Identification of an Amino Acid in the ATP Binding Site of Na^+/K^+-ATPase after Photochemical Labeling with 8-Azido-ATP†

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Received November 12, 1993; Revised Manuscript Received January 28, 1994®

ABSTRACT: [α-^{32}P]-8-N_3-ATP, [2-^{3}H]-8-N_3-ATP, and non-radioactive 8-N_3-ATP have been used as photoaffinity probes of the ATP binding site of dog kidney Na^+/K^+-ATPase. 8-N_3-ATP has previously been shown to bind to Na^+/K^+-ATPase with high affinity, to be a substrate for Na^+/K^+-ATPase, and to inactivate the enzyme upon ultraviolet irradiation [Scheiner-Bobis, G., & Schoner, W. (1985) *Eur. J. Biochem. 152*, 739–746]. 8-N_3-ATP competitively inhibits the high-affinity binding of [2,8-^{3}H]-ATP to Na^+/K^+-ATPase with a K_i of 3.4 μM, which is comparable to the reported K_D of 3.1 μM for the binding of 8-N_3-ATP to the enzyme. The extent of inhibition of ATP hydrolysis by 8-N_3-ATP was linearly correlated with the stoichiometry of covalent incorporation of 8-N_3-ATP into Na^+/K^+-ATPase up to about 50% inhibition of activity; however, the linkage between the protein and 8-N_3-ATP was unstable, and the maximum incorporation of 8-N_3-ATP was less than the nucleotide binding capacity of the protein. After photolysis with ultraviolet light, 8-N_3-ATP was specifically incorporated into the carboxy-terminal 58-kDa fragment of the α-subunit of Na^+/K^+-ATPase generated by limited trypsin digestion in the presence of KCl, and the β-subunit was not labeled. 8-N_3-ATP-labeled Na^+/K^+-ATPase was digested with trypsin, and a single peak containing the nucleotide was identified after HPLC fractionation of the digest. The peptide in this peak was purified and sequenced and was found to have the amino acid sequence, Ile-Val-Glu-Ile-Pro-Phe-Asn-Ser-Thr-Asn-Lys-Tyr-Gln-Leu-Ser-Ile-His-Lys-Asn-Pro-Asn-Thr-Ser-Glu-Pro-Arg. This sequence corresponds to amino acids 470–495 of the Na^+/K^+-ATPase α-subunit and is also highly conserved among other P-type ion pumps. The yield from the sequencer at cycle 11, corresponding to lysine 480, was substantially reduced in the sequence of the photochemically labeled peptide compared to the same sequence of an unlabeled peptide. These results indicate that 2-N_3-ATP labels lysine 480 of Na^+/K^+-ATPase from within the ATP binding site of the protein.

Na^+/K^+-ATPase[1] (EC 3.6.1.37) is an intrinsic membrane-embedded enzyme that transports Na^+ and K^+ ions across cell membranes against their electrochemical gradients (Skou & Esmann, 1992; Glynn, 1993). The enzyme consists of a catalytic α-subunit (M_r = 112 000) and a glycosylated β-subunit (M_r = 35 000). The amino acid sequence of the α-subunit of Na^+/K^+-ATPase has 20–75% identity with polypeptides of similar size found in other ion-translocating ATPases. β-Subunits have been identified only for Na^+/K^+-ATPase and the gastric H^+/K^+-ATPase in this family of enzymes.

The translocation of Na^+ and K^+ ions by Na^+/K^+-ATPase requires the enzymatic hydrolysis of ATP and also that the γ-phosphate of ATP be transiently transferred to the α-subunit at Asp-369 during the catalytic cycle (Bastide et al., 1973). Identification of amino acids that are located within the ATP binding site of Na^+/K^+-ATPase is essential in order to understand the structural basis for ATP-coupled ion transport. Many chemical reagents with different structural characteristics have been used in attempts to label amino acids within the nucleotide binding site of this protein. The structures of some of these reagents, such as FITC (Farley et al., 1984; Kirley et al., 1984), do not resemble the structure of ATP, and others that are derived from nucleotides, such as AP_2PL (Hinz & Kirley, 1990), FSBA (Ohta et al., 1986), and ClR-ATP (Ovchinnikov et al., 1987) are not substrates for the enzyme. As a result, some uncertainty exists about the identities of amino acids that interact with nucleotides in these proteins. 8-N_3-ATP or 8-N_3-ADP has been used as a photoaffinity active-site probe for Na^+/K^+-ATPase in red blood cells (Haley & Hoffman, 1974), for Na^+/K^+-ATPase from pig kidney (Scheiner-Bobis & Schoner, 1985), and for the Ca^{2+}-ATPase of sarcoplasmic reticulum (Campbell & MacLennan, 1983; Lacapere et al., 1993). 8-N_3-ATP is hydrolyzed as a substrate by these enzymes, and upon irradiation with UV light, 8-N_3-ATP inhibits each of the ATPase activities. From these results, it appears likely that

† Supported by Grant GM28673 from the National Institutes of Health (R.A.F.) and by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 249 "Pharmakologie Biologischer Makromolekuele" (Giessen) and the Fonds der Chemischen Industrie (Frankfurt/Main) (W.S.).

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® Abstract published in *Advance ACS Abstracts,* March 15, 1994.

[1] Abbreviations: Na^+/K^+-ATPase, sodium- and potassium-dependent adenosine triphosphatase (EC 3.6.1.37); FITC, fluorescein 5′-isothiocyanate; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; AP_2PL, adenosine 5′-diphosphopyridoxal; 2-N_3-ATP, 2-azidoadenosine 5′-triphosphate; 8-N_3-ATP, 8-azidoadenosine 5′-triphosphate; FSBA, [*p*-(fluorosulfonyl)benzoyl]adenosine; ClR-ATP, γ-[[4-(*N*-(2-chloroethyl)-*N*-methylamino)benzyl]amido]adenosine 5′-triphosphate; kDa, kilodalton; HPLC, high-pressure liquid chromatography; TNP-8-N_3-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl)-8-azidoadenosine 5′-triphosphate.

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8-N_3-ATP is labeling the proteins from within the high-affinity ATP binding site. McIntosh et al. recently used TNP-8-N_3-ATP to label the sarcoplasmic reticulum Ca^{2+}-ATPase and concluded that lysine 492 is located within the ATP binding site of this enzyme (McIntosh et al., 1992). In the experiments described in this report, 8-N_3-ATP was used to photochemically label renal Na^+/K^+-ATPase, and a tryptic peptide from the high-affinity ATP binding site was identified, purified, and sequenced. Lysine 480, which occupies the position in Na^+/K^+-ATPase that is analogous to lysine 492 in Ca^{2+}-ATPase, was identified as the amino acid that is labeled by 8-N_3-ATP. A preliminary account of this work has previously been reported (Tran et al., 1988).

