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Any of Several Lysines Can React with 5′-Isothiocyanatofluorescein To Inactivate Sodium and Potassium Ion Activated Adenosinetriphosphatase†

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ABSTRACT: Determinations of reaction stoichiometry demonstrate that the covalent incorporation of one molecule of 5′-isothiocyanatofluorescein can inactivate one molecule of sodium and potassium ion activated adenosinetriphosphatase in agreement with earlier determination of this stoichiometry. Several different modified peptides are produced, however, when the modified enzyme is digested with trypsin. One of these peptides has been identified as HLLVMK(thioureidylfluorescein)GAPER by use of a specific immunoadsorbent. The modified lysine is lysine 501 in the amino acid sequence of the α polypeptide of (Na^+ + K^+)-ATPase. This peptide has been previously isolated from such digests [Farley, R. A., Tran, C. M., Carilli, C. T., Hawke, D., & Shively, J. E. (1984) *J. Biol. Chem. 259*, 9532-9535]. The other specifically modified peptides have been purified and identified by amino acid sequencing. Their sequences identify lysine 480 and lysine 766 from the α polypeptide as amino acids modified by 5′-isothiocyanatofluorescein in reactions sensitive to the addition of ATP and responsible for inactivation of the enzyme.

Sodium and potassium ion activated adenosinetriphosphatase [(Na^+ + K^+)-ATPase][1] (Skou, 1957) is the enzyme responsible for the coupled, active transport of sodium and potassium across the plasma membranes of all animal cells (Kyte, 1981). The enzyme is composed of one α subunit and one β subunit. Recent advances in recombinant DNA technology have facilitated the determination of the primary structure of the two polypeptides. The α subunit, composed from a polypeptide with a length of 1016 amino acids (Shull et al., 1985; Ovchinnikov et al., 1986), is responsible for catalysis (Kyte, 1981). The β subunit is a glycoprotein (Kyte, 1972) composed of a polypeptide with a length of 302 amino acids (Shull et al., 1986; Ovchinnikov et al., 1986). The physiological role of this β subunit in cation transport, however, still remains unknown.

One of the major problems to be solved in the understanding of active transport is the molecular mechanism by which the hydrolysis of a chemical bond of high energy is coupled to the unidirectional movement of a small molecule. Its solution requires information about the structure of the protein and the relative locations of the sites for hydrolysis and transport as well as an understanding of subunit interactions and conformational changes accompanying transport. Little is known about the structural features of the active site of (Na^+ + K^+)-ATPase, which is a participant in the coupling between hydrolysis and ion transport. One approach to this problem has been to use reagents specific for particular functional groups to investigate the active site of this protein. Treatment of purified (Na^+ + K^+)-ATPase with such reagents can demonstrate the presence of essential functional groups and may enable us to elucidate the relationships between structure and function.

The active site of (Na^+ + K^+)-ATPase has been investigated with a variety of chemical reagents. It has been shown that an aspartate is the amino acid within this active site that accepts the phosphate of ATP during the first step of its hydrolysis, and the tripeptide containing this essential residue has been shown to be SD(PO_3^{2-})K (Post et al., 1973; Bastide et al., 1973). This identifies it as aspartate 369 of the α polypeptide (Shull et al., 1985). Nucleotide affinity reagents, which might react covalently with amino acid residues within the active site, have been used in an effort to identify other sequences surrounding the active site (Munson, 1981; Patzelt-Wenczler et al., 1975; Haley et al., 1974; Cooper et al., 1983; Ohta et al., 1986). A cysteine residue (Schoot et al., 1978), an arginine residue (De Pont et al., 1977), and a tyrosine residue (Cantley et al., 1978) have been suggested to play an important role in the active site. Dzhandzhugazyan et al. (1988) have suggested that aspartate 710 and aspartate 714 both are functionally important residues because they are modified by adenosine 5′-[*N*-[4-[*N*-(2-chloroethyl)-*N*-methylamino]benzyl]-γ-amidotriphosphate] in native (Na^+ + K^+)-ATPase. All of this information suggests that the active site is formed from amino acids distant from each other in the

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[1] Abbreviations: TUF, 5′-thioureidylfluorescein; (Na^+ + K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate; HPLC, high-pressure liquid chromatography; PTH-amino acid, phenylthiohydantoin of the amino acid; (H^+ + K^+)-ATPase, proton and potassium ion activated ATPase; Ca^{2+}-ATPase, calcium ion activated ATPase; H^+-ATPase, proton-activated ATPase; K^+-ATPase, potassium ion activated ATPase.

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primary structure rather than from only a small domain in the tertiary structure.

Karlish (1980) introduced the use of 5′-isothiocyanatofluorescein as a specific probe of the active site of $(Na^+ + K^+)$-ATPase. He demonstrated that this compound completely inhibits ATPase activity and that the inhibition can be prevented by ATP. 5′-Isothiocyanatofluorescein reacts with a stoichiometry of 1 mol incorporated for each site for ATP or ouabain, and it was suggested that the polycyclic reagent binds to the part of the site for ATP that is normally occupied by adenosine (Karlish, 1980; Cantley et al., 1983). A kinetic study of the inhibition caused by 5′-isothiocyanatofluorescein indicates that this inhibition is an irreversible, pseudo-first-order reaction, and the variation in rate as a function of the concentration of the reagent is consistent with the formation of a specific and reversible complex between it and the enzyme before the covalent modification occurs (Karlish, 1979; Farley et al., 1984). A labeled tryptic peptide derived from the α polypeptide $(Na^+ + K^+)$-ATPase that had been modified with 5′-isothiocyanatofluorescein has been isolated. The amino acid sequence of this peptide is HLLVMK(TUF)GAPER, and the lysine, lysine 501 in the amino acid sequence of the α polypeptide, bears the fluorescein. This lysine resides in a conserved region of the sequences found in the ATPases responsible for the primary active transport (Farley et al., 1984). Furthermore, the changes that occur in the rate of incorporation of [^{3}H]acetyl from [^{3}H]acetic anhydride into lysine 501 when ATP is bound by the intact native enzyme suggest that lysine 501 of the α polypeptide is directly involved in binding ATP within the active site of $(Na^+ + K^+)$-ATPase (Xu & Kyte, 1989).

During recent experiments (Xu & Kyte, 1989) studying the reaction of 5′-isothiocyanatofluorescein with $(Na^+ + K^+)$-ATPase, a determination of the yield of incorporation of the reagent into lysine 501 was made. The present paper describes the result of this assessment and observations made consequent to it. Although one molecule of 5′-isothiocyanatofluorescein was able to inactivate one molecule of $(Na^+ + K^+)$-ATPase, only half of the incorporation was into lysine 501. It has been found that, during specific incorporation of thioureidylfluorescein into the enzyme, lysine 480 and lysine 766 also react, and the results indicate that these lysines are also within or near the active site at which ATP is hydrolyzed. A significant number of identities in the amino acid sequences around these lysines in the enzymes catalyzing active transport indicate that these lysines are involved in the hydrolysis of ATP catalyzed by these enzymes.

