + K^+)$*-ATPase.* $(Na^+ + K^+)$-ATPase was purified from canine kidney by the method of Jørgensen (1974) with the modifications described by Winslow (1981). Enzymatic activity was determined as described by Kyte (1971). Protein concentration was determined by the method of Lowry et al. (1951) as described by Kyte (1971). The specific activity the enzyme in these preparations was between 800 and 1000 μmol of P_i mg^{-1} h^{-1}.

Labeling of $(Na^+ + K^+)$*-ATPase with N-*[^3H]*Ethylmaleimide.* Prior to each experiment, purified $(Na^+ + K^+)$-ATPase (5–20 mg, 2–10 mg mL^{-1}) was dialyzed overnight at 4 °C against 0.25 M sucrose–30 mM histidinium chloride, pH 7.5, to remove the 2-mercaptoethanol that had been present during the purification and storage of enzyme. The solution containing the enzyme was removed, and small aliquots (5–25 μL) of concentrated solutions of NaCl, ATP (sodium or potassium salts), *trans*-1,2-diaminocyclohexanetetraacetic acid (CDTA), or $MgCl_2$ were added to poise the enzyme in the desired conformations. For the sample submitted to the conditions of case I, NaCl was added to a final concentration of 100 mM, ATP to 10 mM, and $MgCl_2$ to 5 mM. For case II, NaCl was added to 100 mM and CDTA to 10 mM. For case III, KCl was added to 20 mM, ATP to 10 mM, and CDTA to 10 mM. The enzyme and the ligands were preincubated at 37 °C for 5 min. *N*-[^{3}H]Ethylmaleimide [(4–25) × 10^7 cpm], which had been prepared as described by Winslow (1981), was added to 5 mM. An aliquot (5 μL) was immediately removed and added to 100 μL of iced 50 mM 2-mercaptoethanol, 0.25 M sucrose, and 30 mM histidinium chloride, pH 7.5, for later determination of initial enzymatic activity. The reaction mixture was flushed with N_2 and then incubated at 37 °C. After 30 min, another aliquot (5 μL) was removed for a determination of enzymatic activity. The main reaction mixture was quenched by adding 2-mercaptoethanol to 15 mM and incubating for 10 min at 37 °C. The solution was then dialyzed against 0.25 M sucrose–30 mM histidinium chloride for 2 h at room temperature to remove excess unattached radioactivity.

[2] J. Kyte, personal communication.

Preparation of the α Polypeptide of $(Na^+ + K^+)$*-ATPase.* Samples were prepared for the isolation of the α polypeptide from these alkylation mixtures by adding unradioactive *N*-ethylmaleimide to 25 mM and then a 4-fold weight excess of $NaDodSO_4$. The mixture was incubated at 37 °C for 30 min, and 2-mercaptoethanol was added to 50 mM. The α-polypeptide of the enzyme was isolated by gel filtration of these samples on either a column of Sepharose 4B (5 cm × 120 cm) or a column of Agarose A-5m (2.5 cm × 85 cm), each equilibrated with 0.1% $NaDodSO_4$–40 mM Tris–SO_4, pH 8.0 (Kyte, 1972). The α polypeptide was located by absorbance at 220 nm and identified by electrophoresis on 5% polyacrylamide–$NaDodSO_4$ gels (Weber & Osborn, 1969). Pools were routinely concentrated by lyophilization and redissolved in small quantities (1–5 mL) of H_2O. The concentration of α polypeptide was determined by quantitative amino acid analysis.

Cyanogen Bromide Digestion. The procedure of Rodriguez et al. (1983) was followed. When desired, the labeled α polypeptide was mixed with 10–20 mg of previously purified α polypeptide that had been alkylated with unradioactive *N*-ethylmaleimide (25 mM) in the presence of excess $NaDodSO_4$. In every instance, however, the α polypeptide was then dialyzed against 0.1% $NaDodSO_4$–5 mM sodium phosphate, pH 7.0. An equal volume of 30 mg mL^{-1} cyanogen bromide in 0.2 M HCl was added, and the mixture was sealed under N_2. After 16 h at room temperature, the digest was diluted with water and lyophilized. The extent of digestion was always greater than 90% when this was assessed by the disappearance of methionine, determined by amino acid analysis of the whole digest. The α polypeptide of the enzyme contains about 25 methionine residues (Kyte, 1972); therefore, about 25 fragments of average length of 40 residues would be expected. A stacked urea–dodecyl sulfate slab gel cast from 20% polyacrylamide resolves about 15 fragments from such digests of the α polypeptide (Kyte & Rodriguez, 1983). Such slab gels were routinely run to check the completion of the digestion.

Purification of Cyanogen Bromide Peptides. The digest was redissolved in 1.4 mL of 88% formic acid, 3.3 mL of 95% ethanol, and 20 μL of 2-mercaptoethanol and then applied to a column of Sephadex LH-60 (4 cm × 96 cm) equilibrated with 95% ethanol–88% formic acid, 4:1 (Khorana et al., 1979; Takagaki et al., 1980). The peptides were eluted with the same solvent at a flow rate of 18 mL h^{-1}.

Pools from the elution profile of the column of LH-60 were lyophilized, redissolved in 88% formic acid, and chromatographed on an HPLC 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 for detection of absorbance at 229 nm. The solvents from which gradients were constructed were 0.1% (v/v) aqueous trifluoroacetic acid (solvent A) and 0.1% (v/v) trifluoroacetic in acetonitrile (solvent B) (Mahoney & Hermodson, 1980). Pools from the column of LH-60 were redissolved in 88% formic acid (50–200 μL) and injected into a Beckman Ultrapore RPSC-C_3 column (4.6 mm i.d. × 7.5 cm) equilibrated with solvent A, and peptides were eluted with a linear gradient system by increasing the concentration of solvent B in solvent A as follows: 0–5 min, 0% B; 5–15 min, 0–15% B; 15–135 min, 15–75% B. The total flow rate was maintained at 0.5 mL min^{-1}.