MATERIALS AND METHODS

Materials

[2,8-^{3}H]-ATP of specific activity 39 Ci/mmol and [α-^{32}P]-8-N_3-ATP of specific activity 7.3–9.8 μCi/μmol were from ICN. Non-radioactive 8-N_3-ATP, Na_2ATP, Tris–ATP, phenylmethanesulfonyl fluoride, and soybean trypsin inhibitor were from Sigma. Electrophoretic grade acrylamide, methylene bis(acrylamide), sodium dodecyl sulfate, *N,N,N′,N′*-tetramethylenediamine, and ammonium persulfate were obtained from Bio-Rad Laboratories. HPLC-grade acetonitrile was from Fisher Scientific. Trifluoroacetic acid (Sequenal grade) was from Pierce Chemical Co. L-1-(Tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin was purchased from Cooper Biomedical. All HPLC columns were from Vydac/The Separations Group (Hesperia, CA). Na^+/K^+-ATPase was purified from dog kidney or pig kidney outer medulla according to Jorgensen (1974). The specific activity of the Na^+/K^+-ATPase was between 20 and 26 μmol of PO_4/mg/min (Cantley et al., 1978).

Experimental Methods

Synthesis of 8-N_3-ATP. 8-N_3-AMP was synthesized from AMP according to the method of Haley and Hoffman (1974). Phosphorylation of the 8-N_3-AMP to 8-N_3-ATP was done using tributylammonium pyrophosphate (Hoard & Ott, 1965) as described by Michelson (1964), except that the solvent was dimethylformamide rather than dioxane. [α-^{32}P]-8-N_3-ATP (specific activity 45 μCi/μmol) was prepared as described by Schoner and Scheiner-Bobis (1988). [2-^{3}H]-8-N_3-ATP was synthesized from [2-^{3}H]ATP (Ikehara & Uesugi, 1969; Scharer et al., 1978). The identities of the products were established by thin-layer chromatography with known samples and by spectrophotometric scanning of azido compounds before and after photolysis at 254 nm.

Binding of [^{3}H]ATP to Na^+/K^+-ATPase. Purified Na^+/K^+-ATPase (10 mg) was washed with 20 mM Tris-HCl and 3 mM EDTA (acid form) (pH 7.4) at 200000*g* for 60 min; the pellets were suspended in the same buffer to a final protein concentration of 2 mg/mL. Aliquots containing 100 μg of protein were incubated with various concentrations of Tris–ATP (0.05–5.0 μM) in the same buffer at 0 °C, either in the absence or in the presence of 25 μM 8-N_3-ATP. Each sample also contained 0.25 μCi of [2,8-^{3}H]-ATP. The bound and unbound ligands were separated by centrifugation at 200000*g* for 90 min. After centrifugation, each pellet was dissolved in 0.20 mL of 1% SDS and counted with 4.5 mL of scintillation fluid.

Photoinactivation and Photolabeling. Na^+/K^+-ATPase was suspended in 10 mM Tris-HCl, 10 mM NaCl, 2.5 mM $MgCl_2$, and 0.2 mM Na_2EDTA (pH 7.4). In most experiments, the enzyme suspension (final protein concentration = 0.1 mg/mL), together with 8-N_3-ATP in the absence or presence of 0.1–2 mM Na_2ATP, was irradiated at 310 nm for 1 h at an incident energy of 200 μW/cm^2. Irradiation was done in a water vapor-saturated chamber to prevent sample evaporation. At the end of the photolysis period, the samples were immediately assayed for remaining Na^+/K^+-ATPase activity. A control sample of Na^+/K^+-ATPase was also irradiated without 8-N_3-ATP in order to determine the nonspecific inhibition of activity due to UV light. In some experiments, the samples were irradiated at 254 nm for 1 h at room temperature at an incident energy of 5–50 μW/cm^2. Nonspecific photoinactivation was slightly higher with 254-nm illumination than with 310-nm illumination.

Limited Proteolytic Digestion and SDS–Polyacrylamide Gel Electrophoresis. [α-^{32}P]-8-N_3-ATP-labeled Na^+/K^+-ATPase was suspended in 20 mM Tris-HCl, 3 mM EDTA, and 50 mM KCl (pH 7.4) to a final concentration of 1 mg/mL. Trypsin digestion was carried out as previously described (Jorgensen & Farley, 1988), and fragments were separated on SDS–polyacrylamide gels prepared according to Laemmli (1970). After staining and destaining, the gels were either dried and put on Kodak XAR-5 film for autoradiography or sliced into 1-mm slices and dissolved in H_2O_2/NH_4OH (95:5, v/v) at 70 °C overnight. The samples were neutralized afterward with 0.5 mL of 1 M acetic acid and were counted for ^{32}P with 5 mL of scintillation fluid.

Stoichiometry of Labeling. Na^+/K^+-ATPase (100 μg) in 0.5 mL of buffer was illuminated with light at 310 nm for 60 min in the presence of 5–50 μM [^{3}H]-8-N_3-ATP with a specific activity of 10 μCi/μmol, in the absence or presence of 0.1 mM ATP. The incident energy was 130 μW/cm^2. After photolysis, an aliquot was withdrawn from each sample for measurement of the remaining activity, and the remainder of each sample was separated from the buffer by centrifugation at 40 000 rpm in a Ty 65 rotor for 60 min. The supernatants were discarded, and the pellets were blotted dry, dissolved in 1 mL of 1% SDS, and counted for radioactivity. The radioactivity in samples that contained 0.1 mM ATP was subtracted from the corresponding samples without ATP, and the remaining radioactivity was used to calculate the amount of 8-N_3-ATP incorporated within the ATP binding site. The result was plotted against the ATP-protectable inhibition of activity, which was the difference in the residual activity of the samples photolyzed in the absence or presence of 0.1 mM ATP.

Complete Trypsin Digestion. Na^+/K^+-ATPase (15–30 mg) was labeled by [2-^{3}H]-8-N_3-ATP, [α-^{32}P]-8-N_3-ATP, or non-radioactive 8-N_3-ATP either in the presence or absence of 2 mM Na_2ATP. After photolysis, the membranes were removed from the suspension by centrifugation at 200000*g* for 90 min, and the pellets were washed with 20 mM Tris-HCl and 3 mM Na_2EDTA (pH 7.4) and were stored in this buffer at −75 °C until further use. Complete trypsin digestion of labeled Na^+/K^+-ATPase was carried out either in 20 mM Tris-HCl and 3 mM Na_2EDTA (pH 7.4) at 37 °C for 3 h with a trypsin:ATPase ratio of 1:10 (w/w) or in 25 mM imidazole hydrochloride and 1 mM Tris–EDTA (pH 7.25) at 37 °C overnight with a trypsin:ATPase ratio of 1:12 (w/w). The digestion was stopped by adding excess soybean trypsin inhibitor. The soluble peptides were separated from the membranes by centrifugation at 200000*g* for 90 min. The supernatant was lyophilized overnight and was fractionated later by HPLC.

