Catalytic Activity of an Isolated Domain of Na,K-ATPase Expressed in *Escherichia coli*

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 USA

ABSTRACT Fusion proteins of glutathione-*S*-transferase and fragments from the large cytoplasmic domain of the sheep Na,K-ATPase α_1-subunit were expressed in *Escherichia coli*. The Na,K-ATPase sequences begin at Ala^{345} and terminate at either Arg^{600} (DP600f), Thr^{610} (DP610f), Gly^{731} (DP731f), or Glu^{779} (DP779f). After affinity purification on glutathione-Sepharose, the fusion proteins were labeled with [α-^{32}P]-2-N_3-ATP, and incorporation of the radiolabel into the fusion proteins was measured by scintillation counting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. K_d values of 220–290 μM for 2-N_3-ATP binding to the fusion proteins were obtained from the photolabeling experiments. Approximately 1 mol of 2-N_3-ATP was calculated to be incorporated per mole of fusion protein after correction for photochemical incorporation efficiency. Labeling of all of the fusion proteins by 25 μM 2-N_3-ATP was reduced in the presence of MgATP, Na_2ATP, $MgCl_2$, 2′,3′-*O*-(2,4,6-trinitrophenyl)-ATP, and *p*-nitrophenylphosphate, and K_i values of 2–11 mM for Na_2ATP, 0.2–5 mM for $MgCl_2$, 0.1–5 mM for MgATP, and 20–300 μM for *p*-nitrophenylphosphate were calculated for these ligands. All of the fusion proteins catalyze the hydrolysis of *p*-nitrophenylphosphate. The reaction requires $MgCl_2$ and is inhibited by inorganic phosphate, which is similar to the hydrolysis of *p*-nitrophenylphosphate by native Na,K-ATPase. Based on these observations, it appears that the soluble fragments from the large cytoplasmic domain of Na,K-ATPase expressed in bacterial cells are folded in an E_2-like conformation and are likely to retain much of the native structure.

INTRODUCTION

P-type ATPases are integral membrane proteins that utilize energy derived from the hydrolysis of ATP to transport ions across cell membranes. All of the P-type ATPases contain a large catalytic subunit that spans the membrane several times. The Na,K-ATPase and H,K-ATPases also require a second subunit for their enzymatic and transport functions. Structural models of the catalytic subunit of P-type ion pumps include a cytoplasmic domain of ~400 amino acids located near the middle of the polypeptide, between membrane-spanning segments of the protein that may be involved in the pathway for ion translocation (Lutsenko and Kaplan, 1995). This cytoplasmic domain contains several sequences of amino acids that are highly conserved among the different ATPases, including the sequence Asp-Lys-Thr-Gly-Ser/Thr, which contains an invariant aspartate residue that is phosphorylated by ATP as part of the catalytic mechanism of these proteins. Transfer of the γ-phosphoryl group from ATP to the proteins is an essential step in the catalysis of ion transport by all P-type ion pumps.

Other amino acids that are important for ATP binding or hydrolysis have also been identified within the central cytoplasmic domain of the catalytic subunit of P-type ATPases. In Na,K-ATPase, inactivation of enzymatic activity occurs by modification of Lys^{480} (Hinz and Kirley, 1990; Xu, 1989; Tran et al., 1994b), Lys^{501} (Ellis-Davies and Kaplan, 1993; Pedemonte and Kaplan, 1990; Hinz and Kirley, 1990; Farley et al., 1984; Kirley et al., 1984), Gly^{502} (Tran et al., 1994a), Asp^{710} and Asp^{714} (Dzhandzhugazyan et al., 1988), and Lys^{719} (Ohta et al., 1986) and is prevented by the presence of ATP or ADP during the reaction. Site-directed mutagenesis of several of these amino acids reduces the rate of ATP hydrolysis or the affinity of Na,K-ATPase for ATP (Lane et al., 1993; Wang and Farley, 1992; Ohtsubo et al., 1990).