Amino-Terminal Sequencing. The manual dansyl Edman procedure was carried out as outlined by Weiner et al. (1972) with the modifications described by Nicholas (1984).

For solid-phase sequencing, peptides were coupled[2] to isothiocyanate glass beads (Laursen, 1972). Pools from the HPLC (~5 mL) were lyophilized after addition of 20 μL of 20% $NaDodSO_4$. The peptides, accompanied by the $NaDodSO_4$, were redissolved in 100 μL of water, the pH was adjusted to 9, solid imidazole was added to 5%, and the solution was transferred to a small tube containing 2.5 mg of isothiocyanate glass beads for every microliter of the solution. Water was added to just cover the beads, and the sample was sealed under N_2 in a humid chamber. After 6 or more hours, the beads were washed several times with 0.5% $NaDodSO_4$ and then water and transferred to 1 M ethanolamine, pH 9.0. After 1 h at room temperature, the beads were washed several times with 0.5% $NaDodSO_4$, then water, and finally methanol and dried.

A solid-phase, automated sequencer was used that was very similar to the one described by Doolittle et al. (1977). *N,N*-Dimethylbenzylamine (DMBA) was used as buffer for the coupling reaction (Hermodson et al., 1972). The coupling buffer was prepared by adding 3 mL of DMBA (redistilled and stored under N_2 at 4 °C) to 21 mL of 1-propanol (HPLC grade), followed by 28 mL of water. The solution was then carefully titrated to pH 9.4 with a solution of 200 μL of trifluoroacetic acid diluted in 700 μL of 1-propanol–water, 3:4. The coupling reagent was prepared by diluting 5 mL of phenyl isothiocyanate (redistilled and stored in 5-mL sealed ampules under Ar at −70 °C) with acetonitrile to 50 mL. Both solutions were always freshly made and bubbled with Ar before being used. In the sequencer, equal volumes of the two were mixed to initiate coupling. Cyclization of the *N*-phenylthioureas was performed with redistilled trifluoroacetic acid, and the thiazolinones were collected in the trifluoroacetic acid. The temperature of the sequencing chamber was kept at 45 °C, and each cycle took 125 min to complete.

Identification of PTH-Amino Acids. The consecutive solutions of the phenylthiazolinone amino acids in trifluoroacetic acid were dried down under vacuum in a Speed Vac concentrator (Savant, Model RH20-12). To each tube, 200 μL of 25% (v/v) aqueous trifluoroacetic acid, which had been flushed with N_2, was added. The tubes were sealed with parafilm under N_2 and incubated for 30 min at 55 °C (Hunkapiller & Hood, 1978). The solutions were dried down again in the concentrator for 4–5 h to ensure the complete evaporation of solvents. Samples were dissolved in 100–200 μL of 40 mM sodium acetate, pH 5.0–acetonitrile, 5:1 (solvent A), and an appropriate portion was loaded onto a Waters Novapak-C_{18} column (3.9 mm × 15 cm) equilibrated with solvent A and jacketed at 39 °C. PTH-amino acids were eluted with the gradient system developed by Waters Associates (1983) in which solvent B was 60% aqueous 2-propanol. Solvent A was filtered through a Sepak cartridge (Waters Associates) before use.

Results

Conditions for Labeling of $(Na^+ + K^+)$-ATPase with N-[^{3}H]Ethylmaleimide. On the basis of preliminary experiments, three of the combinations of ligands described in Table IV of Winslow (1981) were chosen for the majority of the experiments described herein. In case I, the ligands were 100 mM NaCl, 10 mM ATP, and 5 mM $MgCl_2$. Under these conditions the enzyme is in the E_2–P state, only about 10% of the activity is lost during the labeling, and only background incorporation should have occurred. In case II, the ligands were 100 mM NaCl and 10 mM CDTA. Under these conditions, the enzyme is in the E_2 state (Koepsell, 1979; Winslow, 1981), about 40% of the activity is lost, and maximum incorporation occurs into a conformationally sensitive cysteine other than the one that inactivates the enzyme. For case III, the ligands were 20 mM KCl, 10 mM ATP, and 10 mM CDTA. Under these conditions the enzyme is in the E_1 state, about 70% of the activity is lost, and incorporation into the cysteine of the active site predominates.

Table I: Distribution of Radioactivity Incorporated into Cyanogen Bromide Peptides A and B of the α Polypeptide Labeled with *N*-[^{3}H]Ethylmaleimide under Different Ligand Conditions[a]

expt	condition	inactivation (%)[b]	^{3}H cpm (pmol of α polypeptide)$^{-1}$ total	peak A	peak B
A	I	13	28	1.8	10.1
	II	46	48	14.6	12.7
	III	77	50	0.9	23
B	II	53	48	8.4	9.7
	III	68	41	3.9	10.3
C	I	17	14.7	2.8	2.4
	II	47	22	4.0	3.8
	III	61	22	0.8	3.9
D	I	5	170	23	22
	II	27	200	36	42
	III	56	280	3.5	93
E	I	7	88	29	6.4
	II	29	91	26	11.9
	III	65	149	45	20