Experimental Procedures

Materials. 5′-Isothiocyanatofluorescein was purchased from Molecular Probes Inc.; soybean trypsin inhibitor, adenosine 5′-triphosphate, tris(hydroxymethyl)aminomethane (Tris), 2-mercaptoethanol, soybean trypsin inhibitor, and histidine were from Sigma Chemical Corp.; acetonitrile (high-pressure liquid chromatographic grade) was from Fisher Chemical Corp.; trypsin treated with L-1-(tosylamino)-2-phenylethyl chloromethyl ketone was from Worthington Corp.; dimethyl sulfoxide was from Mallinkrodt Chemical Corp. Imidazole was recrystallized from benzene and then acetone, and trifluoroacetic acid was redistilled. Anthroylouabain (Fortes, 1977) was the gift of Dr. P. A. George Fortes, University of California, San Diego.

Preparation of $(Na^+ + K^+)$-ATPase. Purified, membrane-bound $(Na^+ + K^+)$-ATPase was prepared from canine kidney by the method of Jørgensen (1974) with the modifications described by Munson (1981). The enzymic activity was determined as described by Kyte (1971). The specific activity of the enzyme used in these studies was 1200 μmol of P_i mg^{-1} h^{-1}. The molar concentration of purified $(Na^+ + K^+)$-ATPase was determined by a titration of binding sites for anthroylouabain as described by Moczydlowski and Fortes (1981). The purified enzyme had 240000 g of protein (mol of binding sites)$^{-1}$ and a turnover number of 80 s^{-1}.

Labeling of $(Na^+ + K^+)$-ATPase with 5′-Isothiocyanatofluorescein. Purified membrane-bound $(Na^+ + K^+)$-ATPase was submitted to centrifugation at 45000 rpm for 60 min in a 50 Ti rotor (Beckman Corp.). The pellet was resuspended in 100 mM NaCl, 5 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetate (EDTA), and 50 mM Tris-HCl, pH 9.2. The resuspended membranes were divided into two portions. Adenosine 5′-triphosphate was added to one portion at a final concentration of 3 mM. 5′-Isothiocyanatofluorescein was dissolved in dimethyl sulfoxide and diluted into the mixtures. Its concentration was calculated from its absorbance at 499 nm with an extinction coefficient of 75000 (Carilli et al., 1982).[2] The reaction was performed at room temperature with stirring in the dark. The final concentration of dimethyl sulfoxide was always less than 1%. To check the enzymic activity of samples from the reaction, each was removed and quenched by a 10-fold dilution into ice-cold 1 mM EDTA, 1 mM 2-mercaptoethanol, and 25 mM imidazolium chloride, pH 7.5. These samples were appropriately diluted and assayed for strophathindin-sensitive ATPase activity. At the completion of the modification with 5′-isothiocyanatofluorescein, the reaction mixture was diluted 10-fold into cold 1 mM EDTA, 1 mM 2-mercaptoethanol, and 25 mM imidazolium chloride, pH 7.5, and centrifuged at 45000 rpm in the 50 Ti rotor for 60 min to remove unattached fluorescein. The pellets were washed again with 1 mM EDTA and 25 mM imidazolium chloride, pH 7.5 (Farley et al., 1984), and the washed pellets were resuspended in the same buffer.

Three separate modifications at three different molar ratios between $(Na^+ + K^+)$-ATPase and 5′-isothiocyanatofluorescein were performed. When enzyme and reagent were mixed at a molar ratio of 1:1, the final concentrations of each were 12 μM, the reaction (in 1.8 mL) was allowed to proceed for 10 h, and the final enzymic activity was 35% of the control. When enzyme and reagent were mixed at a molar ratio of 1:1.7, the final concentrations were 12 and 20 μM, respectively, the reaction (7 mL for −ATP and 7 mL for +ATP) was allowed to proceed for 3 h, and the final enzymic activity was 10% of the control. When enzyme and reagent were mixed at a molar ratio of 1:2.7, the final concentrations were 12 and 33 μM, the reaction (20 mL for −ATP and 3.3 mL for +ATP) was allowed to proceed for 1 h, and the final enzymic activity was <1% of the control.

Tryptic Digestion. Washed and resuspended preparations of $(Na^+ + K^+)$-ATPase, labeled with 5′-isothiocyanatofluorescein, were digested with trypsin at a ratio of 0.1 mg of trypsin (mg of enzyme)$^{-1}$, for 3 h at 37 °C. Soybean trypsin inhibitor was added at a 2-fold excess (w/w) over trypsin to stop the reaction (Farley et al., 1984). Following the digestion the mixtures were centrifuged at 45000 rpm for 1 h. The supernatant was removed and centrifuged again at 45000 rpm for 30 min and kept at −20 °C.

Immunoadsorption. An immunoadsorbent, specific for the carboxy-terminal sequence, −GAPER, was described in detail earlier (Kyte et al., 1987). The peptide KGAPER was synthesized and then coupled to serum albumin. The resulting

[2] Myun Han and P. A. George Fortes, personal communication.

covalent complex was injected into rabbits as an antigen. Polyclonal immunoglobulins against the sequence -GAPER were purified by immunoadsorption with an immunoadsorbent made from the synthetic peptide. These polyclonal immunoglobulins were, in turn, coupled to agarose to produce the immunoadsorbent. Its capacity was assessed by its ability to bind and release the synthetic peptide.

Portions of the supernatants from tryptic digests of (Na^+ + K^+)-ATPase that had been labeled with 5′-isothiocyanatofluorescein were passed directly over a column (1.1 cm × 2.7 cm) of the immunoadsorbent (14-nmol capacity). The liganded immunoadsorbent was washed with 0.15 M NaCl, 0.1 mM EDTA, and 20 mM sodium phosphate, pH 7.4, and eluted with 0.1 M sodium phosphate, pH 2.5. The absorbance at 499 nm, and hence the molar concentration of fluorescein, of each fraction was determined after the pH was adjusted to between 7 and 8.

High-Pressure Liquid Chromatography (HPLC). The columns used for HPLC were a TSK-G2000SW molecular exclusion column (0.9 cm × 33.2 cm) manufactured by Toyo Soda Corp., a Vydac C_4 reverse-phase column (0.46 cm × 25 cm) and a Vydac C_{18} reverse-phase column (0.46 cm × 25 cm) both manufactured by Sep/a/ra/tions Group, and a μBondapak C_{18} reverse-phase column (0.6 cm × 31.5 cm) manufactured by Waters Associates. Detection was carried out sequentially with in-line flow cells of a 440 UV detector equipped with an extended wavelength module (Water Associates) and a Spectra/Glo fluorometer (Gilson Medical Electronics) with an excitation filter of 350 ± 30 nm and an emission filter of 500 ± 70 nm. All chromatography was carried out at room temperature in dim light.

Sequencing. Peptides were sequenced on either one of two Applied Biosystems Model 470 Gasphase sequencers equipped with Model 120 on-line high-pressure liquid chromatographic systems. The peptides were applied to polybrene-treated discs of glass fiber. The fluorescein appeared to bind tightly to these discs because they remained yellow through the entire run of the sequencer when fluorescein-modified peptides were adsorbed to them. The normal program supplied by the manufacturer (Applied Biosystems FIL 470-1) incorporates several washes of the filters with trifluoroacetic acid before the first cycle is initiated with phenyl isothiocyanate.