Although the structure of the ATP molecule within the nucleotide-binding site of Na,K-ATPase has been determined by nuclear magnetic resonance (Stewart and Grisham, 1988; Stewart et al., 1989; Klevikis and Grisham, 1982), little is known about the structure of the protein. Secondary structure prediction algorithms have been used to suggest that the large cytoplasmic loop of P-type ion pumps contains a structure of alternating β-strands and α-helices that may be similar to the nucleotide-binding domain of adenylate kinase (Taylor and Green, 1989). Determination of the structure of P-type ATPases by crystal structure analysis or high-resolution magnetic resonance methods has been hampered by the difficulties associated with application of these techniques to integral membrane proteins. These techniques might be used to determine the structure of the nucleotide-binding domain of P-type ATPases, however, if these domains could be obtained in soluble form.

High-affinity nucleotide binding to isolated domains of adenylate kinase (Hamada et al., 1979), the 70-kDa bovine heat-shock cognate protein (Flaherty et al., 1990; DeLuca-Flaherty et al., 1988), the Rec A protein of *Escherichia coli*

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Address reprint requests to Dr. Robert A. Farley, Department of Physiology and Biophysics, USC School of Medicine, 1333 San Pablo St., MMR250, Los Angeles, CA 90033. Tel.: 323-442-1241. Fax: 323-442-2283; E-mail: rfarley@hsc.usc.edu.

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(Knight and McEntee, 1986), and elongation factor Tu (Parmeggiani et al., 1987) has been described, and crystals have been obtained from some of these preparations. Among the P-type ATPases, the large cytoplasmic domains of the sarcoplasmic reticulum Ca^{2+}-ATPase (Moutin et al., 1994) and the yeast H^+-ATPase (Capieaux et al., 1993) catalytic subunits have been expressed in bacterial cells as fusion proteins. The fusion proteins enhance the fluorescence intensity of TNP-ATP, and this enhancement is reduced in the presence of ATP, suggesting that these domains can still bind nucleotides. Several preliminary reports of the expression of the ATP binding domain of Na,K-ATP as a fusion protein have been published (Wang et al., 1991; Farley et al., 1993; Tran et al., 1996), and it was recently reported that the large cytoplasmic domain of Na,K-ATPase had been expressed in bacterial cells with a histidine tag (Gatto et al., 1998). Because determination of high-resolution structures is more easily accomplished with smaller proteins than with larger proteins, we attempted to identify the smallest fragment of P-type ion pumps that retains nucleotide binding capacity. Several fusion proteins beginning at the amino-terminal end of the large cytoplasmic domain of Na,K-ATPase and ending at different amino acids within the domain were prepared. The interaction of these fusion proteins with nucleotides was examined quantitatively, using [α-^{32}P]-2-N_3-ATP labeling. The results of these experiments indicate that the expressed cytoplasmic domain of Na,K-ATPase is folded in a manner similar to that of the conformation of this region in the native enzyme, and that nucleotide binding is retained in a fragment containing as few as ~250 amino acids.

EXPERIMENTAL PROCEDURES

Materials

Cloning enzymes and restriction enzymes were purchased from Boehringer Mannheim, Pharmacia, or New England Biolabs. Sequenase was from United States Biochemical Corp. Reagent chemicals were obtained from Sigma or Fisher, unless indicated otherwise, and were of the highest grade available.