HPLC Separation. The lyophilized, trypsin-digested Na^+/K^+-ATPase supernatant was dissolved in a minimum volume

of distilled water or 0.1% trifluoroacetic acid and was fractionated on a Vydac C_{18} reverse-phase HPLC column with a linear gradient of 0–60% solvent B over 90 min at a flow rate of 1.5 mL/min. Solvent A was 0.1% trifluoroacetic acid, and solvent B was 0.1% trifluoroacetic acid in 95% acetonitrile and 5% water. For [α-^{32}P]-8-N_3-ATP-labeled ATPase, 0.5-min fractions were collected and were counted for radioactivity. For non-radioactive 8-N_3-ATP-labeled Na^+/K^+-ATPase, two HPLC UV absorbance detectors were connected in series. One detector was set at 280 nm and the other at 254 nm. Both detector outputs were fed to a dual pen recorder, and the attenuation was adjusted so that the ratio of A_{254}:A_{280} of all peptides in a tryptic digest of unlabeled Na^+/K^+-ATPase was less than 1. With this system, peptide peaks from the labeled ATPase that had an A_{254}:A_{280} ratio greater than 1 were collected, lyophilized, and reapplied to an HPLC C_4 column and then to an HPLC phenyl column. The columns were developed with a gradient of 10–40% solvent B. Peaks with an A_{254}:A_{280} ratio greater than 1 were again collected and lyophilized and then desalted on a C_4 column developed with the same gradient.

Amino Acid Sequence Determination. Samples of purified, labeled peptides were lyophilized before being subjected to amino acid sequencing. Microsequence analysis was performed on an Applied Biosystems 470A gas-phase sequenator using the program "01 run h" on the standard tape supplied with the instrument. This is a triple-coupling, single-cleavage program. Phenylthiohydantoin derivatives were separated and quantitated on a Perkin-Elmer series 4 HPLC; the average repetitive yield from two sequence runs was greater than 95%. [2-^{3}H]-8-N_3ATP-labeled peptides were analyzed manually by a micro modification of the Edman degradation using diaminobenzyl isothiocyanate (Chang, 1981).

RESULTS

Photoinactivation of Na^+/K^+-ATPase. Photolysis of dog kidney Na^+/K^+-ATPase with ultraviolet light in the presence of 8-N_3-ATP resulted in the irreversible inhibition of the ATPase activity of the enzyme. The photoinhibition of Na^+/K^+-ATPase activity by 8-N_3-ATP required both Mg^{2+} and Na^{2+}, and the extent of inhibition increased with increasing concentrations of 8-N_3-ATP (Figure 1). Maximum inhibition of activity was achieved with about 50 μM 8-N_3-ATP and was 40–50% of the initial activity. Nonspecific UV inhibition of the enzyme irradiated in the absence of 8-N_3-ATP was 10–15%. The inclusion of K^+ in the irradiation buffer abolished the inhibition by 8-N_3-ATP (data not shown). Upon irradiation with actinic light, 8-N_3-ATP is converted into a reactive nitrene. The incomplete inhibition of Na^+/K^+-ATPase activity by 8-N_3-ATP may be due to reactions of the nitrene other than insertion into the active site of the protein (Bayley & Knowles, 1977, 1978). Water molecules in the ATP binding site, for example, will react with 8-N_3-ATP and limit the reaction of 8-N_3-ATP with the protein. Alternatively, the incomplete inhibition may be related to an unstable linkage between the protein and the nucleotide, as discussed below. Inactivation of Na^+/K^+-ATPase activity by 8-N_3-ATP was prevented when 100 μM ATP was present during photolysis and the illumination wavelength was 310 nm (Figure 1) or when the incident energy at 254 nm was 5–50 μW/cm^2 (data not shown). When the incident energy at 254 nm was very high, however, irreversible inhibition of activity was observed in the presence of 2 mM ATP, in either the presence or absence of 8-N_3-ATP.

Stoichiometry of Labeling. If the inhibition of Na^+/K^+-ATPase activity by 8-N_3-ATP is a consequence of the reaction

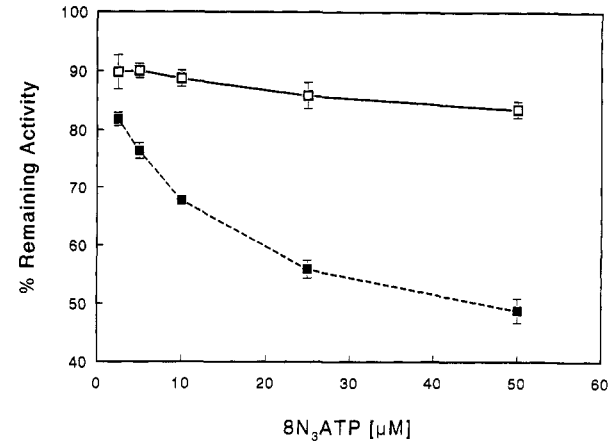

FIGURE 1: Photoinhibition of Na^+/K^+-ATPase activity by 8-N_3-ATP. Na^+/K^+-ATPase (0.1 mg/mL) was suspended in 10 mM Tris-HCl, 10 mM NaCl, 2.5 mM $MgCl_2$, 0.2 mM Na_2EDTA (pH 7.4), and various concentrations of 8-N_3-ATP as indicated on the abscissa. 50-μL samples were irradiated in a water-saturated chamber at 310 nm with an energy level of 200 μW/cm^2 for 60 min at room temperature. At the end of the photolysis period, the samples were assayed for ATPase activity using an enzyme-linked coupled assay (Cantley et al., 1978). The remaining activities are plotted (±SEM, n = 9) against 8-N_3-ATP concentrations for samples in the presence (□) and absence (■) of 0.1 mM ATP, with the activity of a nonirradiated sample equal to 100%.

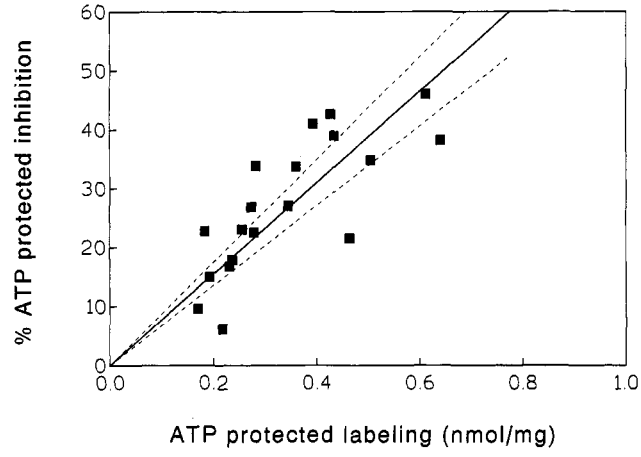

FIGURE 2: Stoichiometry of photochemical incorporation of [α-^{32}P]-8-N_3-ATP into Na^+/K^+-ATPase and inhibition of Na^+/K^+-ATPase activity by 8-N_3-ATP. 100 μg of Na^+/K^+-ATPase was photolyzed at 310 nm in the presence of different concentrations of [α-^{32}P]-8-N_3-ATP, as described in Materials and Methods. After photolysis, an aliquot of each sample was removed for the measurement of Na^+/K^+-ATPase activity, and the remainder of each sample was separated by centrifugation into supernatant and membrane pellet. The supernatants were discarded, and the amount of [α-^{32}P]-8-N_3-ATP recovered in each pellet was measured. Differences in Na^+/K^+-ATPase activity in the absence and presence of 0.1 mM ATP and in the amount of 8-N_3-ATP incorporated in the absence and presence of 0.1 mM ATP are plotted. — is the least-squares fit to the data, and - - - show the 95% confidence interval.