Results

Stoichiometry between Inactivation of (Na^+ + K^+)-ATPase and Incorporation of 5′-Isothiocyanatofluorescein. The molar concentration of purified (Na^+ + K^+)-ATPase was determined by a titration of binding sites for anthroylouabain as described by Moczydlowski and Fortes (1981). From this value and the concentration of protein, it could be calculated that there was 240 000 g of protein (mol of ouabain binding sites)$^{-1}$ in the preparation of enzyme used for these studies. This preparation of (Na^+ + K^+)-ATPase was labeled with 5′-isothiocyanatofluorescein at room temperature under the following conditions: 21 nmol of (Na^+ + K^+)-ATPase and 21 nmol of 5′-isothiocyanatofluorescein in 1.8 mL of 1 mM EDTA and 50 mM Tris-HCl, pH 9.2. The activity of the enzyme was followed by assaying the strophanthidin-sensitive ATPase activity of samples withdrawn at various times. The enzymic activity decreased monotonically over the first 4 h and decreased no further between 6 and 10 h. Presumably, the aryl isothiocyanate had either modified the protein or been hydrolyzed by 6 h. The maximum inactivation was 65%. The membrane-bound enzyme that had been modified with 5′-isothiocyanatofluorescein was extensively washed to remove unattached fluorescein and then digested with trypsin (Farley et al., 1984) at a ratio of 0.1 mg of trypsin (mg of protein)$^{-1}$. Soluble peptides were separated from fragments of membrane by centrifugation. The pellet, containing these fragments of membrane, was resuspended in the same buffer as the supernatant. The absorbances of the superantant and the resuspended pellet were assessed as a function of wavelength (Figure 1). Fluorescein has a maximum absorbance at 500 nm. Peptides with a maximum absorbance at 499 nm were present in the supernatant (Figure 1A), and no peak of absorbance (<20% of the absorbance of the supernatant) was observed in the spectrum of the pellets (Figure 1B). Therefore, digestion with trypsin released all (>80%) of the fluorescein covalently attached to the membrane-bound enzyme.

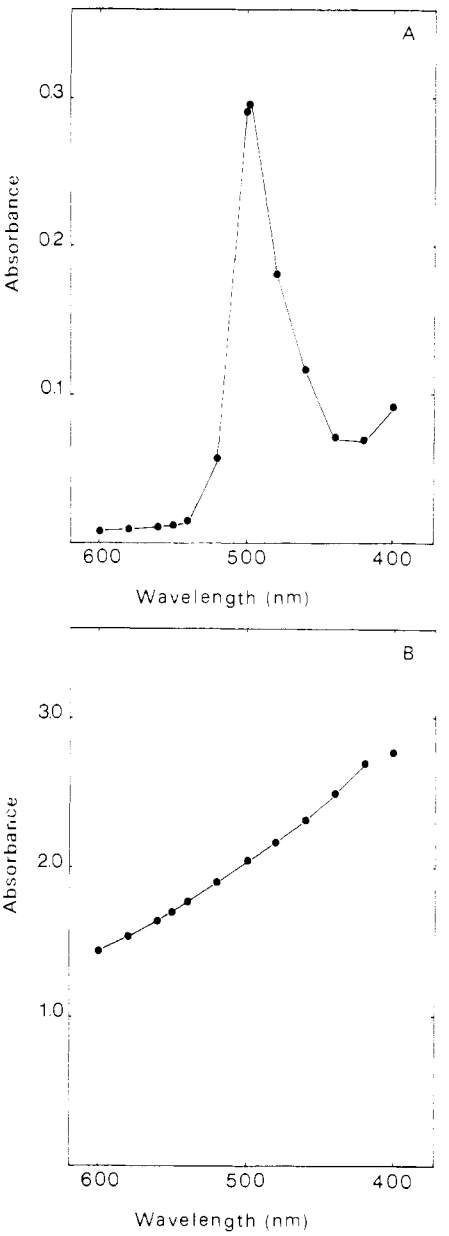

FIGURE 1: Release of fluorescein from modified (Na^+ + K^+)-ATPase by trypsin. Following modification of (Na^+ + K^+)-ATPase by 5′-isothiocyanatofluorescein, the membrane-bound protein was washed and resuspended in 1 mM EDTA and 25 mM imidazolium chloride, pH 7.5. The resuspended membranes were digested with trypsin at a ratio of 1:10 (w/w) for 3 h at 37 °C. Membranes were removed by centrifugation, and the pellet was resuspended in the same buffer. The absorbance of the supernatant (A) and the pellet (B) was determined as a function of wavelength.

The amount of labeled peptides in the supernatant was calculated from an extinction coefficient of 75 000 (Carilli et

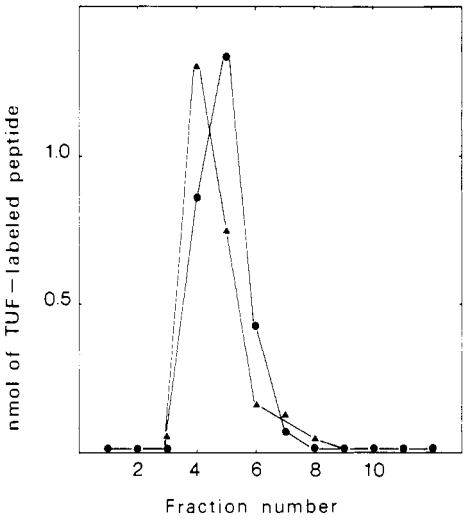

FIGURE 2: Immunoadsorption of tryptic digests of $(Na^+ + K^+)$-ATPase labeled with 5′-isothiocyanatofluorescein. A portion of the supernatant from a tryptic digest of modified enzyme was added directly to a column (1.1 cm × 2.7 cm) of beaded agarose to which immunoglobulin directed against the carboxy-terminal sequence –GAPER had been attached (14-nmol capacity). The nanomoles of fluorescein in each fraction of the peptides passing directly through the column (▲) and in each fraction of the peptides released by lowering the pH to 2.5 (●) were calculated from their absorbance at 499 nm after the pH was adjusted to between 7 and 8. Fraction 1 of the wash was collected as the sample was added, and fraction 1 of the eluate was collected as the pH of the entering solution was dropped.

al., 1982)[2] and found to be 12 nmol, or 57% of the 21 nmol of fluorescein initially present. This result, the complete removal of fluorescein by trypsin, and the results of the inactivation together demonstrate that the incorporation of one molecule of 5′-isothiocyanatofluorescein can inactivate one molecule of $(Na^+ + K^+)$-ATPase. This agrees with earlier determinations of this stoichiometry (Carilli et al., 1982; Fortes & Han, 1985).

Not All Tryptic Peptides Have the Carboxy-Terminal Sequence –GAPER. Farley et al. (1984) were able to isolate from a similar digest of $(Na^+ + K^+)$-ATPase labeled with 5′-isothiocyanatofluorescein the peptide HLLVMK(TUF)-GAPER, in which lysine 501 from the α polypeptide had been covalently modified to form the thioureidyl derivative. It was of interest to determine whether or not this peptide accounted for all the fluorescence in the initial digest. When 6 nmol of fluorescein, attached to the peptides from $(Na^+ + K^+)$-ATPase that had been labeled with 5′-isothiocyanatofluorescein, was passed through an immunoadsorbent directed against the carboxy-terminal sequence, –GAPER, only 3.0 nmol of fluorescein was retained by the immunoadsorbent and could be released with acid while 2.9 nmol of fluorescein passed through in the wash pror to the elution with acid (Figure 2). This result and the equimolar stoichiometry between incorporation and inactivation suggest that modification of lysines other than lysine 501 also contributes to the inactivation of the enzyme caused by 5′-isothiocyanatofluorescein.

