Photoaffinity Labeling of the Active Site of the Na^+/K^+-ATPase with 4-Azido-2-nitrophenyl Phosphate†

Chinh M. Tran‡ and Robert A. Farley*,‡,§

Department of Physiology and Biophysics and Department of Biochemistry and Molecular Biology, University of Southern California School of Medicine, Los Angeles, California 90033

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ABSTRACT: Na^+/K^+-ATPase will hydrolyze small acylphosphates such as *p*-nitrophenyl phosphate (pNPP) in addition to ATP and can derive sufficient energy from the hydrolysis of these small molecules to catalyze active ion transport. In this report, 4-azido-2-nitrophenyl phosphate (ANPP), a photoreactive analog of pNPP, was used as a probe of the substrate binding site of dog renal Na^+/K^+-ATPase. ANPP was slowly hydrolyzed by Na^+/K^+-ATPase with a V_{max} of 0.19 μmol mg^{-1} min^{-1} and with an apparent K_m of 1.0 mM. The K_m for hydrolysis of pNPP was 1.7 mM. ANPP competitively inhibited the hydrolysis of pNPP with a K_i of 0.37 mM. Both the ATPase and pNPPase activity of the Na^+/K^+-ATPase were irreversibly inhibited after photolysis of the enzyme and ANPP with UV light, although neither activity was completely inhibited by up to 200 μM ANPP. Inhibition of activity was prevented by including 0.2 mM ATP in the reaction or by excluding Mg^{2+} from the photolysis buffer. Photolysis with [^{32}P]ANPP labeled only the α subunit of the Na^+/K^+-ATPase, and the amount of labeling was substantially reduced by 0.2 mM ATP or in the absence of Mg^{2+}. The stoichiometry of labeling extrapolated to a maximum of about 1.2 nmol/mg of protein at 100% inhibition of Mg^{2+}-dependent activity. Limited proteolytic digestion showed labeling sites on nonoverlapping tryptic peptides derived from the α subunit of Na^+/K^+-ATPase, and two radiolabeled peptides were purified from an exhaustive tryptic digest of [^{32}P]ANPP-labeled Na^+/K^+-ATPase. One peptide contained amino acids Met-379 to Lys-406, and the second contained amino acids Ala-655 to Lys-676. Amino acids corresponding to Asn-398 and Pro-668 were missing from the sequences and may represent residues derivatized by ANPP from within the substrate binding site of Na^+/K^+-ATPase.

Na^+/K^+-ATPase[1] (3.6.1.37) is an integral membrane protein that uses energy derived from the hydrolysis of ATP to transport Na^+ and K^+ ions across animal cell membranes. The Na^+/K^+-ATPase, also called the sodium pump, consists of two polypeptide subunits, α and β, that are required for enzymatic activity. The α subunit of Na^+/K^+-ATPase is structurally similar to polypeptide subunits found in other P-type ion-transporting ATPases, such as Ca^{2+}-ATPase of sarcoplasmic reticulum, H^+/K^+-ATPase of gastric mucosa, and H^+-ATPases of fungal plasma membranes. At this time, only Na^+/K^+-ATPase and gastric H^+/K^+-ATPase have been shown to require a β subunit for activity, although the function of the β subunit is not well understood.

The energy for active ion transport by P-type ATPases is derived from the hydrolysis of ATP, which binds to the α subunit of these enzymes. The identities of several amino acids suggested to participate in the interaction between these proteins and ATP have been determined after either chemical modification of the proteins or site-directed mutagenesis. In Na^+/K^+-ATPase, Lys-480[2] (Hinz & Kirley, 1990; Tran et al., 1994b), Lys-501 (Farley et al., 1984; Kirley et al., 1984), Gly-502 (Tran et al., 1994a), Asp-710 (Ovchinnikov et al., 1987), Lys-719 (Ohta et al., 1986), and possibly Cys-656 (Ohta et al., 1986) have been modified by reagents whose inhibition of Na^+/K^+-ATPase activity is consistent with reaction at the nucleotide binding site of the protein. Lys-480, Lys-501, and Gly-502 appear to be in proximity to the purine part of ATP, and Lys-480, Asp-710, Lys-719, and Cys-656 appear to be located in the vicinity of the phosphate groups of the nucleotide. Mutagenesis of Lys-480 (Wang & Farley, 1992; Lane et al., 1993) has shown that this amino acid is not essential for ATP hydrolysis, although substitution of lysine with glutamic acid reduced the apparent affinity of the enzyme for both ATP and phosphate. Mutagenesis of Cys-656 had no effect on the apparent affinity of the Na^+/K^+-ATPase for ATP, but mutagenesis of Asp-710 and Asp-714 to Asn inactived the enzyme (Lane et al., 1993).

In addition to nucleotides, Na^+/K^+-ATPase can hydrolyze small acyl phosphates such as acetyl phosphate and *p*-nitrophenyl phosphate (pNPP). The hydrolysis of acetyl phosphate and *p*-nitrophenyl phosphate is stimulated by Mg^{2+} and K^+ ions and is inhibited by Na^+ ions, and acetyl phosphate can serve as a substrate for Na^+ transport by Na^+/K^+-ATPase (Beauge & Berberian, 1984). Like the nucleotide analogs used in the studies cited above, chemical probes

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* Address correspondence to this author at Department of Physiology and Biophysics, University of Southern California School of Medicine, 2025 Zonal Avenue. Los Angeles, CA 90033. Telephone: (213) 342-1241. Fax: (213) 342-2283.

‡ Department of Physiology and Biophysics.

§ Department of Biochemistry and Molecular Biology.

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[1] Abbreviations: Na^+/K^+-ATPase, sodium- and potassium-dependent adenosinetriphosphatase (EC 3.6.1.37); ANPP, 4-azido-2-nitrophenyl phosphate; pNPP, *p*-nitrophenylphosphate; FITC, fluorescein-5′-isothiocyanate; HPLC, high-pressure liquid chromatography.

[2] Amino acid numbers correspond to sheep kidney α_1 subunit.

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of the active site of Na^+/K^+-ATPase based on the structures of small acyl phosphate substrates of Na^+/K^+-ATPase have the potential to identify additional amino acids within the nucleotide binding site of the enzyme. In this regard, 4-azido-2-nitrophenyl phosphate (ANPP) has recently been shown to photochemically label the Ca^{2+}-ATPase of sarcoplasmic reticulum and to inhibit the hydrolysis of ATP by this protein (Lacapere & Garin, 1994). A 6 kDa radiolabeled peptide, beginning at Ala-506 (corresponding to Ala-491 of Na^+/K^+-ATPase), was identified as the labeled peptide, but the labeled amino acids were not identified. The interaction of ANPP with Na^+/K^+-ATPase and the photochemical labeling of the enzyme are described in this report. ANPP is hydrolyzed by Na^+/K^+-ATPase, indicating that it binds within the substrate binding site of the enzyme, and, after illumination with ultraviolet light, ANPP is covalently linked to the protein and enzyme activity is irreversibly inhibited. On the basis of an analysis of the amino acid sequences of peptides labeled by [^{32}P]ANPP, two amino acids not previously identified by nucleotide binding-site probes, Asn-398 and Pro-668, are suggested to be located within the active site of the protein.