[a] For each experiment, A–E, respectively, three equal amounts of purified $(Na^+ + K^+)$-ATPase (4.8–20 mg in 0.6–2.0 mL) were labeled with equal amounts of *N*-[^{3}H]ethylmaleimide [(4–25) × 10^7 cpm] under the three ligand conditions of cases I–III. The labeled α polypeptide was then purified by gel filtration. For each experiment, an equal amount of purified α polypeptide (5–20 mg), which had been fully alkylated with unradioactive *N*-ethylmaleimide, was mixed with each of the three samples of labeled α polypeptide. The specific radioactivity of each of these ^{3}H-labeled α polypeptide mixtures, presented as total ^{3}H cpm (pmol of α polypeptide)$^{-1}$, was then determined from the ^{3}H cpm recovered with the labeled α polypeptide pool and the total protein content of each α polypeptide mixture. Each mixture was then lyophilized, digested with cyanogen bromide, and submitted to gel filtration on a column of LH-60. The total yield of ^{3}H cpm across each elution profile was calculated. These numbers were divided by the specific radioactivity of the α polypeptide mixture to give an estimate of the actual nanomoles of digested, labeled α polypeptide recovered from the LH-60 column (usually greater than 80%). The amount of ^{3}H cpm incorporated into peaks A and B per picomole of α polypeptide recovered in each experiment is presented. [b] The enzymatic activity of the alkylation mixtures was determined immediately following addition of *N*-[^{3}H]ethylmalimide and after a 30-min incubation. The percentage of inactivation was determined from these two numbers.

Purification of the α Polypeptide of $(Na^+ + K^+)$-ATPase Labeled with N-[^{3}H]Ethylmaleimide. $(Na^+ + K^+)$-ATPase was preincubated in the presence of the ligands necessary to generate the conformations found in cases I, II, and III. *N*-[^{3}H]Ethylmaleimide was then added to 5 mM, and the mixture was incubated for 30 min at 37 °C and quenched with 25 mM 2-mercaptoethanol. Enzymatic activity was assayed after the alkylation had been quenched, and the tabulated losses of enzymatic activity during each alkylation (Table I) were based on controls that had been quenched immediately after addition of the *N*-[^{3}H]ethylmaleimide. Unattached radioactivity was removed from the alkylated samples by dialysis, the enzyme was denatured by addition of $NaDodSO_4$, and all remaining unreacted sulfhydryl groups were alkylated with 25 mM unradioactive *N*-ethylmaldeimide. This strategy was based on the assumption that all of the cysteines in the protein would become alkylated and chemically identical while only those that had reacted during the labeling reaction would be radioactive. This would permit radioactively labeled protein to be mixed with unlabeled but fully alkylated carrier at any step.

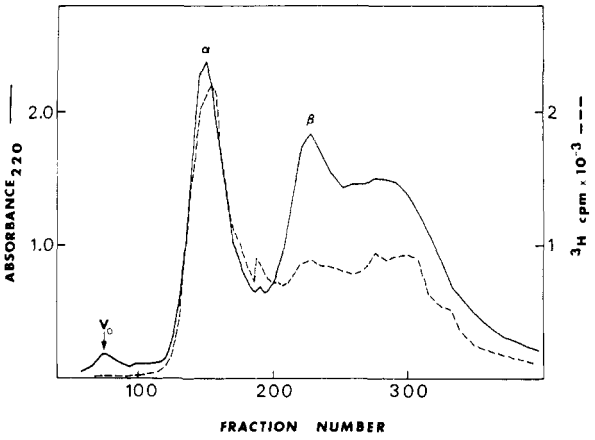

FIGURE 1: Purification of the α polypeptide from $(Na^+ + K^+)$-ATPase that had been labeled with *N*-[^{3}H]ethylmaleimide. Purified $(Na^+ + K^+)$-ATPase (20 mg) was preincubated in 3.6 mL of 0.25 M sucrose, 30 mM histidininum chloride, and 1 mM EDTA, pH 7.1, for 5 min at 37 °C. *N*-[^{3}H]Ethylmaleimide (1.3×10^8 cpm) was then added to a final concentration of 5 mM, and the mixture was incubated under nitrogen for 30 min at 37 °C. To quench the reaction, 2-mercaptoethanol was added to 15 mM (4.2 μL), and the mixture was incubated for 5 min at 37 °C. Excess *N*-[^{3}H]ethylmaleimide was removed by dialysis. Unradioactive *N*-ethylmaleimide was added to the labeled enzyme at a final concentration of 25 mM followed by 40 μL of 20% $NaDodSO_4$, and the mixture was incubated for 15 min at 37 °C. The entire solution was dialyzed twice against 250 mL of 0.1% $NaDodSO_4$–40 mM Tris–SO_4, pH 8.0, and loaded onto a column of Bio-Gel A-5m (2.7 cm × 85 cm) equilibrated with the same Tris–SO_4 buffer. The proteins were eluted with the same buffer at a flow rate of 10 mL h^{-1}. Fractions of 2.0 mL were collected, the absorbance at 220 nm was determined (solid line), and 50-μL aliquots were removed for measurement of ^{3}H cpm (dashed line). The positions of the void volume (V_0), the α polypeptide (α), and the β polypeptide (β) are indicated.

As reported earlier (Winslow, 1981), when samples of this labeled material were subjected to electrophoresis in 0.1% $NaDodSO_4$, it was observed that the majority of the radioactivity had been incorporated into the α polypeptide of $(Na^+ + K^+)$-ATPase. To prepare the large quantity of this radioactively labeled α polypeptide needed for subsequent studies, the products of the entire alkylation were loaded onto a column of Bio-Gel A-5m equilibrated with 0.1% $NaDodSO_4$–40 mM Tris–SO_4, pH 8.0 (Kyte, 1972). A typical elution profile from such a column is shown in Figure 1. It can be seen that the α polypeptide is well separated from the β polypeptide and other impurities. Also, there is a large peak of radioactivity that coelutes with the α polypeptide. The radioactivity that coincides with the β polypeptide and impurities of lower molecular weight varied with the extent of the dialysis that had preceded this step.