of the azido nucleotide at the ATP binding site of the protein, the extent of photoinhibition may be related to the stoichiometry of incorporation of 8-N_3-ATP into the protein. Examples are known, however, in which the loss of a photoinserted radiolabel has resulted in an inactivated enzyme that no longer contains the photolabel (Lewis et al., 1989). In the absence of dissociation of the photoprobe, maximum inhibition of activity might also be expected to occur at a stoichiometry of labeling that is about the same as the stoichiometry of ATP binding to the protein. Figure 2 shows the relationship between the stoichiometry of photochemical incorporation of [α-^{32}P]-8-N_3-ATP into Na^+/K^+-ATPase and

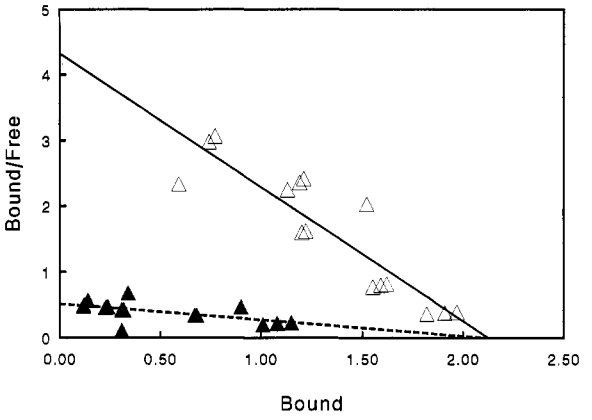

FIGURE 3: Equilibrium binding of ATP to the Na^+/K^+-ATPase in the absence and presence of 8-N_3-ATP. Samples of Na^+/K^+-ATPase (0.1 mg/mL) in 20 mM Tris-HCl and 3 mM EDTA (pH 7.4) were incubated with various concentrations of Tris-ATP (0.05–5.0 μM) in the presence (▲) or absence (△) of 25 μM 8-N_3-ATP. The final volume of each sample was 1 mL. All samples contained 0.25 μCi of [2,8-^{3}H]-ATP. After centrifugation for 90 min at 200000g to separate the bound and unbound ligands, the supernatants were aspirated and the pellets were blotted dry with strips of Whatman 3MM filter paper. The pellets were dissolved in 0.2 mL of 1% SDS and were counted for ^{3}H with 4.5 mL of scintillation fluid. The data collected are plotted according to Scatchard (1949), and the lines through the data points are the best fits for a single-site model. The units on the abcissa are nanomoles/milligram.

the inhibition of ATPase activity in the same samples. A linear relationship between ATP-protectable inhibition of activity and ATP-protectable incorporation is seen for inhibition up to about 50%. As indicated above, inhibition greater than about 50% was not observed under the conditions used in these experiments. Extrapolation of the incorporation stoichiometry to complete inhibition of activity indicates that complete inhibition of activity would occur at about 1.3 nmol of 8-N_3-ATP/mg of protein. This is significantly less than the ATP binding stoichiometry of about 2 nmol/mg for this sample (Figure 3). The difference between the incorporation stoichiometry and the ATP binding capacity is due both to some 8-N_3-ATP-independent inactivation of activity by the ultraviolet light and also to an underestimate of the incorporation, due to instability of the linkage between the protein and 8-N_3-ATP, as discussed below.

Equilibrium Binding of [2-^{3}H]ATP to Na^+/K^+-ATPase. Equilibrium binding of [2-^{3}H]ATP to Na^+/K^+-ATPase was characterized by a K_D of 0.49 μM and a maximum binding stoichiometry of about 2 nmol/mg of protein (Figure 3). The binding of ATP to Na^+/K^+-ATPase was inhibited competitively by 8-N_3-ATP, and a K_i of 3.4 μM for 8-N_3-ATP inhibition of ATP binding was calculated using the equation $K_{D(app)} = \{(K_D/K_i)[8\text{-}N_3\text{-ATP}]\} + K_D$, where K_D and $K_{D(app)}$ are obtained from Scatchard plots in the absence or presence of 8-N_3-ATP, respectively. This K_i value is similar to the reported K_D of 3.1 μM for 8-N_3-ATP binding to Na^+/K^+-ATPase (Scheiner-Bobis & Schoner, 1985).

Labeling of Na^+/K^+-ATPase with [α-^{32}P]-8-N_3-ATP and Limited Trypsin Digestion. After UV irradiation, [α-^{32}P]-8-N_3-ATP was incorporated into the α-subunit of Na^+/K^+-ATPase. The β-subunit was not labeled (Figure 4, lanes 1a and b). The small amount of radiolabel at the position of the β-subunit in the figure is associated with a 58-kDa tryptic fragment of the α-subunit that comigrates on these gels with the β-subunit. The labeling of the α-subunit by 8-N_3-ATP was nearly completely prevented when ATP was included in the photolysis buffer (Figure 4, lanes 2a and b). After limited trypsin digestion of the labeled Na^+/K^+-ATPase in the presence of 150 mM KCl, the sites of labeling are localized to the 58-kDa tryptic fragment derived from the carboxy-terminal half of the α-subunit (Figure 4, lanes 3a and b) (Castro & Farley, 1979; Jorgensen & Farley, 1988). The amino-terminal 41-kDa tryptic fragment is not labeled by 8-N_3-ATP. The trypsin cleavage site between these two nonoverlapping fragments occurs after arginine 438 (Jorgensen & Collins, 1986).

HPLC Separation of Tryptic Peptides from 8-N_3-ATP-Labeled Na^+/K^+-ATPase. Dog kidney Na^+/K^+-ATPase was labeled with [α-^{32}P]-8-N_3-ATP, and after removal of unreacted 8-N_3-ATP from the membranes by centrifugation, the Na^+/K^+-ATPase was digested with trypsin as described under Materials and Methods. Most of the radioactivity incorporated into the protein was released from the membranes by trypsin digestion, and the lyophilized water-soluble peptides were dissolved in a minimum volume of distilled water and fractionated on a Vydac C_{18} column. About 90% of the radioactivity released from the sample by trypsin digestion did not bind to the C_{18} column, and about 7% of the radioactivity eluted in a single peak at about 43.5 min after starting the gradient. The remainder of the radioactivity was distributed fairly uniformly throughout the whole chromatogram. The major peak of radioactivity was not detected if the Na^+/K^+-ATPase was labeled with [α-^{32}P]-8-N_3-ATP in the presence of 2 mM Na_2ATP. Radioactivity continued to be released from the peptides during subsequent purification steps, and a purified peptide containing about 0.5% of the initial radioactivity was recovered. The amino-terminal amino acid sequence of this peptide was Ile-Val, after which the yield was too low to permit the identification of additional amino acids.