EXPERIMENTAL PROCEDURES

Materials. 4-Amino-2-nitrophenol was from Aldrich, $H_3{}^{32}PO_4$ in 0.02 N HCl was from ICN, trypsin-TPCK and α-chymotrypsin were from Worthington, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride, and DEAE-Sephadex A-25 were from Sigma, and Silica GF was from J. T. Baker. The reagents were the highest grade available. Dog kidney membranes containing purified Na^+/K^+-ATPase were prepared as described by Jorgensen (1974).

Synthesis of ANPP and [^{32}P]ANPP. ANPP was synthesized as described by Lauquin et al. (1980), and the synthesis of [^{32}P]ANPP was done according to Garin et al. (1989), except that the final separation step was done on a 2.5 cm × 60 cm DEAE-Sephadex A25 column in the bicarbonate form. The column was washed with about 500 mL of distilled water, and a 2 L linear gradient of 0−500 mM triethylamine/HCO_3^- was used to separate the products. The identity of the [^{32}P]ANPP was verified by comparing the R_f of the product on TLC with nonradioactive ANPP. The concentration of the product was calculated using an extinction coefficient $\epsilon_{335} = 1.81$ mM^{-1} cm^{-1} (Lauquin et al., 1980). To determine the extinction coefficient for the azidonitrophenol, an accurately weighted sample of azidonitrophenol dried over P_2O_5 was dissolved in methanol to a concentration of 14.07 mM. This sample was diluted further either 30- or 60-fold with 0.5 N NaOH and 0.05% Triton X-100, and the absorbance of each sample was scanned from 550 to 300 nm. The peak absorbance at 452 nm from each sample was used to calculate the extinction coefficient for azidonitrophenol, $\epsilon_{452} = 5.595$ mM^{-1} cm^{-1} in 0.05 N NaOH.

Photolysis of the Na^+/K^+-ATPase with ANPP. The purified enzyme (0.1 mg/ml) was photolyzed in a final volume of 1 mL in 25 mM Hepes/triethylamine, pH 7.4, 3 mM $MgCl_2$, and 0.2 mM EDTA (acid form). The photolysis was done as described earlier (Tran et al., 1994a) except that the samples were in a nine-well Coors spotting plate. Samples were photolyzed with various amount of ANPP for 30 min, and from each sample a 20 μL aliquot was used to measure the remaining Na^+/K^+-ATPase activity and *p*-nitrophenyl phosphatase activity. When samples were photolyzed in the presence of Na_2-ATP, the ANPP used was converted to Na form on an AG50 × 8 ion exchange column in the Na form. Photolysis was also done in the absence of Mg^{2+}. The amount of protection by ATP was comparable to photolysis in the absence of Mg^{2+}. The photolysis time was chosen to be 30 min because longer photolysis times did not increase the specific inhibition and also increased nonspecific damage by UV light. The concentrations of ANPP were from 1 to 200 μM. Higher concentrations of ANPP up to 1 mM were used in some experiments, but the specific inhibition did not increase, probably due to the absorption of incident light by high concentrations of ligand. The nonspecific inhibition by UV in these experiment was from 10 to 15% of the nonphotolyzed sample.

Na^+/K^+-ATPase Activity Assays. After photolysis, 10 or 20 μL aliquots from each sample were assayed for 30 min at 37 °C for remaining activity. The assay buffer contained 100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM Na_2EDTA, and 3 mM Na_2ATP, with or without 1 μM sodium orthovanadate. The reactions were initiated by addition of enzyme to 1 mL of the incubation buffer and were stopped by addition of 0.2 mL of 30% trichloroacetic acid. The samples were incubated on an ice slurry for 30 min, and precipitates were removed by centrifugation. A 0.5 mL aliquot of the supernatant from each sample was used to assay for the inorganic phosphate released. For *p*-nitrophenylphosphatase activity, the same amounts of enzyme were incubated for 45−60 min in 20 mM Hepes/triethylamine, pH 7.4, 25 mM KCl, 3 mM EDTA (acid form), 2.5 mM *p*-nitrophenyl phosphate (cyclohexylammonium salt), and 3 mM $MgCl_2$ in the presence or absence of 1 μM sodium orthovanadate. The reactions were initiated by addition of enzyme to 1 mL of reaction buffer at room temperature and were stopped by addition of 1 mL of 1 N NaOH and 0.1% Triton X-100. Precipitates were removed by centrifugation for 5 min at about 2500*g*, and the absorbance at 410 nm of the supernatants was measured. An extinction coefficient of 18.5 mM^{-1} cm^{-1} was used for the *p*-nitrophenol released. The difference in absorbance of samples with and without 1 μM sodium orthovanadate was used to calculate the remaining activity.

ANPP as a Competitive Inhibitor of p-Nitrophenyl Phosphate. Samples of 2 μg of enzyme each were incubated in various concentrations of *p*-nitrophenyl phosphate or *p*-nitrophenyl phosphate in the presence of 0.2 mM ANPP, in a buffer identical to that used in the *p*-nitrophenyl phosphatase activity assay. The concentrations of *p*-NPP were from 0.1 to 4 mM. The samples were incubated for 30 min (*p*-nitrophenyl phosphate only or *p*-nitrophenyl phosphate in the presence of 0.2 mM ANPP) at room temperature and were stopped by addition of 1 mL of 1 N NaOH and 0.1% Triton X-100. Precipitates were removed as described above, and the absorbance of the supernatants was measured at 410 nm. From the differences in samples with and without 1 μM vanadate, the specific activity at each concentration was calculated. V_{max} and apparent K_M values were obtained from plots of $1/v$ vs 1/[substrate].

ANPP as a Substrate for Na^+/K^+-ATPase. Aliquots of 10 μg each of Na^+/K^+-ATPase were added to tubes containing 1 mL of 20 mM Hepes/triethylamine, pH 7.4, 25 mM KCl, 3 mM EDTA (acid form), and 3 mM $MgCl_2$ with and without 1 μM sodium orthovanadate. The concentration of

ANPP in each tube was between 0.1 and 2 mM. The reactions were started by addition of the enzyme, samples were incubated at 37 °C for 1 h, and the reactions were stopped by addition of 1 mL of 0.1% Triton X-100 and 1 N NaOH to each tube. The samples were centrifuged in a clinical bench top centrifuge for 5 min, and the supernatants were used for measurement of absorbance at 460 nm. The differences in absorbance of samples without and with vanadate were used to calculate the amount of azidonitrophenol produced, using an extinction coefficient $\epsilon_{460} = 5.27$ mM^{-1} cm^{-1}.

Limited Proteolytic Digestion and SDS−Polyacylamide Gel Electrophoresis. [^{32}P]ANPP-labeled Na^+/K^+-ATPase was digested with trypsin in 100 mM KCl to generate tryptic fragments of 60 and 46 kDa and with α-chymotrypsin in the presence of ouabain to generate chymotryptic fragments of 77, 40, and 35 kDa (Castro & Farley, 1979). The membrane-embedded fragments were collected by centrifugation at 60000*g* for 90 min, and the supernatants were discarded. Membrane pellets were dissolved in electrophoresis sample buffer, were incubated at 37 °C for 1 h, and were separated on a 10% SDS−polyacrylamide gel (Laemmli, 1970). After staining and destaining, an autoradiogram was obtained from the dried gel.