Peptides Produced by Digestion with Cyanogen Bromide from the α Polypeptide Labeled with N-[^{3}H]Ethylmaleimide. The purified α polypeptide, labeled with *N*-[^{3}H]ethylmaleimide, was mixed with previously purified, unlabeled α polypeptide that had been alkylated with unradioactive *N*-ethylmaleimide in the presence of $NaDodSO_4$. This mixture was digested with cyanogen bromide (Rodriguez et al., 1983), and the digest was then submitted to gel filtration on a column of Sephadex LH-60 equilibrated with 95% ethanol–88% formic acid, 4:1 (Khorana et al., 1979; Takagaki et al., 1980). The distributions of the radioactive cyanogen bromide peptides from α polypeptide that had been labeled while it was in native $(Na^+ + K^+)$-ATPase under each of three conditions established in cases I–III, respectively, contain two major peaks of radioactivity, denoted A and B (Figure 2). Calibration of this column with standards demonstrated that peaks A and

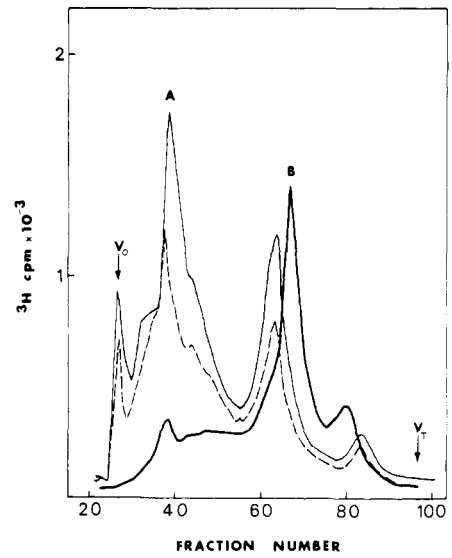

FIGURE 2: Gel filtration on Sephadex LH-60 of peptides produced by cyanogen bromide digestion of the α polypeptide labeled with *N*-[^{3}H]ethylmaleimide. Three samples of purified enzyme (each 5 mg in 0.45 mL) were labeled with *N*-[^{3}H]ethylmaleimide (each 4×10^7 cpm) under the three different conditions of cases I–III. Preparations of purified α polypeptide from each reaction [2.4–3.3 mg, $(1.3–2.2) \times 10^5$ cpm of ^{3}H] were dialyzed against 5 mM NaP_i–0.1% $NaDodSO_4$, pH 7.0, and then subjected to cyanogen bromide digestion. Each digest was redissolved in 1.4 mL of 88% formic acid, 3.3 mL of 95% ethanol, and 20 μL of 2-mercaptoethanol and loaded onto a column of Sephadex LH-60 (4 cm × 96 cm) equilibrated with 95% ethanol–88% formic acid, 4:1. The peptides were eluted with the same solvent at a flow rate of 18 mL h^{-1}. Fractions of 7.4 mL were collected, and 4.0-mL aliquots were removed for measurement of ^{3}H cpm. To present the distribution of radioactivity for all three cases in one figure, the total yield of ^{3}H cpm across the entire elution profile was determined for each case. This information was used to normalize (see Table I) the three elution profiles. α polypeptide labeled under case I (––); α polypeptide labeled under case II (light line); α polypeptide labeled under case III (heavy line). The positions of peaks A and B, as well as the void (V_0) and the included (V_T) volumes, are indicated.

B had mobilities corresponding to peptides of 80 and 20 residues, respectively. Summations of the total cpm in each of these peaks, normalized to the original picomoles of α polypeptide in the respective cyanogen bromide digests, are presented in Table I. The total cpm per picomole of undigested α polypeptide in each case is also included.

As can be seen, the amount of radioactivity within these two peaks varied according to the conformation in which the enzyme had been poised during the labeling with *N*-[^{3}H]-ethylmaleimide. In each set of results from a given experiment, the amount of radioactivity incorporated into peak B per picomole of α polypeptide increased as the degree of inactivation of the enzyme increased (Table I). To combine all of the results in Table I that support this correlation and display them simultaneously required that each individual experiment be normalized to the same scale because both the specific radioactivity of the *N*-[^{3}H]ethylmaleimide (Winslow, 1981) and the proportions in which radioactive and unradioactive α polypeptide were mixed varied among the experiments. The cpm per picomole of α polypeptide that would have been incorporated into peak B in each experiment at an inactivation of 50% was determined by linear interpolation. This value was used to normalize the actual observations from the respective experiments to the same scale. When these normalized values are plotted against the extent of inactivation, a linear correlation is found (Figure 3). This demonstrates that peak B is the peptide that contains the sulfhydryl residue whose reaction with *N*-ethylmaleimide causes inactivation of the en-

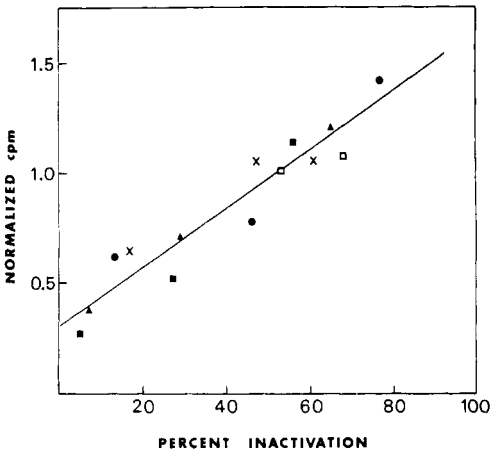

FIGURE 3: Relationship between the incorporation of *N*-[^{3}H]ethylmaleimide into peak B and the percentage of enzyme inactivation. For each set of experiments presented in Table I, the ^{3}H cpm per picomole of α polypeptide that would have been incorporated into peak B at an inactivation of 50% was determined by linear interpolation. This value was then used to normalize the actual ^{3}H cpm incoporated from the respective experiments to the same scale. Thus, the value of incorporation for 50% inactivation was arbitrarily assigned as 1.00. All data were taken from Table I: (●) experiment A; (□) experiment B; (×) experiment C; (■) experiment D; (▲) experiment E.

zyme (Skou et al., 1965; Fahn et al., 1968).