Construction of expression plasmids

Plasmid pNKAα containing a full-length cDNA for sheep kidney Na,K-ATPase α-subunit in the *Pst*I site of pBR322 (Shull et al., 1985) was obtained from J. Lingrel (University of Cincinnati). The coding region for the α-subunit was subcloned into the plasmid pKS$^-$ (Stratagene). A synthetic oligonucleotide (3′) GTAAGGACTCATCCCCGGGAAGGACT (5′) was used to introduce a stop codon and an *Apa*I site into the cDNA sequence immediately after the codon for Glu^{779}. The presence of the mutations was confirmed by *Apa*I digestion and DNA sequencing. The plasmid was digested with *Sal*I and *Xmn*I, and the resulting 1.9-kb fragment was purified by agarose gel electrophoresis. Subsequent digestion with *Bal*I yielded a 1.4-kb fragment that was ligated to *Eco*RI linkers. The fragment was then ligated to pGEX-3X (Pharmacia) that had been digested with *Eco*RI and treated with calf intestinal alkaline phosphatase to generate the expression plasmid pGEX-DP779f. The fusion protein encoded by pGEX-DP779f contains a Factor Xa cleavage site between glutathione-*S*-transferase and DP779. Cleavage at this site results in a DP779 polypeptide that begins at Ala^{345} in the sheep kidney α-subunit. The sequence from Na,K-ATPase in the fusion protein, including the junction region, was confirmed by dideoxy-DNA sequencing.

To generate expression plasmids for other fusion proteins, stop codons were inserted into pGEX-DP779f. pGEX-DP779f was digested with *Eco*RI, and a 1.3-kb fragment was ligated into *Eco*RI-cut pKS$^+$. Oligonucleotide primers were used to introduce a stop codon after amino acid 731 of the Na,K-ATPase sequence, and the mutant plasmid was then cut with *Eco*RI and ligated to *Eco*RI-cut pGEX-3X to make plasmid pGEX-DP731f. For all other expression plasmids, a two-step polymerase chain reaction procedure (Horton et al., 1989) was used to insert stop codons after amino acids 518, 600, and 610 in pGEX-DP731f. All mutations were confirmed by dideoxy-DNA sequencing, and only plasmids in which no unexpected mutations were detected were used for domain protein expression.

Expression and isolation of domain fusion proteins

DH5α cells (Life Technologies) [F$^-$ φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-,m_K^+)*sup*E44λ- *thi*-1 *gyr*A *rel*A1] were transformed with the appropriate pGEX-domain protein plasmid and were grown overnight at 30°C in LB medium containing 50 μg/ml of ampicillin and 0.002% thiamin. The culture was diluted 10-fold into LB medium with ampicillin and thiamin previously equilibrated at 30°C, and isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM when the OD_{600} of the culture was between 0.2 and 0.4. The culture was incubated at 30°C for 4–6 h, the cells were collected by centrifugation and washed with cold distilled water, and the cell pellets were stored at −80°C. Cell pellets were suspended in phosphate-buffered saline (20 mM sodium phosphate, 150 mM NaCl, pH 7.1) and were lysed on ice, using 20 mg lysozyme/g cells. Phenylmethylsulfonyl fluoride was added to 0.1 mM, benzamidine was added to 0.2 mM, and the cells were broken by sonication. The cell lysate was spun at 15,000 × *g* for 25 min at 4°C, and the pellet was discarded. The lysate supernatant was applied to a column of glutathione-Sepharose (Pharmacia) that had been equilibrated at 4°C with 25 mM imidazole/HCl (pH 7.4), 100 mM NaCl. The column was washed with the same buffer, and the fusion protein was eluted using 10 mM glutathione in the same buffer. The protein was stored in 25 mM imidazole/HCl (pH 7.4), 100 mM NaCl, 0.3M sucrose, 20% (w/v) glycerol, 1 mM dithiothreitol.

Synthesis of [α-^{32}P]-2-N_3-ATP

[α-^{32}P]-2-N_3-ATP was synthesized as previously described (Tran et al., 1994a).