Photolabeling with [^{32}P]ANPP. Purified enzyme was photolyzed with 0.2 mM [^{32}P]ANPP (specific activity 24 μCi/μmol) for 30 min in the photolysis buffer in the presence or absence of Mg^{2+} at a protein concentration of 0.1 mg/mL. Preparative-scale labeling reactions were done using about 14 mg of Na^+/K^+-ATPase. Because of the large volume of sample, the photolysis was done in Costar six-well culture plates containing 5 mL of sample in each well. After photolysis, aliquots of 20 μL of sample were assayed for remaining Na^+/K^+-ATPase and *p*-nitrophenyl phosphatase activity. The rest of the sample was pelleted in a Ti70 rotor at 100000*g* to collect the labeled enzyme. The supernatant was discarded, and the pellets were washed by suspension in 25 mM imidazole/HCl, pH 7.4, and 1 mM Na_2EDTA, and the centrifugation was repeated. The labeled enzyme was suspended in 25 mM imidazole/HCl, pH 7.4, and 1 mM Na_2EDTA to a concentration of about 2 mg/mL and was stored at −80 °C. To determine the effects on enzymatic activity and on photochemical labeling of Na^+/K^+-ATPase by including ATP in the photolysis buffer or by excluding Mg^{2+}, samples of 2 mg each were labeled with 0.2 mM [^{32}P]ANPP in the presence and absence of 0.2 mM ATP and in the presence and absence of 3 mM $MgCl_2$ under the same photolysis conditions. After analysis for remaining enzymatic activities, samples were digested with trypsin and were analyzed by SDS−polyacrylamide gel electrophoresis, autoradiography, and HPLC analysis.

Trypsin Digestion and HPLC Separation of Tryptic Fragments. [^{32}P]ANPP-labeled enzyme was suspended in 25 mM imidazole/HCl, pH 7.4, and 1 mM Na_2EDTA and was digested by trypsin at a ratio of 1:10 (w/w) for 3 h at 37 °C. Membranes were separated from solubilized peptides by centrifugation at 180000*g* for 90 min. The supernatant was collected and injected on to a C_{18} reverse-phase HPLC column. The column was developed as previously described (Tran et al., 1994a), and fractions of 3 mL were collected and counted for Cerenkov radioactivity. Protection against labeling was evaluated using samples of about 1 mg that were labeled in the either the presence or absence of 1 mM ATP or 3 mM $MgCl_2$, with digestion conditions and HPLC analysis as described above. Including ATP in the labeling reaction or leaving out $MgCl_2$ reduced the incorporation of radiolabeled ANPP into the protein, and the HPLC chromatograms of tryptic digests of the Na^+/K^+-ATPase labeled under each condition were qualitatively similar to each other. Because the extent of labeling was reduced to a greater extent by leaving Mg^{2+} out of the reaction, fractions from the C_{18} fractionation of the tryptic digest of 14 mg of [^{32}P]ANPP-labeled Na^+/K^+-ATPase corresponding to fractions in the 1 mg digest that showed reduced incorporation of label in the absence of $MgCl_2$ were pooled and subjected to further HPLC separation of peptides as described in the legends to Figures 5 and 6.

Stoichiometry of Labeling. Samples (0.1 mg) of Na^+/K^+-ATPase in 1 mL were photolyzed with various concentrations of [^{32}P]ANPP in the presence and absence of 3 mM $MgCl_2$ in a nine-well spotting plate. The samples were photolyzed as described above. After photolysis, aliquots of 5 μl from each sample were used to measure the remaining Na^+/K^+-ATPase and *p*-nitrophenyl phosphatase activity. Aliquots of 200 μL of 30% trichloroacetic were added to the remaining samples in microfuge tubes, and the samples were kept on an ice slurry for about 1 h. Samples were centrifuged in a benchtop Eppendorf centrifuge at 14 000 rpm for 30 min. The supernatants were discarded, and the pellets were blotted dry with cotton tipped applicators. The pellets were dissolved in electrophoresis sample buffer with an additional 10 μL of 2 M Tris base, pH 11, to neutralize the remaining trace of trichloroacetic acid. The samples were separated on a 10% SDS−polyacrylamide gel, and, after staining and destaining, the α-subunit bands from each sample were excised from the gel and were counted for Cerenkov radiation in 1 mL of water. Five microliters of the same solution of [^{32}P]ANPP that was used in the experiment was polymerized in 1 mL of a 10% SDS−polyacrylamide gel and was counted along with the gel slice to get the corrected specific activity. The radioactivity of samples labeled in buffer that did not have $MgCl_2$ was subtracted from identical samples that contained $MgCl_2$. The remaining enzymatic activity of samples that did not have Mg^{2+} was also subtracted from the corresponding samples that contained Mg^{2+}. The amount of labeling was calculated from the specific activity of the [^{32}P]ANPP and plotted against the activity remaining in the same sample. The data were fit by a straight line, and the extent of labeling at 100% inhibition of activity was calculated from the fit parameters.

RESULTS

Hydrolysis of ANPP by Na^+/K^+-ATPase. The hydrolysis of pNPP and the photochemical analog ANPP by dog renal Na^+/K^+-ATPase was measured as described in Experimental Procedures. K^+ stimulated the hydrolysis of both substrates, and Mg^{2+} was required for the hydrolysis of either substrate. Lineweaver−Burk plots showing the dependence of the rate of hydrolysis of each substrate on substrate concentration are shown in Figure 1. The hydrolysis of both ANPP and pNPP follow Michaelis−Menten kinetics, and, from the data shown in the figure, the calculated K_M for ANPP hydrolysis by Na^+/K^+-ATPase is 1.0 mM, and the calculated K_M for pNPP hydrolysis is 1.7 mM. Although the K_M values for both substrates are comparable, the V_{max} for ANPP hydrolysis