Purification of the Labeled Cyanogen Bromide Fragments in Peaks A and B by HPLC. In each experiment, peaks A and B (Figure 2) were pooled separately, lyophilized, redissolved in 88% formic acid, and injected onto a C_3 reverse-phase column equilibrated with 0.1% trifluoroacetic acid in H_2O. Peptides were then eluted with a linear gradient of 0.1% trifluoroacetic acid in H_2O to 0.1% trifluoroacetic acid in acetonitrile. The profile of absorbance at 229 nm and the distribution of radioactivity for peaks A and B are shown in panels A and B of Figure 4, respectively. It can be seen that, upon chromatography, peak A was resolved into two peaks of radioactivity that coeluted with two peaks of absorbance (Figure 4A). The two peaks, denoted A_1 and A_2, were pooled separately for amino-terminal sequencing. Likewise, peak B also yielded two major peaks of radioactivity that coeluted with what appeared to be a collection of incompletely resolved peaks of absorbance (Figure 4B). It was assumed that this chromatographic behavior was due to microheterogeneity in the short peptide (20 residues) being purified (Nicholas, 1984), and fractions were pooled as indicated in Figure 4B for subsequent amino-terminal sequencing.

Amino-Terminal Sequencing. Amino-terminal sequencing by the manual dansyl Edman procedure (Weiner et al., 1972) revealed that both pools A_1 and A_2 contained the same unique peptide with the amino-terminal sequence Lys-Gly-Ala-. Both pools gave this same sequence in two separate experiments, and in six other experiments both gave an amino-terminal Lys. In each analysis of peptide A_1, the dansyl amino acids from this sequence or this amino terminus were 2–3 times brighter than any other dansyl amino acids on the plate. No other dansyl amino acids appeared consistently during amino-terminal sequencing of A_1.

In one particular experiment, purified α polypeptide, radiolabeled under the conditions of case II (60 nmol, 91 000 cpm nmol^{-1}), was mixed with 20 mg (180 nmol) of α polypeptide that had been exhaustively alkylated with unradioactive *N*-ethylmaleimide. The resulting mixture (24 000 cpm nmol^{-1}) was subjected to cyanogen bromide digestion and gel filtration through the column of Sephadex LH-60, and pools A_1 and A_2

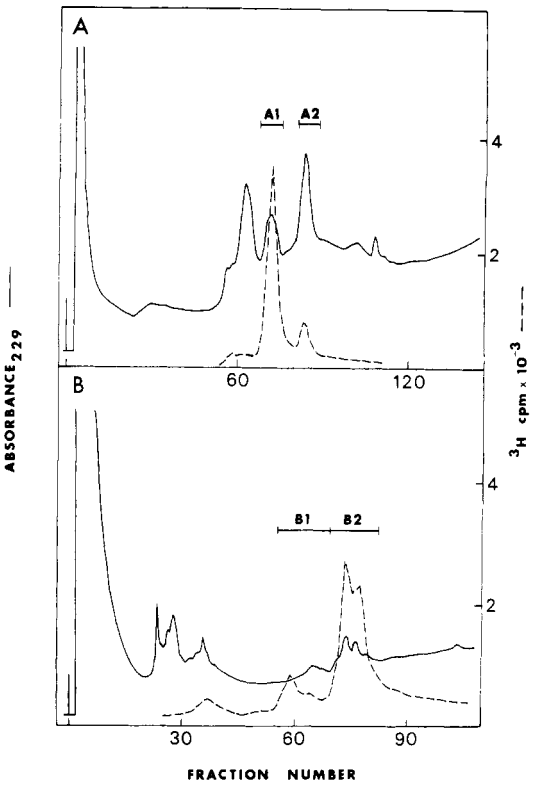

FIGURE 4: Purification of peaks A and B by HPLC on an Ultrapore RPSC-C_3 column (4.6 mm i.d. × 7.5 cm). (A) A sample of peak A from the gel filtration on Sephadex LH-60 was lyophilized, redissolved in 88% formic acid (50 μL, 1.7 × 10^5 cpm), and injected onto the column equilibrated with 0.1% aqueous trifluoroacetic acid (solvent A). A gradient system with 0.1% trifluoroacetic acid in acetonitrile as solvent B was developed as follows: 0–5 min, 0% B; 5–15 min, 0–15% B; 15–135 min, 15–75% B. The total flow rate was 0.5 mL min^{-1}. Fractions of 0.5 mL were collected, and 100-μL aliquots were removed for measurement of ^{3}H cpm (dashed line). (B) A sample of peak B from the gel filtration on Sephadex LH-60 was lyophilized, redissolved in 88% formic acid (100 μL, 1.1 × 10^5 cpm), and injected onto the column equilibrated with 0.1% trifluoroacetic acid. The peptides were eluted with a gradient identical with that described in (A). Fractions of 0.5 mL were collected, and in this particular run the entire sample was used for measurement of ^{3}H cpm (dashed line). In subsequent runs, for both peaks, the regions were pooled as indicated and sequenced.