Isolation of the Peptide α470–α495 Modified by 5′-Isothiocyanatofluorescein. A portion from a digest of a larger amount of $(Na^+ + K^+)$-ATPase (85 nmol) that had been modified with 5′-isothiocyanatofluorescein at a molar ratio of 1:1.7 was submitted to HPLC by molecular exclusion on a column of TSK chromatographic medium (Figure 3A). It can be seen that 5′-isothiocyanatofluorescein had been incorporated into several peptides in the tryptic digest of native, modified $(Na^+ + K^+)$-ATPase. The incorporation of 5′-isothiocyanatofluorescein into four of these components (numbered 2, 3, 4, and 6 in Figure 3A) decreased significantly when the modification was performed in the presence of 3 mM ATP (Figure 3B). Sensitivity of its modification to ATP was the criterion used by Farley et al. (1984) to identify HLLVMKGAPER as a candidate for a peptide from the active site of $(Na^+ + K^+)$-ATPase. Therefore, the amino acids modified by 5′-isothiocynatofluorescein in these other peptides are also candidates for functional groups within the active site of the enzyme.

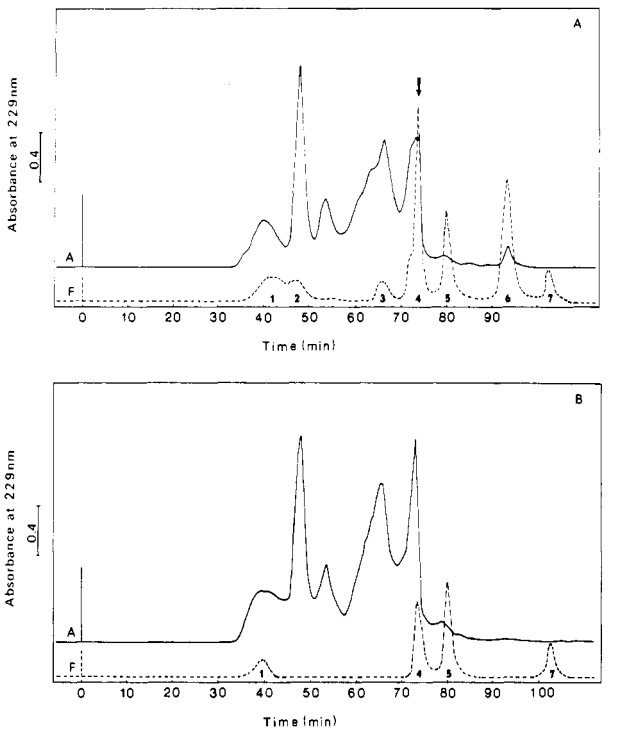

FIGURE 3: Chromatography by molecular exclusion of fluoresceinyl peptides. Supernatants of tryptic digests were prepared from samples of $(Na^+ + K^+)$-ATPase (each 85 nmol) labeled with 5′-isothiocyanatofluorescein at a molar ratio to $(Na^+ + K^+)$-ATPase of 1.7:1 either in the absence (A) or in the presence (B) of 3 mM ATP. Equivalent portions of these supernatants were injected directly onto an HPLC column (0.9 × 33.2 cm) of TSK-G2000SW equilibrated with 0.1% trifluoroacetic acid. The column was developed with the same solvent. Absorbance at 229 nm (—) and fluorescence (– –) were monitored simultaneously. The arrow indicates the peak containing the peptide HLLVMK(TUF)GAPER.

The fluorescent peptides contributing to peak 4 from the chromatographic separation of the digest from $(Na^+ + K^+)$-ATPase labeled in the absence of ATP (Figure 3A) and those contributing to the corresponding peak from the digest of $(Na^+ + K^+)$-ATPase labeled in the presence of ATP (Figure 3B) were separately pooled. From their absorbance, they were found to contain 4.0 and 1.2 nmol of fluorescein, respectively. The difference illustrates the sensitivity to ATP of the incorporation of 5′-isothiocyanatofluorescein into these peptides. These two pools were then passed separately over the immunoadsorbent directed against the carboxy-terminal sequence, –GAPER. Only half (1.9 nmol) of the first pool (–ATP) was adsorbed by the immunoadsorbent and could be eluted with acid while the other half (1.8 nmol) passed directly through the immunoadsorbent. The second pool, derived from $(Na^+ + K^+)$-ATPase modified in the presence of ATP, displayed similar behavior. Half of this pool (0.5 nmol) was adsorbed by the immunoadsorbent and could be eluted with acid while the other half (0.4 nmol) passed through the column. These results identify the location of the peptide HLLVMK-(TUF)GAPER on the chromatographic separation (Figure 3A) and indicate that the yield of the peptide, HLLVMK-

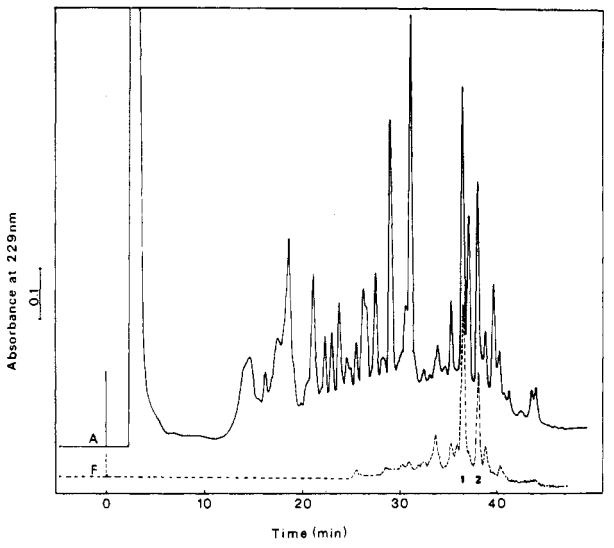

FIGURE 4: Chromatography of fluoresceinyl peptides by C_4 reverse-phase HPLC. Fractions containing peak 4 from the molecular exclusion chromatogram (arrow, Figure 3A) were pooled, evaporated, redissolved in 50% formic acid, and injected onto the Vydac C_4 reverse-phase column (0.46 cm × 25 cm). The chromatogram was then developed with a linear gradient of 0%–60% acetonitrile in 0.1% trifluoroacetic acid delivered at 1 mL min^{-1} over 60 min. Fluorescence (--) and A_{229} (—) were monitored continuously.

(TUF)GAPER, was decreased by 74% when AT was present during the modification. They also demonstrate that peak 4 contains fluorescent peptides in addition to HLLVMK-(TUF)GAPER. The incorporation of 5′-isothiocyanto-fluorescein into the other peptide or peptides present in this pool also decreased (by 78%) when ATP was present.