The release of the radioactive phosphate from the Na^+/K^+-ATPase after labeling with [α-^{32}P]-8-N_3-ATP is likely to have been due to hydrolysis of the phosphoryl groups from the nucleotide. This conclusion was obtained from another experiment in which dog renal Na^+/K^+-ATPase was photolabeled with [2-^{3}H]-8-N_3-ATP, which contains the radiolabeled atom in the purine ring. Although the specific radioactivity of the probe was low, a single HPLC peak containing ATP-protected radioactivity and eluting between 40 and 45 min from the HPLC column was isolated from a tryptic digest of the labeled enzyme. The amino-terminal sequence Ile-Val-Glu-Ile-Pro-Phe-Asn-Ser was obtained from this peptide. The yield of the first Ile was about 25 pmol, and an unambiguous sequence could not be identified after serine. This sequence corresponds to amino acids 470–477 of the sheep (Shull et al., 1985) or dog[2] kidney Na^+/K^+-ATPases; however, no radioactivity was released at any cycle of the sequence, and the identity of the labeled amino acid could not be determined. This experiment was also done using pig kidney Na^+/K^+-ATPase, and the purified peptide was sequence by manual Edman degradation (Chang, 1981). The specific activity of the probe in this experiment was also low, and only three cycles of the Edman degradation could be performed. The same amino-terminal sequence Ile-Val-Glu was obtained from this peptide, and no radioactivity was released in any step.

In order to overcome the problems associated with the loss of the α-^{32}P moiety from [α-^{32}P]-8-N_3-ATP and the low specific activity of [2-^{3}H]-8-N_3-ATP, the chromatographic behavior of the labeled peptide was monitored as the difference

[2] R. A. Farley et al., unpublished observation; Z. Xie et al., personal communication.

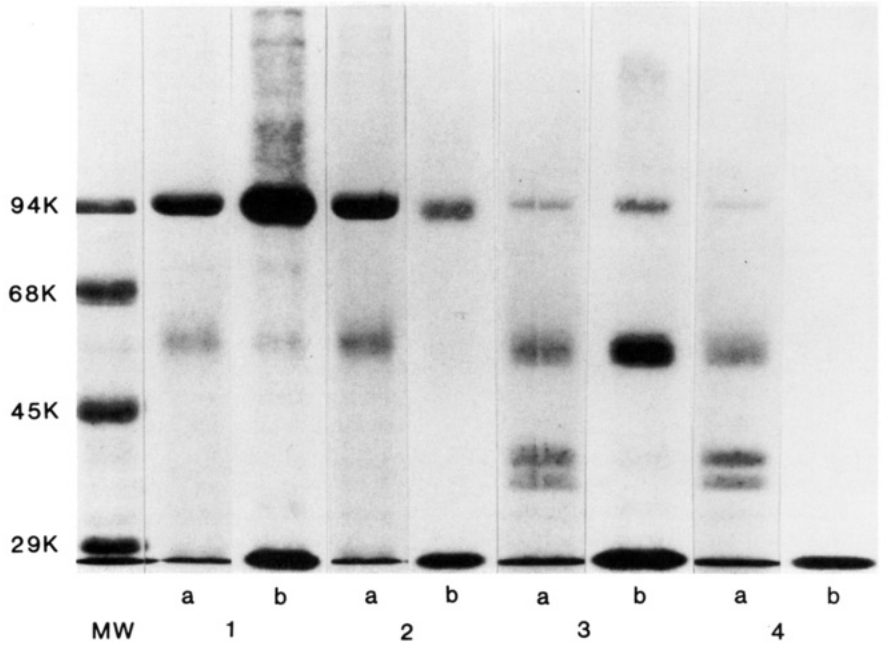

FIGURE 4: SDS–polyacrylamide gel fractionation of [α-^{32}P]-8-N_3-ATP-labeled, trypsin-digested Na^+/K^+-ATPase. Na^+/K^+-ATPase was labeled with [α-^{32}P]-8-N_3-ATP in the presence or absence of 2 mM Na_2ATP and was digested with trypsin in the presence of 100 mM KCl. 100 μg of each sample was applied to a 7.5% Laemmli gel. After being stained, destained, and dried, the gel was exposed with Kodak XAR-5 film for 24 h at −75 °C. The Coomassie Blue-stained gel and autoradiogram of each sample are shown in lanes a and b, respectively. Lanes 1a and b show Na^+/K^+-ATPase labeled with [α-^{32}P]-8-N_3-ATP in the absence of 2 mM Na_2ATP and before trypsin digestion; lanes 2a and b show Na^+/K^+-ATPase labeled in the presence of 2 mM Na_2ATP and before trypsin digestion; lanes 3a and b show the Na^+/K^+-ATPase sample from lane 1 after trypsin digestion; and lanes 4a and b show the Na^+/K^+-ATPase from lane 2 after trypsin digestion.

in absorbance at 254 and 280 nm. The incorporation of 8-N_3-ATP into peptides increases the ratio of the absorbance of light at 254 nm relative to that at 280 nm because of the difference between the extinction coefficients of adenosine and aromatic amino acids at these wavelengths (Knight & McEntee, 1985). In order to identify the 8-N_3-ATP-labeled peptides from Na^+/K^+-ATPase, two HPLC detectors were arranged in series and set to measure the absorbances of peptides at 254 and 280 nm. The detectors were calibrated such that the A_{254}:A_{280} ratio for all tryptic peptides of unlabeled Na^+/K^+-ATPase was less than 1. Dog renal Na^+/K^+-ATPase was labeled with non-radioactive 8-N_3-ATP and digested with trypsin, and the digest was fractionated as for radiolabeled Na^+/K^+-ATPase. Figure 5 shows the chromatogram obtained from each absorbance detector. A peak with an absorbance ratio A_{254}:A_{280} of greater than 1 (Figure 5, asterisk) was observed to elute from the column at the same position as the radioactive peak from [α-^{32}P]-8-N_3-ATP and [2-^{3}H]-8-N_3-ATP-labeled Na^+/K^+-ATPases.