Labeling of Na,K-ATPase domain proteins with [α-^{32}P]-2-N_3-ATP

Thirty micrograms of fusion proteins were incubated (0.5 mg/ml) in 50 mM Tris/HCl (pH 7.4), 50 mM NaCl, 10% (w/v) glycerol, and the different concentrations of [α-^{32}P]-2-N_3-ATP. Photolysis (λ = 310 nm) was done for 5 min in a six-well Coors spotting plate at an incident power of 310 μW/cm^2. Samples were transferred to microcentrifuge tubes, the sample wells were rinsed, and the rinse was added to the microcentrifuge tube. A 10-fold excess of acetone was added, and the samples were frozen in liquid nitrogen and kept at −20°C overnight. After centrifugation at 14,000 × *g* for 30 min, the supernatant was removed and the sample was dissolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer for electrophoresis. After electrophoresis, the proteins were transferred to Immobilon membranes, and radiolabeled bands were identified by autoradiography. Radiolabeled bands were excised from the Immobilon membranes, and the amount of radioactivity in each band was quantified by scintillation counting.

Hydrolysis of *p*-nitrophenylphosphate

One hundred to two hundred micrograms of fusion protein were incubated in 20 mM HEPES/triethylamine (pH 7.4), 0.2 mM EDTA at room temperature for 15–48 h. When present, $MgCl_2$ was 3 mM, KCl was 25 mM, and NaCl was 100 mM. Ionic strength was maintained with choline chloride. Plastic reaction tubes were found to minimize variation from sample to sample and to reduce the nonenzymatic hydrolysis of substrate. In addition, the reaction buffer was prepared fresh on the day of the experiment from stock solutions that had been stored at 4°C. Duplicate samples were incubated in the presence and absence of the fusion proteins, and reactions were stopped by the addition of 1 ml of 1 N NaOH, 0.1% Triton X-100. Insoluble debris was removed by centrifugation at 1500 × *g* for 10 min, and the absorbance of the supernatant at 410 nm was measured. An extinction coefficient for *p*-nitrophenol of 18.5/mM/cm was used to convert absorbance to moles of product.

RESULTS

Design of the domain proteins

DNA encoding the large cytoplasmic domain of sheep Na,K-ATPase α-subunit was joined to glutathione-*S*-transferase by an in-frame ligation of glutathione-*S*-transferase in the vector pGEX-3X to nucleotides encoding Ala^{345} of the Na,K-ATPase. Ala^{345} lies 24 amino acids upstream from Asp^{369}, which is phosphorylated by ATP during the normal functioning of the pump. To investigate the minimum Na,K-ATPase structure required for nucleotide binding, stop codons were inserted into the cDNA encoding the cytoplasmic domain of Na,K-ATPase to generate fusion proteins that terminate at amino acids Gly^{518} (DP518f), Arg^{600} (DP600f), Thr^{610} (DP610f), Gly^{731} (DP731f), and Glu^{779} (DP779f) (Fig. 1). DP518f terminates near Gly^{502}, which is labeled by 2-N_3-ATP (Tran et al., 1994a), and Lys^{501}, which is labeled by fluorescein isothiocyanate (Farley et al., 1984; Kirley et al., 1984) and includes amino acids labeled by 8-N_3-ATP (Tran et al., 1994b) and AP_2PL (Hinz and Kirley, 1990). The sizes of DP600f and DP610f approximate that of an unusually stable 25–30-kDa fragment generated by trypsin from 2-N_3-ATP-labeled Na,K-ATPase (Tran et al., 1994a). DP731f is similar in size to cytoplasmic domains of yeast H^+-ATPase (Capieaux et al., 1993) and sarcoplasmic reticulum Ca^{2+}-ATPase (Moutin et al., 1994) that have previously been expressed in bacterial cells as fusion proteins and have been shown to bind nucleotides. DP779 has previously been expressed by us in *E. coli* as an isolated domain (Wang et al., 1991) and terminates at Glu^{779}. Although Glu^{779} was originally suggested to be located at the junction between the cytoplasmic domain and the fifth transmembrane segment of the Na,K-ATPase α-subunit (Shull et al., 1985), this amino acid may be located within the hydrocarbon interior of the membrane (Arguello and Kaplan, 1994).