Another portion from the same digest of $(Na^+ + K^+)$-ATPase that had been modified in the absence of ATP with 5′-isothiocyanatofluorescein at a molar ratio of 1:1.7 was submitted to molecular exclusion chromatography, and peak 4 (Figure 3A) was pooled. This pool was submitted to HPLC on a C_4 reverse-phase column (Figure 4). Two major peaks of fluorescence (labeled 1 and 2 in Figure 4) were present on the chromatogram. The ability of the immunoadsorbent to bind each of these peptides, respectively, was assessed. All of the fluorescence (>99%) contributing to peak 1 was bound by the immunoadsorbent, and this identified it as HLLVMK(TUF)GAPER. None (<0.5%) of the fluorescence contributing to peak 2 was adsorbed.

The peptide responsible for peak 2 (Figure 4) was further purified by HPLC on a C_{18} column to obtain a product whose distribution of absorbance at 229 and fluorescence coincided (Figure 5). This peptide was sequenced (Figure 6) and found to comprise isoleucine 470 to arginine 495 in the amino acid sequence[3] of the α polypeptide of canine renal $(Na^+ + K^+)$-ATPase (Figure 7). This fluorescent peptide was sequenced twice, and both times lysine 480 gave a very low yield while lysine 487 gave a good yield of the respective phenylthiohydantoin (Figure 6). From this result, it was concluded that lysine 480 bore the modification with 5′-isothiocyanato-fluorescein. The reason trypsin did not cleave after lysine 487, which appears to be an unmodified amino acid (Figure 6), is not known.

Correcting for analytical losses and the intentional division of intermediate samples, the yield of the final peptide, calculated from the picomoles of the phenylthiohydantoin of isoleucine (PTH-isoleucine) and of PTH-valine appearing in the first and second cycle of the sequencing (Figure 6) and the original nanomoles of $(Na^+ + K^+)$-ATPase, was 6%. As this peptide is only one of several modified during the reaction (Figure 3), only a portion of the $(Na^+ + K^+)$-ATPase digested could have been modified at this lysine. This, in part, explains the low yield.

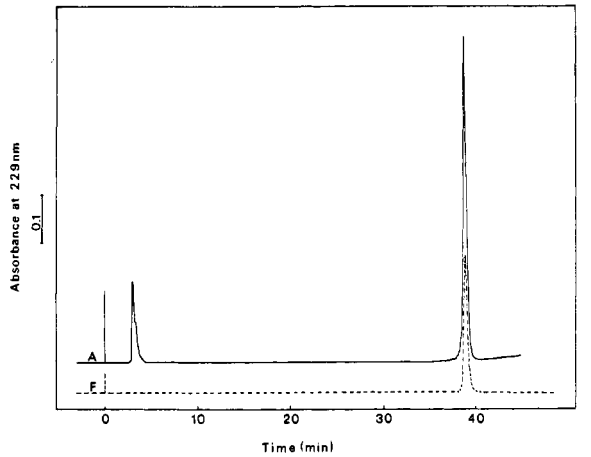

FIGURE 5: Homogeneity of tryptic peptide α470–α495. Fractions containing peak 2 from the C_4 reverse-phase chromatogram (Figure 4) were pooled, evaporated, redissolved in 0.1% trifluoroacetic acid, and injected onto the μBondapak C_{18} reverse-phase column (0.6 cm × 31.5 cm). The chromatogram was then developed with a linear gradient of 0%–60% acetonitrile in 0.1% trifluoroacetic acid delivered at 1 mL min^{-1} over 60 min. Fluorescence (--) and A_{229} (—) were monitored continuously.

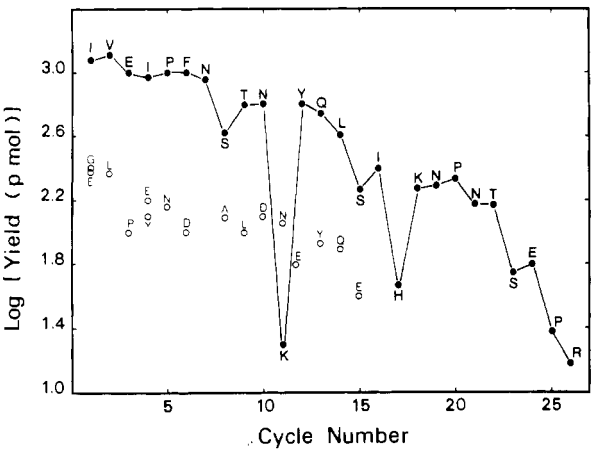

FIGURE 6: Amino acid sequencing of the modified tryptic peptide identified as α470–α495 from canine renal $(Na^+ + K^+)$-ATPase. The logarithms of the yields in picomoles of every phenylthiohydantoin of each amino acid observed (>10 pmol) on a particular cycle of the automated sequencing are plotted. The amino acids assigned as those from the peptide and expected at a particular cycle are in bold face.

Portions of supernatants from tryptic digests of another preparation of $(Na^+ + K^+)$-ATPase (250 nmol), modified with 5′-isothiocyanatofluorescein at a molar ratio of 1:2.7 either in the absence of ATP (Figure 8A) or in the presence of 3 mM ATP (Figure 8B), respectively, were submitted to chromatography on the same C_4 reverse-phase column that had been used in the experiment described in Figure 4. The peak containing HLLVMK(TUF)GAPER (peak 3) was positively identified by amino acid sequencing. The peak containing the peptide α470–α495 (peak 4) was identified by its amino acid composition and its mobility relative to that of peak 3 (compare Figure 8A with Figure 4). When ATP was present during the modification of $(Na^+ + K^+)$-ATPase with 5′-isothiocyanato-

[3] An incomplete sequence of canine renal $(Na^+ + K^+)$-ATPase was available (Robert Farley, personal communication). As this sequence lacked the amino-terminal portions, the numbering of the ovine α polypeptide (Shull et al., 1985) is used herein.