were isolated. The specific radioactivity of pools A_1 and A_2 were 31 000 and 2400 cpm (nmol of peptide)$^{-1}$, respectively. Fifty nanomoles of pool A_2 was then attached to isothiocyanate glass beads and subjected to solid-phase, amino-terminal sequencing (Doolittle et al., 1977). The amino-terminal sequence obtained was X-Gly-Ala-Pro-Glu-Arg-Ile-Leu-Asp-Arg-X-Ser-Ser-Ile-Leu-Leu-His-Gly-X-Glu-Glu-Pro-Leu-Leu-Glu-Tyr-Leu-X-X-Ala-. At the positions denoted X, no identifiable phenylthiohydantoin was collected. The coupling method used in the solid-phase sequencing precludes the identification of the amino terminus. The amount of radioactivity in the output from each cycle and the amount of phenylthiohydantoin recovered were determined (Figure 5). Peaks of radioactivity were observed in steps 11, 19, and 28. By interpolation, the amount of the phenylthiohydantoin that should have been produced at step 11 is 3 nmol. With this interpolated value and the cpm observed, the specific radioactivity of the modification at this position in the sequence can be calculated to be 1700 cpm nmol^{-1}.

Pool A_1 (16 nmol, 400 000 cpm) was also subjected to solid-phase sequencing exactly as A_2 had been, but less than 0.2 nmol of phenylthiohydantoin was recovered in steps 2 and 3 (Gly and Ala, respectively), and no clear sequence could be

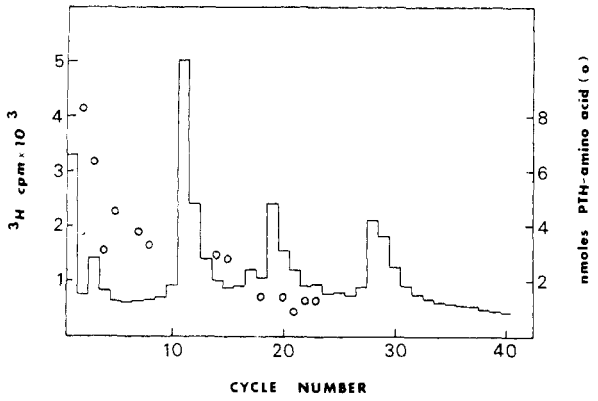

FIGURE 5: Distribution of radioactivity associated with the PTH-amino acids recovered from a sample of peptide A_2 sequenced on a solid-phase, automated sequencer. A sample of peptide A_2, attached to PITC glass beads (55 nmol, 4×10^4 cpm), was loaded into the sequencer. As each cycle was completed, the phenylthiazolinones of the amino acids were collected in 1 mL of trifluoroacetic acid, and one-fourth of the sample was submitted to liquid scintillation. The remaining sample was converted to PTH-amino acids for identification. The radioactivity represents the total ^3H cpm recovered in each cycle. The open circles represent total yield of PTH-amino acid, determined by comparing absorbance at 254 nm with known amount of standard PTH-amino acids.

obtained beyond these steps. Furthermore, no peak of radioactivity was observed in any step up to cycle 24. In a previous experiment, the same results had been obtained; a clear sequence was obtained from peptide A_2 but none from A_1.

Manual dansyl Edman sequencing clearly showed that both pools B_1 and B_2 contained the same unique peptide with amino-terminal sequence Lys-Asp-. Repeated attempts to sequence B_1 and B_2 by solid-phase methods, however, failed to yield any unique sequence even though the dansyl Edman results had produced an expectation. Consequently, the peptide in peak B_2 was further submitted to the manual dansyl Edman procedure; the sequence observed was Lys-Asp-X-Phe-Leu-.

The unidentified amino acid, denoted X in the amino-terminal sequence of peak B_2, had some peculiar characteristics worth mentioning. First, when the sample of dansyl amino acids from step 3 was run on the polyamide plate according to the procedure of Hartley (1970), a single bright spot was observed in the vicinity of the standards Ile and Phe after the second dimension. After the third dimension, however, this spot remained stationary and, thus, moved with neither of the standards. Second, when a sample of pool B_1 (Figure 4B) was subjected to solid-phase sequencing, a significant amount of radioactivity was released by trifluoroacetic acid in cycle 3. Third, on one occasion, the sequencer gave a good phenylthiohydantoin of aspartic acid at cycle 2, but no other amino acid could be identified beyond this cycle. Thus, at the moment, the third amino-terminal residue of peptide B cannot be identified. These observations are described here in the hope that the overall sequence of the α polypeptide will resolve these ambiguities.

Discussion

The reaction between *N*-ethylmaleimide and $(Na^+ + K^+)$-ATPase under conditions that produce all of the known kinetic states and their associated conformations have been studied in detail by Winslow (1981). Under conditions leading to maximum incorporation, 4 mol of *N*-ethylmaleimide was found to be incorporated into each mole of the α polypeptide in the native enzyme. Two of these 4 mol of *N*-ethylmaleimide was rapidly incorporated under all conditions studied. In the experiments described here, there was no indication that either of these 2 mol modify a unique sulfhydryl residue, and it has been assumed that this background incorporation is at random and adventitious. Winslow also found that 1 of the 4 mol of *N*-ethylmaleimide was incorporated only in the absence of ATP. At that time, this incorporation was thought to be peculiar to the E_2 conformation of the enzyme (Winslow, 1981) because the enzyme that was used had been stripped of any tightly bound nucleotide and Koepsell (1979) has shown that in the absence of bound nucleotide the enzyme is always in the E_2 conformation. But if one examines closely the results in Table IV of Winslow, it becomes clear that the major difference in the amount of alkylated external sulfhydryl occurs between conditions with and without ATP present. Therefore, this incorporation will be referred to here as an alkylation of the ATP-protected sulfhydryl. Alkylation of this sulfhydryl does not result in loss of enzymatic activity. Finally, it was assumed by Winslow that at least 1 mol of the maximum incorporation of 4 mol had to have been responsible for the inactivation of the enzyme caused by *N*-ethylmaleimide (Fahn et al., 1968; Skou, 1974). The sulfhydryl alkylated during this inactivation was referred to as the activity-associated sulfhydryl.