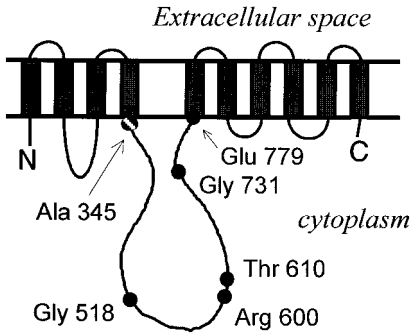

FIGURE 1 Na,K-ATPase α-subunit. The sheep kidney Na,K-ATPase α_1-subunit is shown in schematic form as a 10-transmembrane segment polypeptide. The dark circles indicate the positions along the polypeptide of the carboxy-terminal amino acids of the fusion proteins DP518f, DP600f, DP610f, and DP779f. Glu^{779} may be located within the fifth transmembrane sequence, as discussed in the text. The hatched circle shows the location of Ala^{345}, which is the first amino acid of the Na,K-ATPase polypeptide in the fusion proteins.

All of the fusion proteins were synthesized in *E. coli* after induction with isopropyl-β-D-thiogalactopyranoside, and all except DP518f were found in the soluble fraction after cell disruption. Fusion proteins were purified from the soluble cell lysate by affinity chromatography on glutathione-Sepharose. DP518f was purified after denaturation in urea and renaturation (Moutin et al., 1994). Fig. 2 (*left*) shows a Coomassie blue-stained polyacrylamide gel of the different fusion proteins after purification on glutathione-Sepharose. Protein analysis of the purified fusion proteins and the total protein of the cell lysate supernatant indicated that the fusion proteins constitute between 2% and 10% of the total soluble protein of the cells. The yield of purified fusion protein varies from one fusion protein to another and is usually 5–10 mg/liter cell culture. Occasionally an additional Coomassie-stained band was observed on the gels of purified fusion proteins at $M_r \approx 75$ kDa. This band is attributed to an *E. coli* protein that is not removed by the affinity column, because it did not react with anti-Na,K-ATPase antibodies. The Na,K-ATPase domain could be separated from the glutathione-*S*-transferase by digestion with Factor Xa or by trypsin (data not shown). In experiments in which the two preparations were compared, no significant differences were found in the concentration dependence of labeling of the fusion proteins and the isolated domains by 2-N_3-ATP or in the protection against labeling by different ligands. The results reported here were primarily obtained by using the fusion proteins.

Photochemical labeling of fusion proteins and domain proteins using [^{32}P]2-N_3-ATP

Because 2-N_3-ATP competes with ATP for high-affinity sites on Na,K-ATPase (Tran et al., 1994a), nucleotide binding to the expressed fusion proteins was determined by measuring the amount of [α-^{32}P]2-N_3-ATP covalently incorporated into the proteins after illumination with ultravi-