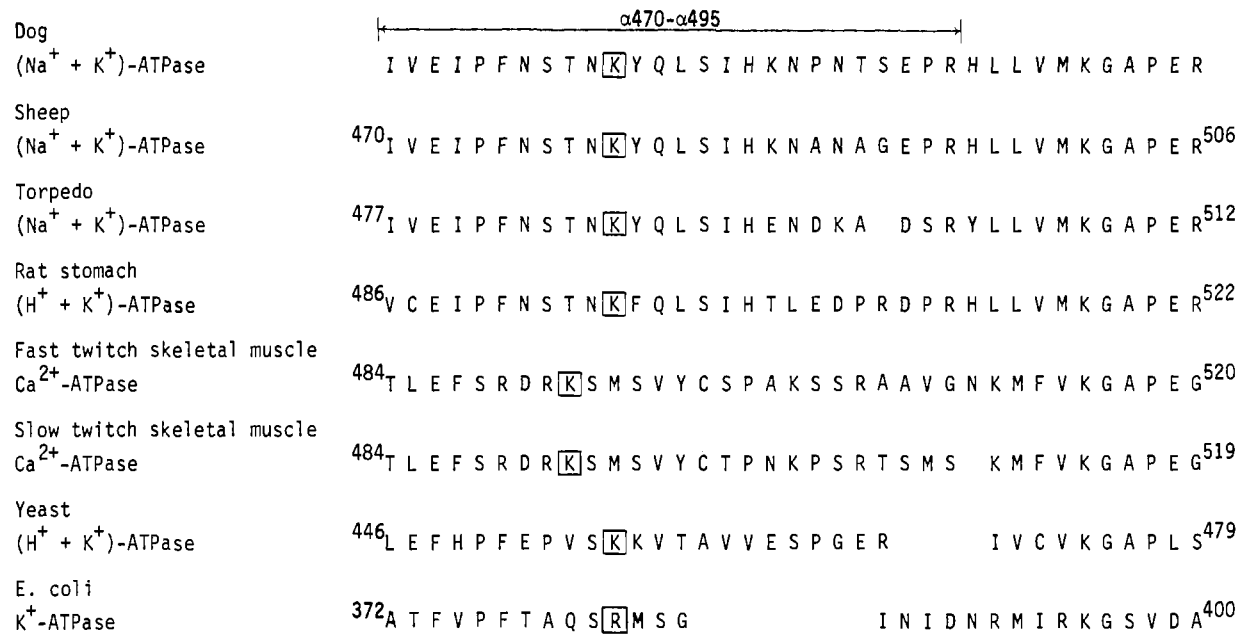

FIGURE 7: Alignment of the sequence of tryptic peptide α470–α495 from canine renal $(Na^+ + K^+)$-ATPase and the homologous regions from the other ATPases responsible for active transport. Sequences aligned are from the α polypeptide of canine renal $(Na^+ + K^+)$-ATPase,[3] α polypeptide of ovine renal $(Na^+ + K^+)$-ATPase (Shull et al., 1985), α polypeptide of $(Na^+ + K^+)$-ATPase from torpedo electric organ (Kawakami et al., 1985), proton and potassium ion activated ATPase [$(H^+ + K^+)$-ATPase] from rat stomach (Shull & Lingrel, 1986), calcium ion activated ATPase (Ca^{2+}-ATPase) from rabbit fast-twitch skeletal muscle (Brandl et al., 1986), Ca^{2+}-ATPase from rabbit slow-twitch skeletal muscle (MacLennan et al., 1985), proton-activated ATPase (H^+-ATPase) from yeast plasma membrane (Serrano et al., 1986), and potassium ion activated ATPase (K^+-ATPase) from plasma membranes of *E. coli* (Hesse et al., 1984). Where no obvious identities existed within the region of interest, the alignment was made on the basis of the highly conserved sequence that lies between positions 500 and 505 in the ovine sequence. This adjacent region of sequence has also been included for reference.

fluorescein, the fluorescence of peak 4 was no longer present on the chromatogram even though several of the other peaks of fluorescence were unaffected. This result confirms the observation that incorporation of 5′-isothiocyanatofluorescein into lysine 480 is sensitive to the presence of ATP.

Other Peptides Modified by 5′-Isothiocyanatofluorescein. The peak of fluorescence numbered 6 in Figure 3A also disappeared when $(Na^+ + K^+)$-ATPase was labeled in the presence of ATP (Figure 3B). It was pooled from a chromatogram of peptides released from $(Na^+ + K^+)$-ATPase modified in the absence of ATP and resubmitted to HPLC on a C_4 reverse-phase column (0.46 cm × 25 cm) eluted with a linear gradient developed between 0.1% trifluoroacetic acid and 60% acetonitrile in 0.1% trifluoroacetic acid (1 mL min^{-1} and 60 min for the complete gradient). A single major peak of fluorescence was seen that coincided with one of several peaks of absorbance at 229 nm. This major peak of fluorescence from the C_4 column (eluting at approximately 27 min) was collected and combined with the same peak from an identical chromatographic separation. The combined fractions were resubmitted to HPLC on a C_{18} reverse-phase column. A single peak of fluorescence was observed that coincided with a single peak of absorbance at 229 nm. This peptide was submitted to sequencing. A low yield of PTH-arginine (95 pmol) appeared at the first cycle of the sequencing, and a high yield of PTH-glycine appeared at the second cycle (350 pmol) and the third cycle (100 pmol).

The peptide or peptides contributing to the fluorescent peak numbered 2 in Figure 8A also displayed incorporation of 5′-isothiocyanatofluorescein that was sensitive to ATP (Figure 8B). This peak was pooled and resubmitted to HPLC on the C_{18} reverse-phase column. A single peak of fluorescence that coincided with a single peak of absorbance at 229 nm was observed (Figure 9). Although the peptide appeared homogeneous, upon amino acid sequencing (Figure 10), two sequences from tryptic peptides of the α polypeptide of canine renal $(Na^+ + K^+)$-ATPase were observed. The peptide present in the highest yield (420 pmol of PTH-glycine in the first cycle) is an arginine-containing tryptic peptide comprising glycine 623 to arginine 640 from the α polypeptide of $(Na^+ + K^+)$-ATPase, but it contains no amino acid that can react with an aryl isothiocyanate (Stark, 1972). The peptide present in lower yield (140 pmol of PTH-leucine in the first cycle) comprises leucine 760 to lysine 767 from the α polypeptide of $(Na^+ + K^+)$-ATPase (Figure 11). The overall yield of this latter peptide was 7%.

Discussion

Sodium and potassium ion activated adenosinetriphosphatase that has been modified with 5′-isothiocyanatofluorescein has been used to follow conformational changes performed by the enzyme during its turnover (Karlish, 1980; Hegyvary & Jørgensen 1981), and the reaction of this reagent with amino acids in the enzyme has been used to identify an important amino acid participating in the active site (Farley et al., 1984; Xu & Kyte, 1989). Because of the large number of studies that have been performed with this reagent, it is important to know how it is covalently bound to the enzyme. The results described in this study identify several of the amino groups with which this reagent reacts.

The reaction between 5′-isothiocyanatofluorescein and $(Na^+ + K^+)$-ATPase is stoichiometric as long as the molar ratio between them is controlled. When enzyme and reagent were mixed in an equimolar ratio, about two-thirds (>57%) of the fluorescein inserted covalently into the protein (Figure 1), and about two-thirds (65%) of the enzymic activity was lost after 10 h. When the molar ratio was raised to 1:1.7, to increase the yield of the reaction, 90% of the enzymic activity had been lost after 3 h of reaction, and most of the incorporation of fluorescein could be eliminated by the addition of ATP during the reaction (Figure 3). When the molar ratio was further increased to 1:2.7, less than 1% of the enzymic activity re-