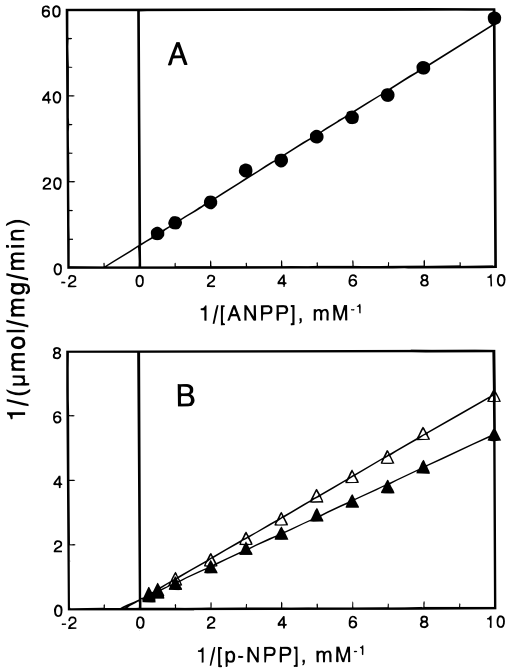

FIGURE 1: Hydrolysis of ANPP and p-NPP by Na,K-ATPase. Ten micrograms of purified Na^+/K^+-ATPase (1 mg/ml) in 20 mM Hepes/triethylamine, pH 7.4, 25 mM KCl, 3 mM EDTA (free acid), and 3 mM Mg^{2+} was added to sample tubes containing 1 mL of the same buffer and various concentrations of ANPP from 0.1 to 2 mM, in the presence and absence of 1 μM sodium orthovanadate (panel A). Samples were incubated at 37 °C for 1 h and quenched with 1 mL of 1 N NaOH, 0.1% Triton X-100, and insoluble debris was removed by centrifugation at 2000*g* for 15 min. The absorbance of the supernatants at 460 nm was obtained, and the differences of between samples incubated in the presence and absence of vanadate were used to calculate the specific activity. For p-NPPase activity (panel B), aliquots of 1 μg of purified Na^+/K^+-ATPase were added to tubes containing 1 mL of buffer with various concentrations of p-NPP from 0.1 to 4 mM in the presence and absence of 1 μM sodium orthovanadate, with (△) and without (▲) 0.1 mM ANPP. Samples were incubated for 30 min at room temperature and quenched as mentioned above. Absorbance at 410 nm was obtained, and specific activities were calculated at each concentration as described in panel A. Double-reciprocal plots of the data are shown, with the best linear fit for each graph. K_M and V_{max} were obtained from the fit, and K_i was calculated from the equation $K_{M\ app} = (K_m/K_i)[I] + K_M$. For ANPP, a V_{max} of about 0.19 μmol mg^{-1} min^{-1} and a K_M of 1.01 mM were obtained. For p-NPP, a V_{max} of 3.36 μmol mg^{-1} min^{-1} and a K_M of 1.7 mM were obtained. Panel B shows that ANPP competitively inhibits p-NPPase activity with a calculated K_i of 0.38 mM. The data shown were from two experiments, and each measurement was done in duplicate. The error bars were smaller than the symbols and are not shown.

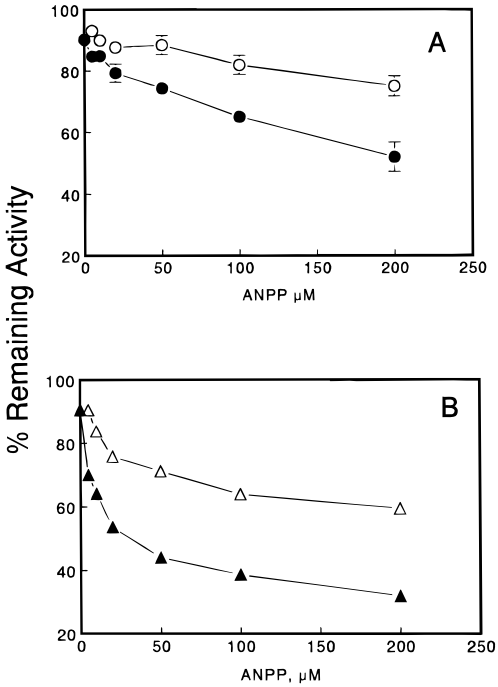

FIGURE 2: Photoinhibition of Na,K-ATPase and p-NPPase activity by ANPP. Purified Na^+/K^+-ATPase was suspended in 20 mM Hepes/triethylamine, pH 7.4, 3 mM EDTA(free acid), and 3 mM $MgCl_2$ to a final concentration of 0.1 mg/mL, and samples of 1 mL each were added to a Coors nine-well spotting plate. Each well contained a difference concentration of ANPP between 5 and 200 μM (closed symbols). The samples were photolyzed at 310 nm with an energy of 310 $\mu W/cm^2$ for 30 min. An identical set of samples was also photolyzed in the presence of 0.1 mM ATP (open symbols) in 20 mM Hepes, TEA, pH 7.4, 150 mM NaCl, 3 mM $MgCl_2$ and 0.2 mM EDTA. The addition of NaCl was made to facilitate ATP binding. After photolysis, aliquots of 2 μg from each sample were assayed for remaining p-NPPase activity (panel A) and Na^+/K^+-ATPase activity (panel B). The data were from four experiments (mean ± SEM), and the error bars are shown unless they are smaller than the symbol.

is only about 6% of the V_{max} for pNPP hydrolysis (0.19 vs 3.36 μmol mg^{-1} min^{-1}). The structural differences between the two molecules, therefore, do not appear to affect the affinity of the protein for each substrate but rather influence the subsequent conformational changes of the protein that accompany hydrolysis of the substrate. As shown in Figure 1, the hydrolysis of pNPP was competitively inhibited by 0.1 mM ANPP with a K_i of 0.37 mM. These data indicate that ANPP binds to Na^+/K^+-ATPase at the site where small acyl phosphates are hydrolyzed as substrates.

Photochemical Inactivation of Na^+/K^+-ATPase Activity by ANPP. When Na^+/K^+-ATPase was illuminated by UV light in the presence of ANPP and Mg^{2+}, both Na^+,K^+-stimulated ATP hydrolysis and K^+-stimulated pNPP hydrolysis were inhibited. No ANPP-dependent inhibition of either activity was observed in the absence of Mg^{2+}. Figure 2 shows the ANPP concentration dependence of inhibition of each activity after UV illumination. It was not practical to use concentrations of ANPP higher than about 0.2 mM because of the formation of colored products both from the hydrolysis of ANPP by Na^+/K^+-ATPase and from the photochemical reaction. These colored products both reduced the effective concentration of ANPP in solution and also served as a filter to reduce the UV dose incident on the sample. For both ATPase and pNPPase activities, inhibition was partially protected by including 0.2 mM ATP in the reaction mix. Surprisingly, no protection against enzyme inactivation by 0.2 mM ANPP was observed after 30 min of irradiation with up to 1 mM pNPP present in the reaction. This observation, however, may be a consequence of the comparable affinities of both pNPP and ANPP for the Na^+/K^+-ATPase and the limited range of concentrations of each that could be used in the experiments. The conditions required for photochemical inhibition of activity by ANPP also favor the rapid hydrolysis of pNPP, and, during a 30 min photochemical reaction, significant hydrolysis of pNPP to *p*-nitrophenol was observed. Inhibition of activity at short times of irradiation was too low and too variable to obtain reliable data regarding a possible effect of pNPP on the initial rate of inactivation by ANPP. Inorganic phosphate also did not provide significant protection against inhibition of activity. As shown in Figure 2, under the conditions of these experiments, neither

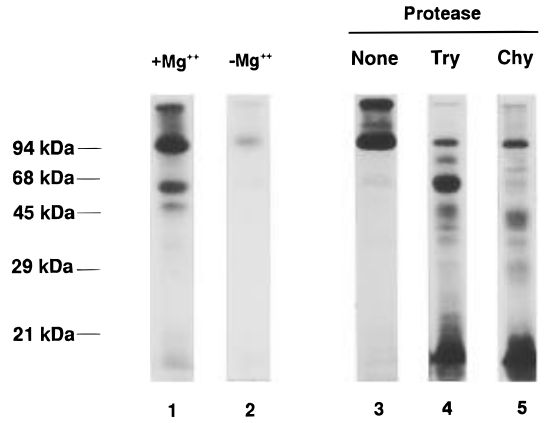

FIGURE 3: Photochemical labeling of Na,K-ATPase with [^{32}P]-ANPP. Purified Na^+/K^+-ATPase was labeled with 200 μM [^{32}P]ANPP in the presence (lanes 1 and 3) and absence (lane 2) of 3 mM $MgCl_2$, as described in Experiment Procedures. Labeled enzyme was digested with TPCK-trypsin with a ratio of 1:50 (w/w) at 37 °C for 90 min in the presence of 150 mM KCl in order to generate 60 and 45 kDa fragments (lane 4), or with α-chymotrypsin at a ratio of 1:3 (w/w) in the presence of 5 mM $MgCl_2$ and 1 mM ouabain in order to generate 40 and 35 kDa fragments (lane 5). The autoradiogram shown was obtained from samples after separation of peptides on a 10% polyacrylamide gel.