To verify that the increased A_{254}:A_{280} ratio of the above peak was due to the incorporation of 8-N_3-ATP into the peptide, a sample of Na^+/K^+-ATPase labeled with 8-N_3-ATP in the presence of 2 mM Na_2ATP was digested with trypsin, and the soluble peptides were fractionated using the same HPLC protocol. The A_{254}:A_{280} absorbance ratio of the peak corresponding to the labeled peptide was reduced to the level of the unlabeled sample (A_{254}:A_{280} < 1; data not shown). The peak with the high A_{254}:A_{280} ratio from the Vydac C_{18} column was collected and applied to an HPLC C_4 column (Figure 6, left panel). The peak that had an absorbance ratio A_{254}:A_{280} of greater than 1 was recovered and then applied to an HPLC phenyl column (Figure 6, center panel). The peak from the C_4 column was separated on the phenyl column into one broad peak, with an absorbance ratio A_{254}:A_{280} of much greater than 1, and one sharp peak, which had an absorbance ratio A_{254}:A_{280} of less than 1. The fractions in the broad peak from the phenyl column were pooled and lyophilized, and the sample was applied again to a Vydac C_4 column (Figure 6, right panel). The final C_4 separation resulted in two overlapping peaks, peak 1 and peak 2. Only peak 1 had a

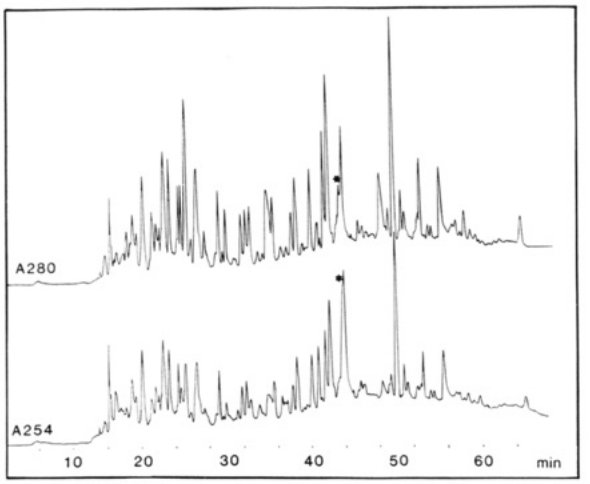

FIGURE 5: HPLC fractionation of tryptic peptides from 8-N_3-ATP-labeled Na^+/K^+-ATPase. Na^+/K^+-ATPase was labeled with non-radioactive 8-N_3-ATP as described in Materials and Methods. Photolysis was done using 256-nm illumination at 50 μW/cm^2. The labeled protein was digested with trypsin at a 1:10 ratio (w/w) for 3 h at 37 °C. After removal of the membranes by centrifugation, the supernatant was lyophilized, dissolved in a small amount of 0.1% trifluoroacetic acid, and applied to an HPLC C_{18} column. Fractionation of peptides was accomplished with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Two HPLC detectors were connected in series to the HPLC column. One detector was adjusted to measure absorbance at 280 nm (upper chromatogram), and the second detector was adjusted to measure absorbance at 254 nm (lower chromatogram). The detectors were adjusted so that the ratio A_{254}:A_{280} for all peaks in a trypsin-digested sample of unlabeled Na^+/K^+-ATPase was less than 1. The asterisk (*) marks the peak eluting from the column at 43.5 min with an absorbance ratio A_{254}:A_{280} greater than 1. A control sample from Na^+/K^+-ATPase labeled in the presence of 2 mM Na_2ATP did not show the increase in the A_{254}:A_{280} ratio (data not shown).

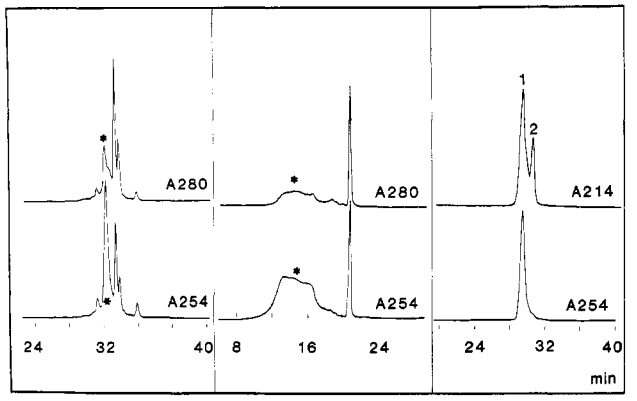

FIGURE 6: Purification of an 8-N_3-ATP-labeled tryptic peptide from Na^+/K^+-ATPase. The peak eluting at 43.5 min from the C_{18} column in Figure 5 was collected and reapplied to a C_4 column (left panel). One peak eluting at 32 min showed an elevated A_{254}:A_{280} ratio (*) and was collected. The peak collected from the C_4 column was applied to an HPLC phenyl column (middle panel), and the broad peak with an elevated A_{254}:A_{280} ratio (*) was collected. The broad peak from the phenyl column was reapplied to a C_4 column for concentration and desalting and was separated into peaks 1 and 2 (right panel). In order to increase detector sensitivity in this chromatogram, one detector was adjusted to measure absorbance at 214 instead of 280 nm.

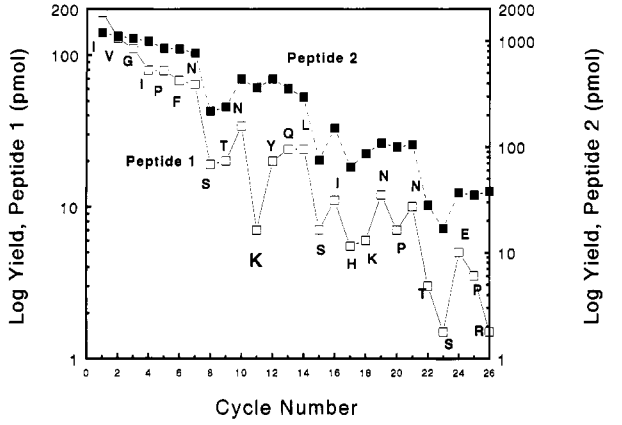

FIGURE 7: Amino acid sequence analysis of peaks 1 and 2 from trypsin-digested, 8-N_3-ATP-labeled Na^+/K^+-ATPase. The logarithm of the recovery of phenylthiohydantoin amino acids from peak 1 (□, left ordinate) and peak 2 (■, right ordinate) is shown for each cycle of analysis. Both peptides have the same amino acid sequence; however, the yield of lysine in the eleventh cycle is reduced by 80% in peptide 1, compared to cycle 10, and by 11% for peptide 2 in the same cycle.

significant absorbance at 254 nm. When peak 1 was collected and applied again to the C_4 column, both peaks 1 and 2 were again recovered. The absorbance of peak 1 at 214 nm was decreased while the absorbance of peak 2 at this wavelength had increased. This change was also accompanied by the loss of absorbance at 254 nm in peak 2. This behavior suggested that peak 2 had been derived from peak 1 by loss of the purine moiety of 8-N_3-ATP.