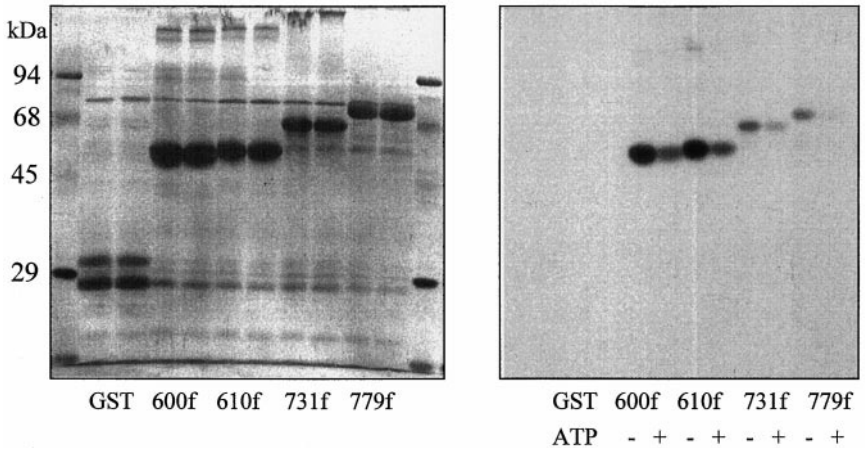

FIGURE 2 Photoaffinity labeling of fusion proteins with [α-^{32}P]2-N_3-ATP. (*Left*) Coomassie blue-stained SDS polyacrylamide gel showing bands corresponding to fusion proteins of glutathione-*S*-transferase and the large cytoplasmic domain of Na,K-ATPase truncated in the positions indicated in Fig. 1. Twenty micrograms of each [α-^{32}P]2-N_3-ATP-labeled fusion protein was applied to two lanes of the gel, as indicated. Molecular size standards are shown on the left and on the right side of the gel. (*Right*) Autoradiogram of the gel shown in the left panel of the figure. Samples were photolabeled by [α-^{32}P]2-N_3-ATP either in the absence (−) or in the presence (+) of 1 mM Na_2ATP. GST, Glutathione-*S*-transferase.

olet light. After labeling, the proteins were separated by SDS polyacrylamide gel electrophoresis and were transferred to Immobilon membranes. Radiolabeled bands were identified by autoradiography and were subsequently cut from the membrane for quantitation of ^{32}P incorporation by scintillation counting. Fig. 2 (*right*) shows that DP600f, DP610f, DP731f, and DP779f are labeled by 2-N_3-ATP, and that ATP prevents photolabeling of the fusion proteins. DP518f was not photolabeled and is not shown in the figure. Fig. 2 also shows that glutathione-*S*-transferase is not labeled by 2-N_3-ATP.

Fig. 3 shows the 2-N_3-ATP concentration dependence of labeling of the fusion protein DP610f in the 2-N_3-ATP concentration range 1–150 μM. The line through the data is the least-squares fit of a rectangular hyperbola to the data.

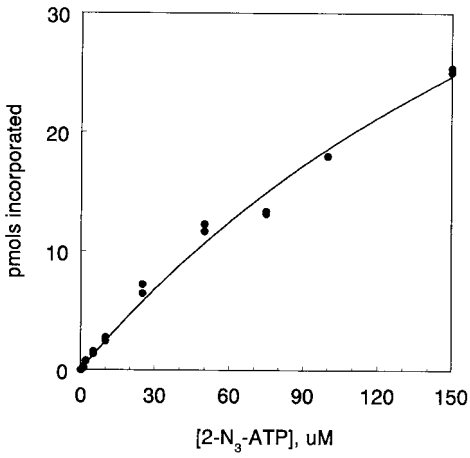

FIGURE 3 Photochemical incorporation of [α-^{32}P]2-N_3-ATP into DP610f. Thirty micrograms of DP610f was photochemically labeled with the concentrations of [α-^{32}P]2-N_3-ATP indicated on the abcissa, as described in Experimental Procedures. Individual data points from duplicate samples are shown. The amount of 2-N_3-ATP incorporated into the fusion protein was calculated after scintillation counting of the radiolabeled band excised from Immobilon membranes that had been prepared by electrophoretic transfer of proteins separated by SDS-polyacrylamide gel electrophoresis. The line is the least-squares fit of a rectangular hyperbola to the data.