The experiments described here represent an effort to isolate and identify, by their amino-terminal sequences, the individual peptides that contain the activity-associated sulfhydryl and the ATP-protected sulfhydryl. This information will be important for the eventual understanding of the three-dimensional structure and the molecular mechanism of the enzyme. When the complete sequence of the α polypeptide of the enzyme becomes available, it will be possible to identify these specific sulfhydryl residues among the 20–25 sulfhydryls present in the α chain (Kyte, 1972). This, along with knowledge of the identity of other specific regions of the enzyme, such as the membrane-spanning segments (Nicholas, 1984) and the phosphorylation site (Post and Kume, 1973), as well as a low-resolution image of the enzyme molecule (Zampighi et al., 1984), should permit construction of a rough three-dimensional model that will provide insight into the mechanism of the enzyme.

Three conformational states of the enzyme, established by cases I–III, were chosen such that under identical labeling conditions with *N*-[^{3}H]ethylmaleimide the $(Na^+ + K^+)$-ATPase activity remaining at the end of incubation varied from 90 to 30% (Table I). Furthermore, in case II the enzyme was poised under conditions in which the ATP-protected sulfhydryl is most reactive toward *N*-ethylmaleimide (Winslow, 1981). When the labeled α polypeptide resulting from these reactions was purified (Figure 1) and subjected to cyanogen-bromide digestion and the digest was resolved on a column of Sephadex LH-60 (Figure 2), it was observed that the incorporation of label into two peaks in the elution profile varied with the conformation in which the enzyme had been reacted with *N*-ethylmaleimide.

An examination of the individual results (Table I) led to the conclusion that the amount of radioactivity incorporated into peak B per picomole of α polypeptide increased as the extent of enzyme inactivation increased. When the results for peak B, taken from Table I, were normalized and plotted against the percent of enzymatic inactivation, a clear correlation was observed (Figure 3). This demonstrates that peak B contained a peptide in which the activity-associated sulfhydryl resides.

In four out of the five sets of experiments presented in Table I, the amount of radioactivity incorporated into peak A per

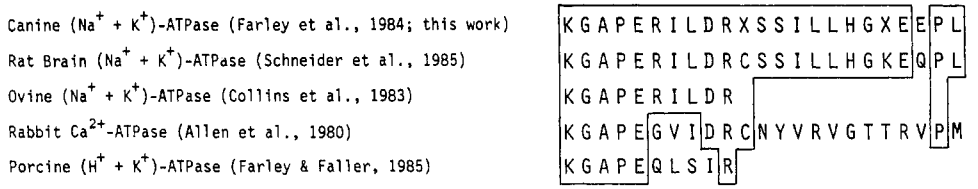

FIGURE 6: Alignment of sequences from various active transport ATPases that are homologous to the amino-terminal sequence of peptide A.

picomole of α polypeptide was largest when the enzyme was under the conditions of case II where ATP was absent. This suggests that peak A represents the peptide that contains the ATP-protected sulfhydryl. The characteristics of this modification reaction also provide support for the suggestion that it is the binding of ATP rather than the specific conformation of the enzyme that is responsible for the changes in reactivity of this cysteine. In case I, where the enzyme was in the E_2–P state and the binding site for ATP was not filled, peak A was only partially protected, but in case III, where the binding site for ATP was fully occupied, the least amount of incorporation into peak A was observed.

The relative values for the incorporation of *N*-[^{3}H]ethylmaleimide into peaks A and B (Table I) suggest strongly that each peak contained only one unique cysteine residue that had been modified to varying degrees during the alkylation of the native enzyme. Because the intercept at the ordinate of Figure 3 is only 20% of the extrapolated incorporation at an inactivation of 100%, it can be concluded that the majority of the incorporation into peak B was due to the modification of only one unique cysteine residue. This is also supported by comparisons of the specific radioactivities of the various peaks B with the respective maximum total incorporations into the α polypeptide, each of which should represent about 3–4 mol of *N*-[^{3}H]ethylmaleimide (mol of α polypeptide)$^{-1}$. The relative values for the incorporation of *N*-[^{3}H]ethylmaleimide into peaks A and B (Table I) are such that, if only one cysteine was being modified to various degrees in peak B, only one was being modified in peak A. Therefore, only two cysteines in the α polypeptide, one in peak A and one in peak B, react significantly under any of these circumstances.

Upon high-pressure liquid chromatography, the radioactivity in peak A was resolved into two peaks that coeluted with two peaks of absorbance at 229 nm (Figure 4A), but amino-terminal analysis repeatedly indicated that both pools A_1 and A_2 contained the same peptide. When the specific radioactivities of both peptide A_1 and peptide A_2 were determined in one of the experiments [31 000 and 2400 cpm (nmol of peptide)$^{-1}$, respectively] and compared to that of the starting α polypeptide [24 000 cpm (nmol of α polypeptide)$^{-1}$], it became clear that peptide A_1 could not have been derived uniformly from the α polypeptide that had been digested. This follows from the fact that this α polypeptide should have contained 3.5 mol of modified cysteine (mol of protein)$^{-1}$ and the specific radioactivity of each modified cysteine must have been less than or equal to 7000 cpm mol^{-1}. The α polypeptide used in this experiment, however, was a mixture of α polypeptide derived from (Na^+ + K^+)-ATPase labeled in its native form with *N*-[^{3}H]ethylmaleimide (60 nmol, 91 000 cpm nmol^{-1}) and unradioactive, pure α polypeptide (180 nmol) as carrier. The specific radioactivity of the unmixed, labeled α polypeptide was sufficient to have yielded peptide A_1. These considerations suggest that peptide A_1 was derived by and large from the α polypeptide modified in its native conformation while A_2 was derived by and large from the carrier α polypeptide that had been modified in its denatured state, even though peptides A_1 and A_2 had the same amino-terminal sequence. The difference between peptides A_1 and A_2 detected by HPLC is unknown.