ATPase activity nor pNPPase activity was inhibited completely at the highest concentrations of ANPP tested.

A comparison of the photochemical inactivation of ATPase activity and pNPPase activity at different concentrations of ANPP indicated that, at all concentrations of ANPP, the inhibition of ATPase activity was greater than the inhibition of pNPPase activity. The inhibition of both activities was measured after different irradiation times and in the presence of different concentrations of ANPP, and an apparent first-order rate constant for inactivation of each activity was obtained. For ATPase activity, the rate constant for inactivation at 200 μM ANPP was 0.14 min^{-1}, and, for pNPPase activity, the rate constant was 0.09 min^{-1}. These rate constants are significantly different from each other ($P < 0.002$) when compared in a two-tailed *t*-test.

Photochemical Labeling of Na^+/K^+-ATPase by [^{32}P]-ANPP. Since the binding and hydrolysis of ANPP by Na^+/K^+-ATPase and also photochemical inhibition of enzyme activity by ANPP depend on the presence of Mg^{2+}, the enzyme was photolabeled with [^{32}P]ANPP in the presence or absence of 3 mM $MgCl_2$ in order to determine whether the enzyme might also be labeled from outside of the substrate binding site. As shown in Figure 3, only the α subunit of the enzyme is labeled by [^{32}P]ANPP under these conditions. Lanes 1 and 2 of Figure 3 show an autoradiogram of Na^+/K^+-ATPase after photochemical labeling by [^{32}P]ANPP in the presence or absence of 3 mM $MgCl_2$ and subsequent separation of the proteolytic fragments on SDS−PAGE. A large amount of radioactivity is incorporated into the α subunit of the enzyme with a molecular mass of about 94 kDa, and this labeling is almost completely prevented by leaving Mg^{2+} out of the reaction. The high molecular weight material present at the top of the gel is photochemically cross-linked Na^+/K^+-ATPase that is too large to enter the separating gel. The β subunit of Na^+/K^+-ATPase is not labeled. The radiolabeled bands in lane 1 at 60 and 45 kDa are proteolytic fragments of the α subunit that were present in this preparation of the enzyme. This is shown more clearly in lane 3, which shows a different preparation of the enzyme that was labeled by [^{32}P]ANPP in the presence of Mg^{2+} in a different experiment. In this sample the proteolytic fragments were not observed, and the [^{32}P]ANPP can be seen to be incorporated only into the α subunit. Since only a small amount of radiolabeled ANPP was incorporated into the Na^+/K^+-ATPase in the absence of Mg^{2+}, it is likely that the enzyme is labeled only from within the active site. The results shown in Figure 3, lanes 4 and 5, however, also demonstrate that the Na^+/K^+-ATPase is labeled at more than one amino acid within this site. Lane 4 shows an autoradiogram of [^{32}P]ANPP-labeled Na^+/K^+-ATPase after limited cleavage by trypsin in the presence of KCl and separation of the proteolytic fragments on SDS−PAGE. Cleavage of Na^+/K^+-ATPase by trypsin in the presence of KCl generates two nonoverlapping fragments of about 60 and 46 kDa (Jorgensen & Farley, 1988). A small amount of a fragment of about 77 kDa that is generated in larger amounts by trypsin digestion in NaCl is also present in the sample in lane 4, as are some smaller fragments that are secondary proteolytic products produced from the larger fragments. The 46 kDa fragment is derived from the amino-terminal region of the α subunit, and the 60 kDa fragment is derived from the carboxy-terminal region of the polypeptide. As shown in the figure, both the 60 and 46 kDa fragments are labeled, indicating that, during the excited-state lifetime of the [^{32}P]ANPP bound within the substrate site of the enzyme, the probe is able to make contact with more than one amino acid located within that site. More label is found in the 60 kDa fragment than in the 46 kDa fragment, possibly reflecting a greater accessibility of the activated probe to this region of the substrate binding site or a greater stability of the photochemical adduct generated with the labeled amino acid from this region. Lane 5 shows an autoradiogram of [^{32}P]ANPP-labeled Na^+/K^+-ATPase after cleavage by chymotrypsin in the presence of Mg^{2+} and ouabain. Two nonoverlapping fragments of approximately 35 and 40 kDa are generated by this cleavage (Castro & Farley, 1979) as well as other fragments that are also found in smaller abundance. The 35 kDa fragment overlaps the site of cleavage between the 46 and 60 kDa tryptic fragments, and the 40 kDa fragment is derived from the carboxy-terminal region of the α subunit. Labeled bands are observed at positions corresponding to both the 35 and 40 kDa fragments in lane 5, but the presence of an additional proteolytic fragment at the position of the 35 kDa chymotryptic fragment (see also lane 4) complicates the interpretation of this observation. The results from the trypsin digest, however, clearly indicate that more than one amino acid can be labeled from within the ATP binding site by [^{32}P]ANPP.

The amount of radioactivity incorporated into the α subunit after UV illumination was measured at different concentrations of [^{32}P]ANPP in the presence and absence of Mg^{2+}, and the Mg^{2+}-dependent incorporation was compared to the Mg^{2+}-dependent inhibition of activity determined from the same samples. A linear correlation between the extent of incorporation of radioactivity and the extent of inhibition is observed for the concentration range 5−200 μM ANPP, although the data were scattered around a straight line fit to them (Figure 4). The extrapolated stoichiometry of incorporation at 100% inhibition of activity is about 1.2 nmol/mg of protein, which is less than the ouabain or ATP binding capacity of the enzyme (2−2.5 nmol/mg). This result suggests either that the protein can be inactivated at less than

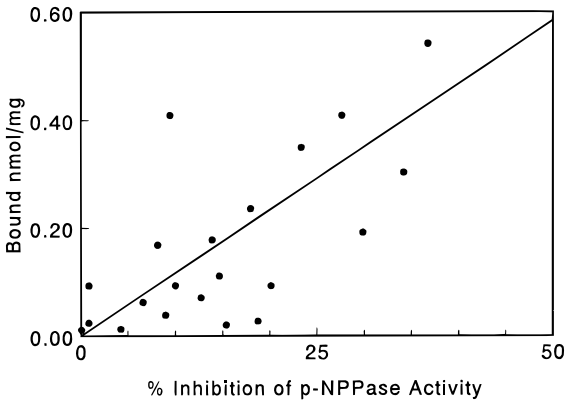

FIGURE 4: Relationship between stoichiometry of photochemical incorporation and photochemical inhibition of activity. Samples (0.1 mg) of Na^+/K^+-ATPase were labeled using different concentrations of [^{32}P]ANPP in the presence and absence of 3 mM $MgCl_2$ as described in Experimental Procedures. Aliquots were removed for measurement of pNPPase and ATPase activity, and the remaining protein was separated by SDS−PAGE. Bands corresponding to the α subunit were excised from the gel and counted for radioactivity. The difference between the amount of ANPP incorporated into each sample in the presence and absence of Mg^{2+} is plotted against the percent inhibition of Mg^{2+}-dependent pNPPase activity for each sample. The regression line for the data is shown ($r^2 = 0.38$).