The incorporation of 2-N_3-ATP into both of the fusion proteins and the isolated domains was also measured after trypsin digestion of the fusion proteins, because both the glutathione-*S*-transferase and the cleaved domains could be separately identified after SDS-PAGE. Only small differences in the 2-N_3-ATP concentration dependence of labeling were found between the fusion proteins and the separated domains, indicating that the affinity of the Na,K-ATPase cytoplasmic domain for 2-N_3-ATP is not affected by glutathione-*S*-transferase. Depending on the fusion protein, [α-^{32}P]-2-N_3-ATP labels between 8% and 31% of the total amount of fusion protein present in the reaction (1.1–5.4 nmol/mg; Table 1). The efficiency of photochemical labeling of proteins by azido nucleotides is usually quite low (Bayley and Staros, 1984), however, and to estimate the actual extent of binding of nucleotides by the fusion proteins, the incorporation of [α-^{32}P]-2-N_3-ATP into the fusion proteins was compared to the photolabeling of Na,K-ATPase purified from renal medulla. The efficiency of photochemical incorporation into the membrane-bound enzyme was calculated as moles of [α-^{32}P]-2-N_3-ATP incorporated into the protein per mole of ouabain bound, and in two experiments the efficiency of labeling of Na,K-ATPase by [α-^{32}P]2-N_3-ATP was 11% and 14%. If the photochemical reactions of 2-N_3-ATP subsequent to binding are the same in both the fusion proteins and the native Na,K-ATPase, this result indicates that close to 100% of the fusion proteins in each preparation are able to bind nucleotides.

Protection by nucleotides and ligands against 2-N_3ATP labeling

The amount of 2-N_3-ATP incorporated into the fusion proteins was reduced when the irradiation was done in the presence of Na_2ATP (0–20 mM), $MgCl_2$ (0–500 μM), MgATP (0–1 mM), *p*-nitrophenylphosphate (0–500 μM), and TNP-ATP (0–500 μM). As an example of this protection, Fig. 4 shows the extent of protection provided by 2 mM and 20 mM Na_2ATP against photolabeling of DP731f. Estimates of the K_d for 2-N_3-ATP binding (K_{2N3ATP}) and

TABLE 1 Photochemical labeling of fusion proteins by [α-^{32}P]2-N_3-ATP and protection by ligands of Na,K-ATPase

	B_{max} (nmol/mg)	K_d 2-N_3-ATP	K_i Na_2-ATP	K_i $MgCl_2$	K_i pNPP
DP600f	5.4 ± 0.3	203 ± 28 μM	11 ± 1 mM	710 ± 202 μM	377 ± 91 μM
DP610f	4.5 ± 0.6	291 ± 43 μM	7 ± 0.9 mM	165 ± 32 μM	22 ± 5 μM
DP731f	2.1 ± 0.5	257 ± 98 μM	2 ± 0.5 mM	ND	95 ± 28 μM
DP779f	1.1 ± 0.3	228 ± 23 μM	11 ± 1 mM	5 ± 5 mM	167 ± 28 μM
NKA	0.1 ± .04	19 ± 4 μM	0.4 ± .1 mM	ND*	46 ± 22 μM

Fusion proteins or native Na,K-ATPase (NKA) purified from dog renal medulla (*left column*) were labeled by 0–150 μM [α-^{32}P]2-N_3-ATP, in the absence or presence of different concentrations of the indicated ligands, as described in the text. Maximum incorporation of 2-N_3-ATP into each protein (B_{max}), the K_d for 2-N_3-ATP binding (K_{2N3ATP}), and the K_i for inhibition of labeling by each ligand were obtained from a global fit of the data as described in the text. Values are shown as mean ± SEM (ND, not determined).
*In the presence of $MgCl_2$, the incorporation of 2-N_3-ATP into membrane-bound Na,K-ATPase increased compared to the absence of $MgCl_2$.

the K_i for Na_2ATP and other ligands were obtained from a global fit of the data obtained at all concentrations of ligand and 2-N_3-ATP, with the equations