As this work was in progress, a partial amino acid sequence from the α polypeptide, derived from the cDNA for (Na^+ + K^+)-ATPase from rat brain, was kindly provided to me by Dr. R. Levenson (Schneider et al., 1985). This sequence (Figure 6), which contains much of the region coinciding to that sequenced here from peak A_2, places a cysteine residue at position 11 of peptide A_2 and a lysine residue at position 19. The specific radioactivity of the labeled cysteine thus identified at position 11 was 1700 cpm nmol^{-1}, which was very close to that of the starting peptide (2400 cpm nmol^{-1}). This, at first glance, suggests that cysteine-11 is the ATP-protected sulfhydryl.

Unfortunately, the situation is more complicated than it seems to be. Because peaks of radioactivity were also observed at cycles 19 (Lys) and 28 (X), it necessarily follows that there must be another cysteine, which also reacted with *N*-[^{3}H]-ethylmaleimide, located to the carboxyl side of lysine 19.[3] Furthermore, it has already been noted that peptide A_1 contains most of the radioactivity, yet this peptide could not be sequenced by the solid-phase methods employed in our laboratory, and no indication of where peptide A_1 had been modified could be obtained. Even though only one cysteine in peptide A is the ATP-protected sulfhydryl, its exact position has not yet been identified.

The elution profiles from pool B, when it was submitted to HPLC, contained two peaks of radioactivity that coeluted with a broad, unresolved collection of small peaks. Nevertheless, when pooled according to their respective peaks of radioactivity and sequenced, the two pools, B_1 and B_2, proved to contain the same unique peptide. This type of chromatographic behavior upon HPLC has been observed many times before and was probably due to microheterogeneity of the short peptide being purified. Only a limited amount of this peptide was ever available, and as with peptide A_1, several attempts to sequence it by solid-phase methods failed. Nevertheless, the partial amino-terminal sequence Lys-Asp-X-Phe-Leu-, obtained from the manual dansyl Edman procedure, should be sufficient to identify this peptide in the complete sequence of the mammalian α polypeptide once it becomes available.

It is of interest that peptide A, in addition to containing the ATP-protected sulfhydryl, has for its amino terminus that lysine residue which is uniquely modified by fluoresceinyl 5′-isothiocyanate in calcium ion activated ATPase (Ca^{2+}-ATPase) from sarcoplasmic reticulum (Mitchinson et al., 1982), (Na^+ + K^+)-ATPase (Farley et al., 1984), and proton and potassium ion activated ATPase [(H^+ + K^+)-ATPase] from gastric mucosa (Farley & Faller, 1985). Incorporation of fluoresceinyl 5′-isothiocyanate leads to complete inhibition of the ATPase activities of these three enzymes. Since this

[3] This conclusion follows from the fact that the peptide is attached to the solid phase through lysine residues. As the Edman cyclization proceeds through position 19, a small fraction of the remaining peptide falls off the solid phase, and it is radioactive. Therefore, there must be significant amounts of radioactivity attached to A_2 on the carboxyl side of lysine-19.

inactivation can be prevented by the addition of ATP (Karlish, 1980), Farley et al. (1984) have concluded that this lysine is present within the active site of $(Na^+ + K^+)$-ATPase. The incorporation of *N*-[^{3}H]ethylmaleimide into this peptide is also prevented by the addition of ATP (Figure 2), but the sulfhydryl that reacts with this reagent cannot be within the active site because the enzyme is not inactivated by its modification. The ability of ATP to protect the enzyme from either of these two reagents could be due solely to the conformational change that occurs upon binding ATP, which might prevent fluoresceinyl 5′-isothiocyanate from reacting with the lysine residue or *N*-ethylmaleimide from reacting with the sulfhydryl even though these residues were both situated at locations distant from the active site. In fact, because this region of the sequence cannot be identified in the Kdp-ATPase of *Escherichia coli* (Hesse et al., 1984), even though this enzyme is homologous in other respects to $(Na^+ + K^+)$-ATPase, is responsible for active transport, and is an ATPase, it is possible that this region of the sequence has nothing to do with the active site.

It is obvious, however, that the sequence of this portion of the α polypeptide is highly conserved among active transport enzymes (Figure 6). These observations, in addition to the fact that the lysine residue exclusively reacts with fluoresceinyl 5′-isothiocyanate, clearly indicate that peptide A is from a region whose structure is highly conserved, and must have an important role in the overall function of the enzyme. It also contains a widely conserved cysteine residue at position 11 that may be the ATP-protected sulfhydryl.

The results presented here, however, do show that peptide A does not contain the sulfhydryl whose alkylation inactivates the enzyme (Fahn et al., 1968; Skou, 1974). In fact, the correlation documented in Figure 3 clearly demonstrates that the sulfhydryl whose alkylation inactivates the enzyme resides in peptide B, which is quite distinct from peptide A. The question of whether peptide B is from a region of the sequence already known to be functionally important or identifies a new functionally important location can only be answered when the complete sequence of $(Na^+ + K^+)$-ATPase becomes available.

ACKNOWLEDGMENTS

I thank Prof. Jack Kyte, in whose laboratory this research was performed, for his advice, support, and guidance. I also thank James Gallagher for preparing the unradioactive α polypeptide. I am grateful to Dr. Robert Levenson for providing the sequence from rat brain prior to publication.

Registry No. ATP, 56-65-5; ATPase, 9000-83-3.

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