The amino acid sequences of the peptides in peaks 1 and 2 were found to be identical, consistent with the suggestion that peak 2 is derived from peak 1 by loss of the purine. The sequence, Ile-Val-Glu-Ile-Pro-Phe-Asn-Ser-Thr-Asn-Lys-Tyr-Gln-Leu-Ser-Ile-His-Lys-Asn-Pro-Asn-Thr-Ser-Glu-Pro-Arg (Figure 7), corresponds to amino acids 470–495 of sheep kidney Na^+/K^+-ATPase (Shull et al., 1985), with differences at positions 489 (Ala), 491 (Ala), and 492 (Gly). In the dog kidney Na^+/K^+-ATPase sequence derived from the cDNA,[2] however, the amino acid sequence is as shown above. Although no amino acids were completely absent from any cycle of the sequencer in either peptide, the yield of lysine in cycle 11, corresponding to lysine 480 in the Na^+/K^+-ATPase sequence, was reduced by 80% (7 pmol) compared to the yield of the previous cycle (35 pmol) in the sample containing the absorbance at 254 nm (peak 1). A similar reduction in yield was not observed for this cycle in the sample that did not have an absorbance at 254 nm (peak 2). Except for this difference, the repetitive yield of each sequence was the same.

DISCUSSION

Considerable data indicate that 8-N_3-ATP reacts at the ATP binding site of Na^+/K^+-ATPase. 8-N_3-ATP has been shown to serve as substrate for the Na^+-dependent ATPase activity of Na^+/K^+-ATPase, with a K_M of 6.7 μM (Scheiner-Bobis & Schoner, 1985), and in this report, 8-N_3-ATP has been shown to act as a competitive inhibitor of the high-affinity binding of [^{3}H]-ATP to Na^+/K^+-ATPase, with a K_i of 3.4 μM (Figure 3). ATP significantly reduced the amount of [α-^{32}P]-8-N_3-ATP photochemically incorporated into the α-subunit of the Na^+/K^+-ATPase and prevented 8-N_3-ATP-dependent inhibition of the enzyme. The extent of inhibition was stoichiometrically related to the extent of incorporation of 8-N_3-ATP into the Na^+/K^+-ATPase up to about 50% inhibition. Although UV-activated 8-N_3-ATP inhibited the ouabain-sensitive activity of Na^+/K^+-ATPase, the maximum inhibition of activity was only about 50% of the initial activity (Figure 1). It was possible to increase the specific inhibition only slightly at higher concentrations of 8-N_3-ATP, even though 8-N_3-ATP binds to Na^+/K^+-ATPase with high affinity (Scheiner-Bobis & Schoner, 1985). There may be several factors that contribute to this observation, and although the mechanism is not completely understood, it is likely that the instability of the bond between the nucleotide and the protein is at least partly responsible for this.

Several investigators have previously reported that the linkage between azido nucleotides and some amino acids is unstable. In the report of Davis and Hammes describing the use of 2-N_3-ATP and 2-N_3-AMP to label yeast H^+-ATPase (Davis et al., 1990), an incorporation stoichiometry of only 0.1 mol/mol of protein was found, and dissociation of the photoprobe from the peptide prevented identification of the labeled amino acids. With Ca^{2+}-ATPase from sarcoplasmic reticulum, McIntosh et al. observed that the bond between the protein and TNP-8-N_3-ATP was also labile, resulting in approximately 50% inhibition of ATPase activity and a less than 10% yield of labeled peptide (McIntosh et al., 1992). Lacapere et al. (1993) were also able to inhibit Ca^{2+}-ATPase by about 40% with 8-N_3-ADP at an incorporation stoichiometry of 1.2 nmol/mg of protein. The instability of the linkage between 8-N_3-ATP and Na^+/K^+-ATPase was most obvious in the conversion of HPLC peak 1 into peak 2, with the loss of absorbance at 254 nm from peak 1 (Figure 6). The instability of the bond between the protein and the nucleotide is the most likely explanation for the low value obtained for the estimated stoichiometry of labeling at complete inhibition (Figure 2). Despite the difference between the expected and observed maximum stoichiometries, however, there is a good correlation between the extent of photochemical inhibition of activity and the stoichiometry of incorporation, up to about 50% inactivation. A similar correlation up to about 40% inhibition was also observed for Ca^{2+}-ATPase and 8-N_3-ADP (Lacapere et al., 1993).

When [α-^{32}P]-8-N_3-ATP was used to label Na^+/K^+-ATPase, greater than 99% of the radioactivity incorporated into the protein was lost from the labeled peptides during

purification. Using dual wavelength detection at 254 and 280 nm, the adenine part of 8-N_3-ATP was found to remain attached to the peptide under the same conditions, and the purine was used as a marker for 8-N_3-ATP-labeled peptides. The absorption of adenosine at 254 nm is significantly higher than the absorption of the aromatic amino acids at this wavelength [$\epsilon_{260}{}^{adenosine} = 14.9 \times 10^3$, $\epsilon_{274}{}^{Tyr} = 1.4 \times 10^3$, and $\epsilon_{257}{}^{Phe} = 0.2 \times 10^3$ (Freifelder, 1976)], whereas at 280 nm the absorption of adenosine is much lower than that at 254 nm (peak height ratio A_{280}:A_{254} of adenosine = 0.084) (Scoble & Brown, 1983). By using a trypsin-digested sample of non-labeled Na^+/K^+-ATPase to adjust the peak height ratio A_{254}:A_{280} to less than 1 for all peptides, an increase in the A_{254}:A_{280} ratio of the peptides from the labeled sample that contained the adenine moiety was easily detected.

Knight and McEntee (1985) have previously shown that a strong absorbance at 254 nm correlates well with the presence of radiolabeled peptides in trypsin- or *S. aureus* V8 protease-digested *E. coli* RecA protein. As shown in Figure 5, a peak that eluted at 43.5 min showed the expected increase in peak height. The elution position of this peak coincided with the position of the single radiolabeled peptide from a tryptic digest of Na^+/K^+-ATPase labeled with [α-^{32}P]-8-N_3-ATP or [2-^3H]-8-N_3-ATP. The increased A_{254}:A_{280} ratio was preserved through subsequent purification steps with the C_4 and phenyl columns. In the final purification step, peak 1, which had a large A_{254}:A_{280} ratio, was gradually converted into peak 2, which had a much smaller A_{254}:A_{280} ratio. The amino acid sequences of these two peaks were identical, except for a large reduction in lysine in the eleventh cycle of peak 1, leading to the conclusion that this conversion is the result of the loss of the adenine moiety of 8-N_3-ATP from the peptide in peak 1 and the subsequent regeneration of lysine in peptide 2. Contamination of the peak 1 peptide with some of the peak 2 peptide is also a reasonable explanation for the appearance of some (20%) lysine at the eleventh cycle of the sequence of peptide 1 (Figure 7). Amino-terminal sequences of peptides purified from Na^+/K^+-ATPase labeled with either [α-^{32}P]-8-N_3-ATP or [2-^3H]-8-N_3-ATP were the same as that of the peptide obtained using dual wavelength detection, in agreement with these results.