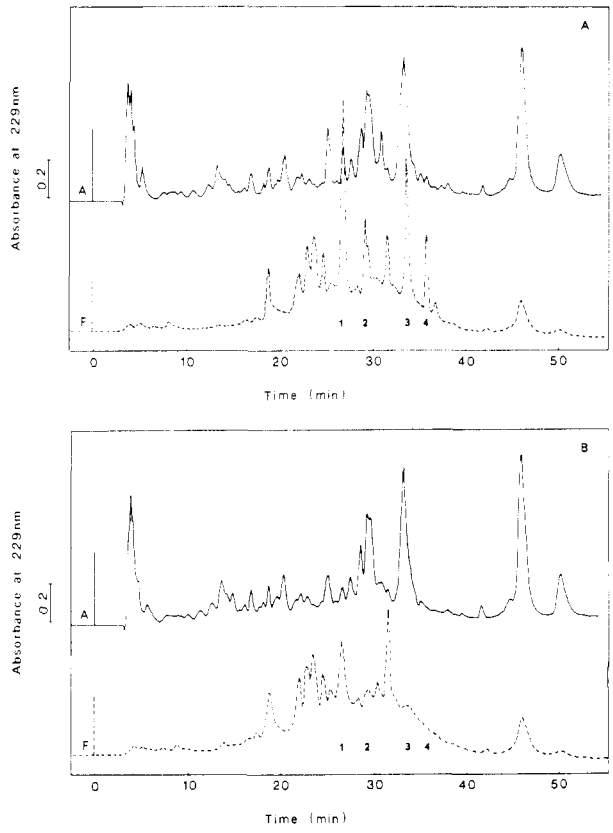

FIGURE 8: Separation on C_4 reverse-phase HPLC of tryptic peptides released from $(Na^+ + K^+)$-ATPase modified with 5′-isothiocyanatofluorescein. Purified $(Na^+ + K^+)$-ATPase was modified with 5′-isothiocyanatofluorescein at a molar ratio of 1:2.7 in the absence (A) or presence (B) of ATP. The membrane-bound enzyme was then washed by centrifugation, resuspended in 1 mM EDTA and 25 mM imidazolium chloride, pH 7.5, and digested with trypsin. The remaining particulate material was removed by ultracentrifugation, and portions of the supernatants were injected directly onto the Vydac C_4 reverse-phase column (0.46 cm × 25 cm). The column was developed with a linear gradient of 0%–60% acetonitrile in 0.1% trifluoroacetic acid delivered at 1 mL min^{-1} over 60 min. Fluorescence (– –) and A_{229} (—) were recorded continuously.

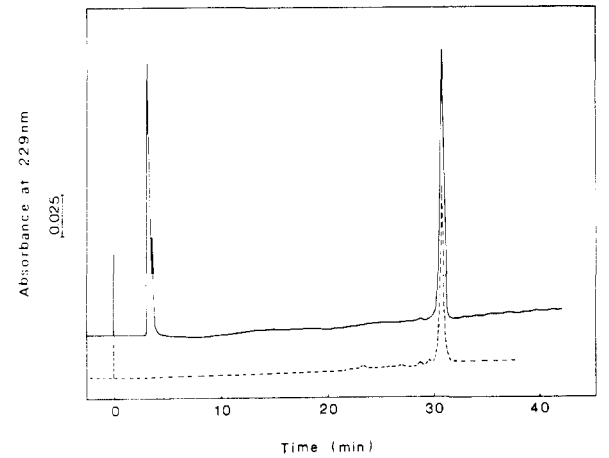

FIGURE 9: Apparent homogeneity of purified tryptic peptides α623–α640 and α760–α767. Fractions including peak 2 from the C_4 reverse-phase chromatogram (Figure 8) were pooled, evaporated, redissolved in 0.1% trifluoroacetic acid, and injected onto the Vydac C_{18} reverse-phase column (0.46 cm × 25 cm) which was then developed with a linear gradient of 0%–60% acetonitrile in 0.1% trifluoroacetic acid delivered at 1 mL min^{-1} over 60 min. Fluorescence (– –) and A_{229} (—) were monitored continuously.

mained after a 1-h reaction, but a significant number of peptides were modified in reactions that were insensitive to the addition of 3 mM ATP (Figure 8). In all cases, ATP

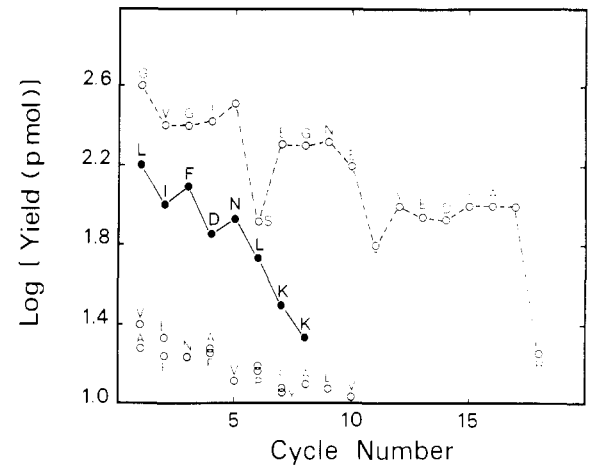

FIGURE 10: Amino acid sequencing of the purified mixture of fluorescent tryptic peptides containing peptide α623–α640 and peptide α760–α767. The logarithms of the yields in picomoles of every PTH-amino acid observed (>10 pmol) on a particular cycle of the automated sequencing are plotted. The amino acids assigned as those from tryptic peptide α760–α767 and expected at a particular cycle are in bold face.

eliminated or attenuated the modification of specific peptides (Figures 3 and 8).

The observation that at least half of the covalent modification by fluorescein, under conditions where each molecule of incorporated fluorescein inactivated one molecule of $(Na^+ + K^+)$-ATPase, was into peptides whose carboxy-terminal sequences were not –GAPER (Figure 2) suggested that lysines other than lysine 501 were being modified by 5′-isothiocyanatofluorescein. It could be shown that lysine 480 also was modified by the reagent in a reaction that was inhibited by the addition of 3 mM ATP (Figures 3, 6, and 8). It was modified to almost the same extent as lysine 501 when inactivation (90%) was performed at a low molar ratio (1:1.7) between enzyme and reagent. Although the frequency of identity in this region of the sequence among the different ATPases responsible for active transport is not remarkable, each ATPase, with the possible exception of K^+-ATPase from *Escherichia coli*, does have a lysine at the same position or close to the position of lysine 480 in the α polypeptide of canine renal $(Na^+ + K^+)$-ATPase (Figure 7). In the amino acid sequence of the protein from *E. coli*, it appears that an arginine substitutes for this lysine.

Although the peptide containing lysine 766 was the minor component of the final purified pools (Figure 10), the major component comprised a sequence of amino acids from the α polypeptide which contained no functionality that could have reacted with an aryl isothiocyanate. A similar observation was made by Ohta et al. (1986), who isolated a pool of tryptic peptides from the α polypeptide of $(Na^+ + K^+)$-ATPase whose major component had the sequence ACVVHGSDLK. None of the amino acids in this peptide could have given a stable derivative upon reaction with the arylsulfonyl fluoride that they used to modify the enzyme. Unfortunately, the sequences of the minor components in their pool were not provided.