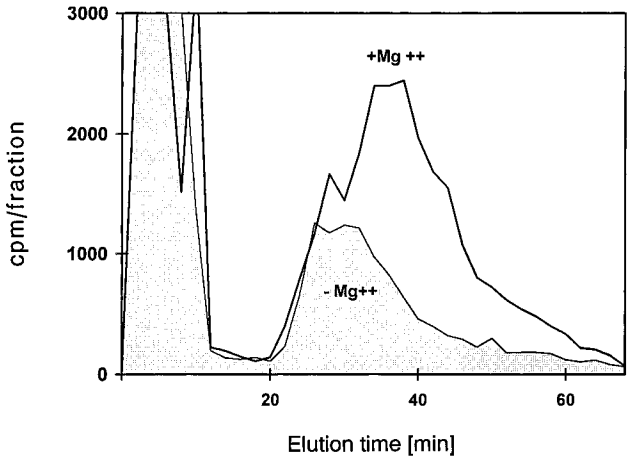

FIGURE 5: HPLC separation of Na^+/K^+-ATPase labeled by [^{32}P]-ANPP in the presence and absence of $MgCl_2$. One milligram each of Na^+/K^+-ATPase labeled with 200 μM [^{32}P]ANPP in the presence of 3 mM $MgCl_2$ or in the absence of $MgCl_2$ was digested with trypsin (1:10) for 3 h at 37 °C. Reactions were stopped with soybean trypsin inhibitor, and soluble tryptic peptides were separated from membranes by centrifugation. Soluble tryptic peptides were separated on a C_{18} HPLC column using a linear gradient of 0 to 70% buffer B in 100 min at a flow rate of 1.5 mL/min. Buffer A was 20 mM sodium phosphate, pH 6.15, and buffer B was 70% acetonitrile in water. The peaks of radioactivity in the initial part of the chromatogram are due to elution of ^{32}P during loading of the column. The gradient was started at about 10 min. Fractions of 3 mL were collected and counted for radioactivity. Radioactivity in fractions eluting between 30 and 45 min show the largest dependence on $MgCl_2$, and these fractions were collected in preparative separations for peptide purification (Figure 6). Column effluent was also monitored by absorbance at 214 nm (not shown).

stoichiometric levels of labeling by ANPP or that the linkage between ANPP and the protein is unstable, causing the stoichiometry to be underestimated in these experiments. An unstable linkage between the probe and the protein is suggested by the disproportionate loss of radioactivity from tryptic peptides after separation by HPLC, as discussed below. An unstable bond between the probe and the protein could also be responsible for the variability observed in data from different experiments reported in Figure 4.

Isolation and Amino Acid Sequence of Tryptic Peptides Labeled by [^{32}P]ANPP. Na^+/K^+-ATPase was photolabeled by [^{32}P]ANPP in the presence or absence of $MgCl_2$, and, after being washed by centrifugation to remove unreacted [^{32}P]ANPP, the sample was digested by trypsin for 3 h at 37 °C. The soluble peptides were separated from the membranes by centrifugation. Most of the radioactivity was recovered in the supernatant, which was applied to an HPLC C_{18} reverse-phase column for fractionation (Figure 5). The fractions in the region of the chromatogram that had the largest differences in radioactivity incorporated in the presence and absence of $MgCl_2$ were pooled and fractionated further by successive HPLC separations (Figure 6). Pools of fractions containing peaks of radioactivity were collected from each chromatogram for successive fractionations. Five peaks of coincident absorbance and radioactivity were eventually isolated for amino acid sequence determination. Sequences were obtained for two of these samples (Figure 6), and Figure 7 shows the identity of amino acids for these peptides and the yield of amino acids for each cycle of the sequencer. The remaining three samples did not contain Na^+/K^+-ATPase sequences, although in some instances amino acids were present in the sample.

Peptide 1 corresponds to amino acids Met-379−Lys-406 from the α subunit of Na^+/K^+-ATPase, and peptide 2 corresponds to the amino acids Ala-655−Lys-676. Although the yield of PTH-methionine at the first cycle of the sequencer for peptide 1 was 845 pmol, the radioactivity in the sample indicated that only 118 pmol was present. This difference is consistent with the loss of radiolabel from the sample that was first observed during measurements of incorporation stoichiometry (Figure 4). Additional amino acids were also present in each sequencer cycle for peptide 1; however, the next most abundant amino acid in the first cycle of peptide 1 was 114 pmol, and no sequences from Na^+/K^+-ATPase other than Met-379−Lys-406 could be identified in the sample. For peptide 1, the yield of PTH-amino acids dropped by 85% at sequencer cycle 20, from 113 to 17 pmol, and remained low thereafter. Cycle 20 corresponds to Asn-398 in the Na^+/K^+-ATPase sequence. This result might be explained if Asn-398 is labeled by ANPP such that derivatization of this amino acid prevents further Edman degradation of the peptide.

The amino acid sequence obtained for peptide 2 corresponded to Na^+/K^+-ATPase amino acids Ala-655−Lys-676, except that cycle 14 was blank. The amino acid expected in cycle 14 is Pro-668, and the sequence following this amino acid returned to expected levels until the end of the peptide. These data suggest that Pro-668 is labeled by ANPP but that the Edman degradation is not blocked by derivatization of proline by the probe. The yield from the sequencer of the first amino acid from peptide 2 was 270 pmol, whereas the radioactivity indicated only 58 pmol. A second peptide corresponding to amino acids Asn-234−Arg-248 was identified in the sample, with a yield of 89 pmol in the first cycle. No amino acids were missing from this sequence, however, indicating either that this peptide was not labeled by ANPP or that any linkage between the probe and the peptide was labile. If this peptide were also labeled by ANPP and if the linkage were hydrolyzed, then the original amino acid must have been regenerated following