The region around the middle of the α-subunits of P-type ATPases is clearly involved in the recognition and binding of nucleotides, and the high degree of amino acid sequence homology among the P-type ATPases in this region is an indication that the structure of the nucleotide binding site in all of these proteins is highly conserved. Several ATP site-directed reagents label these proteins within 20–25 amino acids of each other. In Na^+/K^+-ATPase, FITC labels lysine 501 (Farley et al., 1984; Kirley et al., 1984) and 2-N_3-ATP labels glycine 502 (Tran et al., 1994). In sarcoplasmic reticulum Ca^{2+}-ATPase, McIntosh et al. have shown that lysine 492, which is the amino acid that is homologous to lysine 480 in Na^+/K^+-ATPase, is labeled by TNP-8-N_3-AMP and TNP-8-N_3-ATP (McIntosh et al., 1992). Lysine 492 also participates in a cross-link with arginine 678 of Ca^{2+}-ATPase, and it had been suggested that these two amino acids are close together in the active site, despite their distance apart in the linear sequence of the polypeptide (McIntosh, 1992). Lysine 480 has also been derivatized in Na^+/K^+-ATPase by AP_2PL (Hinz & Kirley, 1990) and was suggested to be involved in the interaction between the protein and the phosphate groups of ATP. Wang and Farley showed, by site-directed mutagenesis of lysine 480, that the side chain of this amino acid does not participate directly in ATP hydrolysis

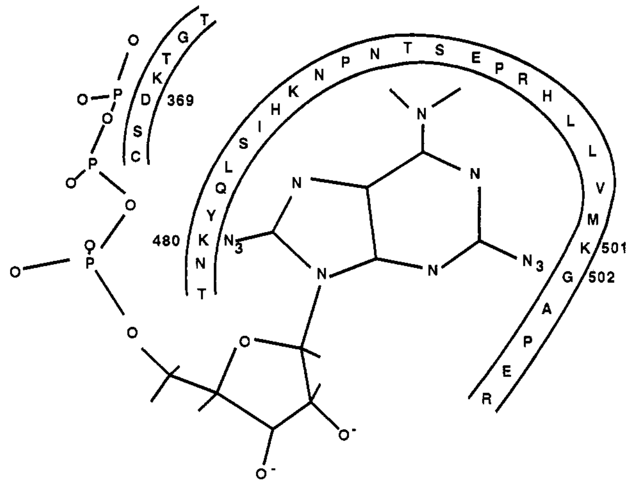

FIGURE 8: Schematic diagram indicating interactions deduced from chemical modification to exist between amino acids in Na^+/K^+-ATPase and ATP. ATP (shown with azido groups at the 2- and 8-positions) is drawn in the conformation indicated by magnetic resonance methods to exist when the nucleotide is bound to P-type ion pumps. The amino acid sequence of Na^+/K^+-ATPase in the region of modification by 8-N_3-ATP and other amino acids previously suggested on the basis of chemical modification or site-directed mutagenesis to interact with the nucleotide is shown. Also shown is the amino acid sequence around D369, which is phosphorylated by ATP.

(Wang & Farley, 1992). Substitution of a glutamate for lysine at this position, however, reduced the apparent affinity of Na^+/K^+-ATPase for both ATP and phosphate, consistent with an interaction with the phosphoryl groups. These results, together with the labeling of this amino acid by 8-N_3-ATP in Na^+/K^+-ATPase and by TNP-8-N_3-ATP in Ca^{2+}-ATPase, indicate that this lysine is accessible to both the purine ring and the phosphate part of the nucleotide. This conclusion is also consistent with the conformation that ATP adopts when bound to Na^+/K^+-ATPase or Ca^{2+}-ATPase, as determined by magnetic resonance (Klevickis & Grisham, 1982; Stewart et al., 1989). In those experiments, it was shown that the phosphate groups of the nucleotide are not extended away from the purine, but fold back toward it in a U-shaped structure.

A diagram of ATP in this conformation, with azido groups at the 2- and 8-positions, is shown in Figure 8 juxtaposed to the amino acids on Na^+/K^+-ATPase that appear to interact with the nucleotide. Both the labeling of glycine 502 by 2-N_3-ATP and the labeling of lysine 501 by FITC suggest that the amino acids around lysine 501 and glycine 502 are accessible to the purine ring. This suggestion differs from the suggestion of Clarke et al. that these amino acids interact with the phosphate moiety of the nucleotide in Ca^{2+}-ATPase (Clarke et al., 1990). The presence of a fluorescein moiety in the position of the adenine ring previously has been seen in lactate dehydrogenase (Wassarman & Lentz, 1971) and yeast hexokinase (Fletterick et al., 1975). Lysine 480 is shown in proximity to both the α- and β-phosphate groups of ATP and the purine ring, on the basis of the results of Hinz and Kirley (1990) and Wang and Farley (1992). Although the structure of the ATP binding site is likely to be very similar in the different P-type ion pumps, the structural domain containing the nucleotide binding sites is not interchangeable among the ion pumps, since no measurable Ca^{2+}-activated ATPase activity was detected in a chimeric molecule containing the cytoplasmic domain of Na^+/K^+-ATPase in place of the same region of Ca^{2+}-ATPase (Sumbilla et al., 1993). The structural basis for the dependence of ATP hydrolysis on the identity of

the transported cations in the different P-type ion pumps has not yet been determined.

The identification of lysine 480 as the site of labeling by 8-N_3-ATP in Na^+/K^+-ATPase is in agreement with the site in Ca^{2+}-ATPase identified by McIntosh et al. (1992) as labeled by TNP-8-N_3-AMP and TNP-8-N_3-ATP, but differs from the suggestion of Lacapere et al. (1993) that amino acids between 530 and 534 of Ca^{2+}-ATPase are labeled by 8-N_3-ADP. The reasons for this discrepancy are not known; however, Lacapere et al. fractionated the tryptic digest of labeled Ca^{2+}-ATPase on Sephadex G-50 before using HPLC and indicated that, without G-50, no radioactive peak was obtained from HPLC. One possibility is that more than one peptide is labeled in each experiment, and the different fractionation procedures used in that report and here have selected different peptides from a collection of labeled peptides. Because of the lability of the bond between 8-N_3-ATP and Na^+/K^+-ATPase that was observed in these experiments, it is not possible to exclude this possibility for Na^+/K^+-ATPase. Alternatively, the presence of different ions and ligands has induced conformational changes in the proteins, such that the amino acids in proximity to the azido groups in the two experiments were different.

ACKNOWLEDGMENT

We thank Dr. Edward Huston for advice regarding the synthesis of 8-N_3-ATP and for many helpful suggestions during the performance of these experiments. We are also grateful to Guy Randolph for providing the purified Na^+/K^+-ATPase used in these experiments. The amino acid sequencing was done by Ms. Lynn Williams at the USC Microchemical Core Facility.

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