The frequency of identity in this region of the amino acid sequence from the various ATPases catalyzing active transport is notable (Figure 11), but H^+-ATPase from fungal plasma membrane and K^+-ATPase from *E. coli* have no lysines within nine amino acids in either direction from the position homologous to that of lysine 766 of canine renal $(Na^+ + K^+)$-ATPase. This lack may simply result from an incorrect alignment of the sequences in this region (Serrano et al., 1986), from the ability of tyrosine or arginine to substitute for lysine,

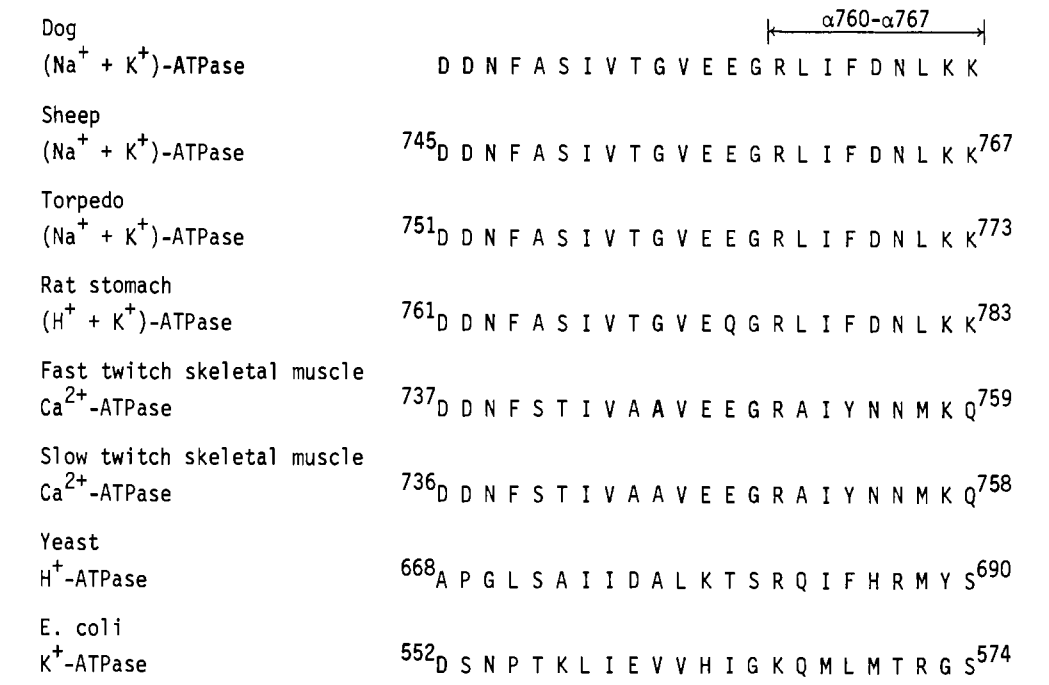

FIGURE 11: Alignment of the sequence of tryptic peptide α760–α767 from canine renal $(Na^+ + K^+)$-ATPase and the homologous regions from other ATPases responsible for active transport. Sequences aligned consecutively are from α polypeptide of canine renal $(Na^+ + K^+)$-ATPase, α polypeptide of ovine renal $(Na^+ + K^+)$-ATPase, α polypeptide of torpedo electric organ $(Na^+ + K^+)$-ATPase, $(H^+ + K^+)$-ATPase from rat stomach, Ca^{2+}-ATPase from rabbit fast-twitch skeletal muscle, Ca^{2+}-ATPase from rabbit slow-twitch skeletal muscle, H^+-ATPase from yeast plasma membrane, and K^+-ATPase from *E. coli*. Regions of a higher frequency of identity preceding α760–α767 are included to support the alignments.

from the movement of a different lysine into the proper position in the active site, or from the unimportance of lysine 766.

In a recent review article (Jørgensen & Andersen, 1988), all of the experimental results implicating particular segments of the amino acid sequence of $(Na^+ + K^+)$-ATPase as participants in the active site were summarized. Neither lysine 480 nor lysine 766 was mentioned in this catalogue. Therefore, the identification of these amino acids as neighbors of lysine 501, an amino acid within the active site (Xu & Kyte, 1989), is an unexpected observation. In most of the models for the folding of $(Na^+ + K^+)$-ATPase (Shull et al., 1985; Jørgensen & Andersen, 1988), these three amino acids are assigned to a large cytoplasmic portion of the protein formed from a long region of the amino acid sequence of the α polypeptide devoid of membrane-spanning segments. Therefore, their presence on the same side of the membrane as the active site has been expected.

The most tenuous suggestion raised by these results is that the amino terminus of the α polypeptide of $(Na^+ + K^+)$-ATPase may also be labeled by 5′-isothiocyanatofluorescein in a reaction inhibited by the addition of ATP. The amino-terminal sequence of the α polypeptide of $(Na^+ + K^+)$-ATPase is GRDKYEP– (Castro & Farley, 1979; Collins et al., 1983; Shull et al., 1985). A peptide that contained an amino acid modified by 5′-isothiocyanatofluorescein in an ATP-sensitive fashion (Figure 3, peak 6) was isolated several times. It was a very short peptide on the basis of its relative mobility on molecular exclusion chromatography (Figure 3). Because it was so short, it could be readily isolated in high yield and high purity. Its sequence has been tentatively assigned as TUF-Gly-Arg. When the peptide was submitted to amino acid sequencing, the first cycle gave the phenylisothiohydantoin of arginine. This would have arisen from cyclization of the 5′-isothiocyanatofluorescein acting in its role as an arylthioureidyl functionality, as does the arylthioureidyl of phenyl isothiocyanate (isothiocyanatobenzene). This would produce free arginine during the pretreatment of the bound peptide with trifluoroacetic acid. The arginine then would have reacted directly with the phenyl isothiocyanate introduced during the first cycle of the sequencing to produce eventually PTH-arginine upon conversion. It was routinely observed that the polybrene discs were yellow at the end of every sequence run, and this suggests that fluorescein has a strong affinity for the polybrene. The fluoresceinylthiazolinone of glycine produced during the initial cyclization would have stuck to the disc and could have reacted with the successive additions of phenyl isothiocyanate to yield the PTH-glycine observed as the product in later cycles. No other PTH-amino acids (<3% of the PTH-glycine) were observed in these later cycles.

It has been reported that the modification of $(Na^+ + K^+)$-ATPase by 5′-isothiocyanatofluorescein behaves as if the reagent binds to the enzyme before it inserts covalently (Farley et al., 1984). It this is the case, it would explain how 5′-isothiocyanatofluorescein can react with several primary amino groups on the protein, and yet each molecule of the protein ends up with only one bound fluorescein. If these primary amines surround the binding site for fluorescein and that site can only bind one fluorescein, once one of the primary amines on a given molecule of protein has reacted, none of the others can. In this way, each bound 5′-isothiocyanatofluorescein could react with lysine 501, lysine 480, lysine 766, or glycine 1, but with only one of them. Therefore, the reaction of 5′-isothiocyanatofluorescein with $(Na^+ + K^+)$-ATPase resembles the reaction of iodoacetamide with ribonuclease (Crestfield et al., 1963). If this is the case, all of these primary amines would have to be clustered very closely together around the binding site for fluorescein.

The inhibition of modification by ATP has been used as a criterion for the specificity of the modification of $(Na^+ + K^+)$-ATPase by 5′-isothiocyanatofluorescein both here and in earlier reports (Karlish, 1980; Farley et al., 1984). The cleavage of the α polypeptide of $(Na^+ + K^+)$-ATPase at specific sites by proteolytic enzymes is known to depend on the conformational state of the enzyme (Jørgensen, 1977;

Jørgensen & Petersen, 1985). This suggests that the addition of ATP could affect the reactivity of lysines distant from the active site. The argument, however, that all of these lysines whose modification is inhibited by ATP surround a unique binding site for 5′-isothiocyanatofluorescein and the conclusion that lysine 501 is within the binding site for ATP (Xu & Kyte, 1989) would place all of these lysines adjacent to or within the active site of $(Na^+ + K^+)$-ATPase.

Acknowledgments

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Registry No. ATPase, 9000-83-3; 5′-isothiocyanatofluorescein, 3326-32-7; L-lysine, 56-87-1.

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