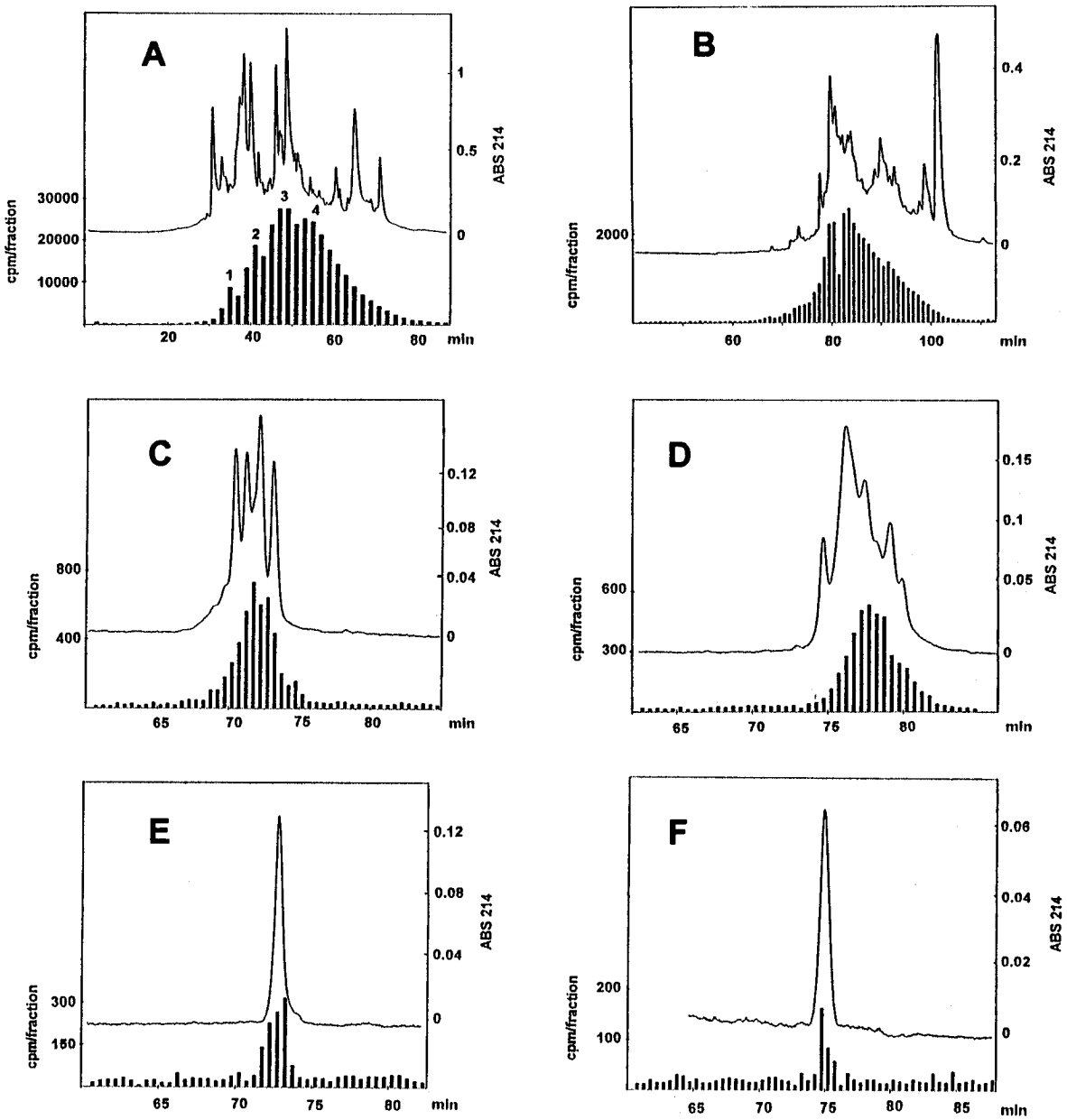

FIGURE 6: HPLC purification of [32P]ANPP-labeled peptides from a tryptic digest of Na^+/K^+-ATPase. Fourteen milligrams of purified Na^+/K^+-ATPase was labeled in the presence of 3 mM $MgCl_2$ and digested with trypsin, as described in Experimental Procedures. Soluble tryptic peptides were separated on an HPLC C_{18} column as described in the legend to Figure 5, and fractions corresponding to peaks between 30−45 min were collected and pooled. (A) HPLC C_4 separation of pooled fractions. A_{214} (solid trace) and radioactivity (bars) are shown for this and subsequent chromatograms. Buffer A was 10 mM sodium phosphate, pH 2.5, and buffer B was 70% acetonitrile in water. A linear gradient of 0−80% buffer B was used to develop the column at a flow rate of 1.5 mL/min. Fractions of 3 mL were collected. Four pools (1−4) were made from fractions as indicated and analyzed by subsequent HPLC fractionation. Identifiable Na^+/K^+-ATPase peptides were obtained only from pool 4 (52−60 min). Fractionation of pool 4 is shown in the other panels of the figure. (B) Pool 4 was separated on an HPLC C_{18} column. Buffer A was 0.05% trifluoroacetic acid and buffer B was 70% acetonitrile/0.05% trifluoroacetic acid. The column was developed with a gradient of 0-80% buffer B at a flow rate of 1.5 mL/min, and fractions were collected every 1 min. Fractions containing peaks of radioactivity centered at about 80 and 83 min were collected and pooled separately. (C and D) Pools from peaks eluting at 80 (C) and 83 min (D) were fractionated again on a C_{18}HS column using the same elution conditions. Thirty second fractions were collected. Fractions eluting from 71.5 to 72.5 min were pooled from C, and fractions eluting from 76.5 to 78 min were pooled from D. Pooled samples were rechromatographed on a C_{18}HS column under identical conditions, and peaks of radioactivity and absorbance were isolated. (E and F) HPLC chromatograms and radioactivity profiles of samples used for amino acid sequencing. The peak in E (peptide 1) is derived from the chromatogram in C, and the peak in F (peptide 2) is derived from the chromatogram in D.

loss of ANPP since no amino acids were missing from the sequence. This possibility cannot be excluded at this time. No other sequences found in Na^+/K^+-ATPase α subunit were identified in the sample.

DISCUSSION

Several experiments described here indicate that photochemically activated ANPP labels the Na^+/K^+-ATPase from within the substrate binding site. The Na^+/K^+-ATPase hydrolyzes ANPP as a substrate, ANPP competitively inhibits the hydrolysis of pNPP by Na^+/K^+-ATPase, and ANPP-dependent photochemical inactivation of Na^+/K^+-ATPase activity is protected by ATP. Hydrolysis of ANPP by Na^+/K^+-ATPase depends on Mg^{2+} and on K^+, and since minimal labeling of the protein by [^{32}P]ANPP occurred in the absence of Mg^{2+}, the amino acids that are labeled by ANPP are likely

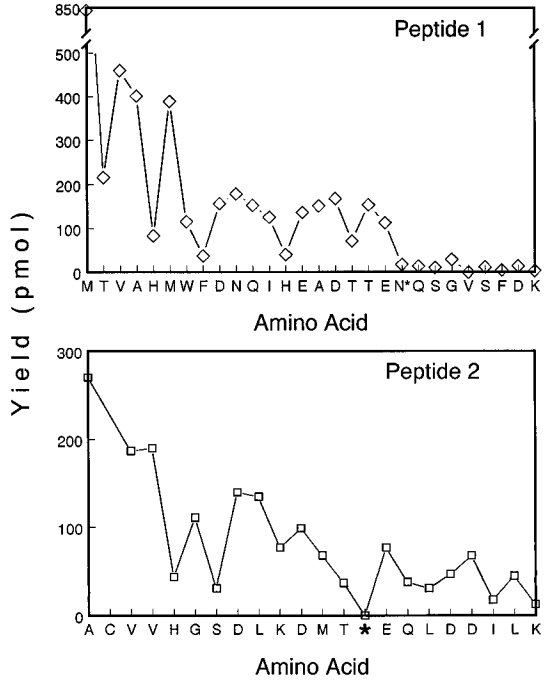

FIGURE 7: Amino acid sequence analysis of peptides from trypsin-digested, [^{32}P]ANPP-labeled Na^+/K^+-ATPase. The yield of PTH-amino acids (pmol) is shown on the left axis, and the amino acid identity obtained at each cycle is shown on the bottom axis for each peptide. In peptide 1 the yield at cycle 20 (Asn-398) dropped to about 85% of cycle 19, and the yield remained low for the rest of the peptide. In peptide 2, cycle 14 corresponds to Pro-668 and was blank (*) during the analysis of this peptide. The yield of amino acids in subsequent cycles returned to normal levels.

to be located within the ATP binding site of the protein.

At all concentrations of ANPP the extent of photochemical inactivation of ATPase activity by ANPP was always greater than the extent of inactivation of pNPPase activity, and this is reflected in the faster apparent first-order rate constant for ATPase inactivation (0.14 min^{-1}) compared to pNPPase inactivation (0.09 min^{-1}). One explanation for this difference is the possibility that, after covalent attachment of ANPP to the protein, pNPP can still bind to the modified protein and be hydrolyzed, although at a slower rate than in the unmodified enzyme. ATP, because of its larger size, may not be able to bind to the modified enzyme. Inactivation of ATPase activity with relatively little effect on pNPPase activity has previously been reported for the reaction of FITC with Na^+/K^+-ATPase (Karlish, 1980; Carilli et al., 1982). FITC is thought to occupy the region of the ATP binding site that is usually occupied by the purine moiety of the nucleotide. ANPP, however, should label the region of the binding site that is usually in proximity to the phosphate groups of the nucleotide. In order for ANPP to label the Na^+/K^+-ATPase and to allow a second molecule of ANPP to bind, the region of the ATP binding site in the vicinity of the phosphate groups must have sufficient volume to accommodate the two ANPP molecules. Some evidence supporting this possibility comes from earlier observations that Na^+/K^+-ATPase can bind both nucleotides and phosphate simultaneously (Askari & Huang, 1982; Scheiner-Bobis et al., 1993).

ANPP was used as a probe for the substrate site because its structure resembles the structure of pNPP, a small acyl phosphate that is hydrolyzed by Na^+/K^+-ATPase in a reaction that may mimic the K^+-dependent hydrolysis of the phosphoenzyme intermediate formed from ATP. This reaction presumably occurs while the enzyme is in the E2 conformation, whereas probes of the nucleotide binding site that have been used previously are expected to label the protein when it has a high affinity for ATP, the E1 conformation. This difference in enzyme conformation may also influence the region of the protein that is labeled by ANPP.

Since two nonoverlapping tryptic fragments from the α subunit of the Na^+/K^+-ATPase are labeled by [^{32}P]ANPP, at least two different amino acids must be accessible to the probe from within the ATP binding site. Amino acid sequence analysis of peptides labeled with [^{32}P]ANPP identified two tryptic peptides from the α subunit of Na^+/K^+-ATPase that contained radioactivity. Asn-398 and Pro-668 were either missing or were present in unexpectedly low abundance in these peptides and may be labeled by ANPP. For Asn-398, the yield from the sequencer dropped by 85%, essentially to background levels, at this cycle. This behavior could occur if the Edman degradation had been terminated by the derivatization of this amino acid. For Pro-668, the output from sequencer was blank at this position, but the sequence of the peptide continued at normal levels in subsequent cycles. This behavior is expected when the Edman degradation is able to cleave a modified amino acid from a peptide, but the elution position of the modified PTH-amino acid from the HPLC column does not correspond to that of the unmodified PTH-amino acid. Subsequent cycles of Edman degradation are unaffected. Positive identification of the derivatized amino acids is needed to confirm these assignments; however, a low stoichiometry of labeling relative to inhibition of activity suggests that the linkage between ANPP and the labeled peptides is unstable. Because of the low recovery of radioactivity in the peptides, it was not possible to recover [^{32}P]ANPP-derivatized amino acids from the sequencer after Edman degradation of the labeled peptides.

These results suggest that Asn-398 and Pro-668 may be located within the ATP binding site of Na^+/K^+-ATPase where they can react with photoactivated ANPP. Asn-398 is 29 amino acids downstream in the amino acid sequence of Na^+/K^+-ATPase from Asp-369, which is phosphorylated by ATP during ATP-dependent ion transport. Using ATP site-directed reagents based on the structure of ATP, previous investigations have not identified any amino acid in the ATP binding site that is closer to Asp-369 in the amino acid sequence than Lys-480 (Farley et al., 1984; Kirley et al., 1984; Tran et al., 1994a,b; Hinz & Kirley, 1990). Many of these reagents label the α subunit in the region around amino acid 500; however, on the basis of a genetic analysis of the FITC binding site of yeast H^+-ATPase, it was recently suggested that there is a direct interaction between this region of P-type ATPases and the phosphorylation domain (Maldonado & Portillo, 1995). An allele-specific suppressor of a mutation in Lys-474 in yeast H^+-ATPase (equivalent to FITC-labeled Lys-501 in Na^+/K^+-ATPase) was identified 18 amino acids downstream from the phosphorylated aspartate residue. The labeling by ANPP of Asn-398 of Na^+/K^+-ATPase would be in agreement with the conclusion that this region of P-type ATPases is in close contact with other amino acids already suggested to be located within the ATP binding site. Pro-668 is in a region of the α subunit that has previously been implicated in interactions with the phosphate groups of other nucleotide substrates. Cys-656 and Lys-

719 have been reported to be labeled by FSBA (Ohta et al., 1986), and Asp-710 and Asp-719 have been labeled by ClR-ATP (Ovchinnikov et al., 1987). The results of labeling Pro-668 of Na^+/K^+-ATPase with ANPP, therefore, also support previous suggestions (McIntosh, 1992; Tran et al., 1994b) that nucleotide binding sites of P-type ATPases must fold into a conformation that brings together into spatial proximity several amino acids that are widely separated in the linear sequence of the proteins.

The results that are reported here using ANPP to label Na^+/K^+-ATPase are different from results reported recently for the interaction of ANPP with sarcoplasmic reticulum Ca^{2+}-ATPase (Lacapere & Garin, 1994). In that report, [^{32}P]ANPP was found to label a 6 kDa peptide that begins at Ala-506 and includes amino acids such as Lys-515 that is modified by FITC. The amino acids labeled by ANPP, however, were not identified. An important difference between the photolabeling procedures for Na^+/K^+-ATPase and Ca^{2+}-ATPase that may account for the difference in results was the absence of Mg^{2+} from the labeling reaction for Ca^{2+}-ATPase. Although hydrolysis of ANPP by Ca^{2+}-ATPase also requires Mg^{2+}, no Mg^{2+} was present during labeling of Ca^{2+}-ATPase by ANPP. For Na^+/K^+-ATPase, the data indicate that there is essentially no labeling of the protein by ANPP in the absence of Mg^{2+}. Another difference between the interaction of ANPP and the two ATPases is the lack of a protective effect of P_i on inactivation of Na^+/K^+-ATPase by ANPP, whereas Pi in the presence of Mg^{2+} was a more efficient protector than ADP against photolabeling of Ca^{2+}-ATPase. The structures of the nucleotide binding sites of all P-type ATPase are thought to be similar to each other (Taylor & Green, 1989), and it is unlikely, therefore, that the differences reported for Na^+/K^+-ATPase in this report and for Ca^{2+}-ATPase (Lacapere & Garin, 1994) reflect significant structural differences between the two proteins. Although differences in the labeling conditions used in the two studies may account for the identification of different labeled amino acids in Na^+/K^+-ATPase and Ca^{2+}-ATPase, it is also possible that ANPP labels the same amino acids in the two proteins but that incomplete analysis of the data has not yet demonstrated this. The peptide labeled in Ca^{2+}-ATPase and Asn-398 in Na^+/K^+-ATPase correspond to the two regions of the active site suggested to interact in yeast (Maldonado & Portillo, 1995). Three additional peaks of coincident A_{214} and radioactivity were isolated from Na^+/K^+-ATPase during this investigation, but no amino acid sequences were obtained for these peptides. It is possible that one of those peaks contains the labeled peptide reported for Ca^{2+}-ATPase. It is not known whether additional labeled peptides are also present in ANPP-labeled Ca^{2+}-ATPase.

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