$$K_{app} = K_{2N3ATP}\left(1 + \frac{[\text{ligand}]}{K_i}\right)$$

and

$$\text{Bound} = B_{max}\left(\frac{[2N3ATP]}{K_{app} + [2N3ATP]}\right)$$

These results are shown for the fusion proteins and for membrane-bound renal Na,K-ATPase in Table 1. These results show that the effects of the ligands on the photolabeling of the fusion proteins and on the membrane-bound Na,K-ATPase are qualitatively similar, although the nucleotides appear to bind to the fusion proteins with a lower affinity than to the native enzyme. The apparent affinity of both the native Na,K-ATPase and the fusion proteins for *p*-nitrophenylphosphate is unexpectedly high, because the K_M for *p*-nitrophenylphosphate hydrolysis by native Na,K-ATPase is ~1 mM (Tran and Farley, 1996). During the normal catalytic cycle of Na,K-ATPase, the enzyme undergoes transitions between E_1 and E_2 conformations. Small acylphosphates such as *p*-nitrophenylphosphate and acetyl phosphate bind to Na,K-ATPase and are hydrolyzed in the E_2 conformation, which has a lower affinity for ATP than the E_1 conformation. A possible explanation for the high apparent affinity of the fusion proteins for *p*-nitrophenylphosphate shown in Table 1 is that the ATP binding domains exist in an E_2-like conformation in the presence of either Na^+ or K^+.

An increase in the intensity of TNP-ATP fluorescence has been used as an indicator of binding of the fluorescent nucleotide to membrane-bound ion pumps (Moczydlowski and Fortes, 1981; Watanabe and Inesi, 1982; Faller, 1989) and to expressed ATP binding domains (Capieaux et al., 1993; Moutin et al., 1994; Gatto et al., 1998) of Ca^{2+}-ATPase, H^+-ATPase, and Na,K-ATPase. The fluorescence of TNP-ATP also increased when it was incubated with the fusion proteins described in this report, and this increase in fluorescence intensity did not occur when Na_2ATP was present (data not shown). TNP-ATP binds to the P-type ATPases with a higher affinity than does ATP (Moczydlowski and Fortes, 1981), and the IC_{50} value for TNP-ATP protection against photolabeling of DP731f by 2-N_3-ATP is 10–15 μM (data not shown).

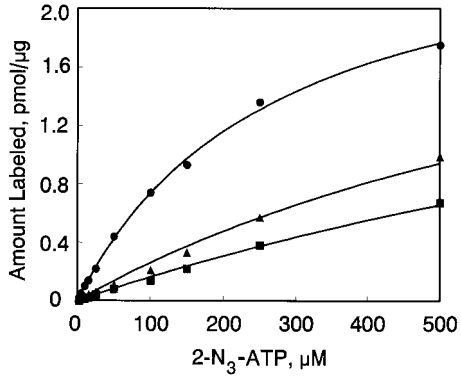

FIGURE 4 ATP prevents labeling of DP731f by [α-^{32}P]2-N_3-ATP. Thirty micrograms of DP731f was labeled as described in Experimental Procedures, with the concentrations of [α-^{32}P]2-N_3-ATP indicated on the abcissa (●). In parallel samples, Na_2ATP was included at 2 mM (▲) or 20 mM (■). Additional samples were also labeled in the presence of 5, 10, or 15 mM Na_2ATP (data not shown). The lines are the least-squares fit of a rectangular hyperbola to the data.

Hydrolysis of *p*-nitrophenylphosphate

The fusion proteins were incubated with *p*-nitrophenylphosphate to test whether the expressed domains were capable of catalyzing the hydrolysis of this pseudosubstrate of Na,K-ATPase. Fig. 5 shows the amount of *p*-nitrophenol obtained from hydrolysis of *p*-nitrophenylphosphate by each fusion protein in a 24-h reaction. Enzymatic hydrolysis was distinguished from nonenzymatic hydrolysis by comparing the formation of *p*-nitrophenol in the presence and absence of protein; the nonenzymatic hydrolysis of substrate is also shown in the figure. For membrane-bound Na,K-ATPase the *p*-nitrophenylphosphatase reaction requires both Mg^{2+} and K^+, and when Mg^{2+} was not included in the reaction with the fusion proteins, the hydrolysis of *p*-nitrophenylphosphate was the same as the hydrolysis of substrate in the absence of protein (data not shown). Hydrolysis of

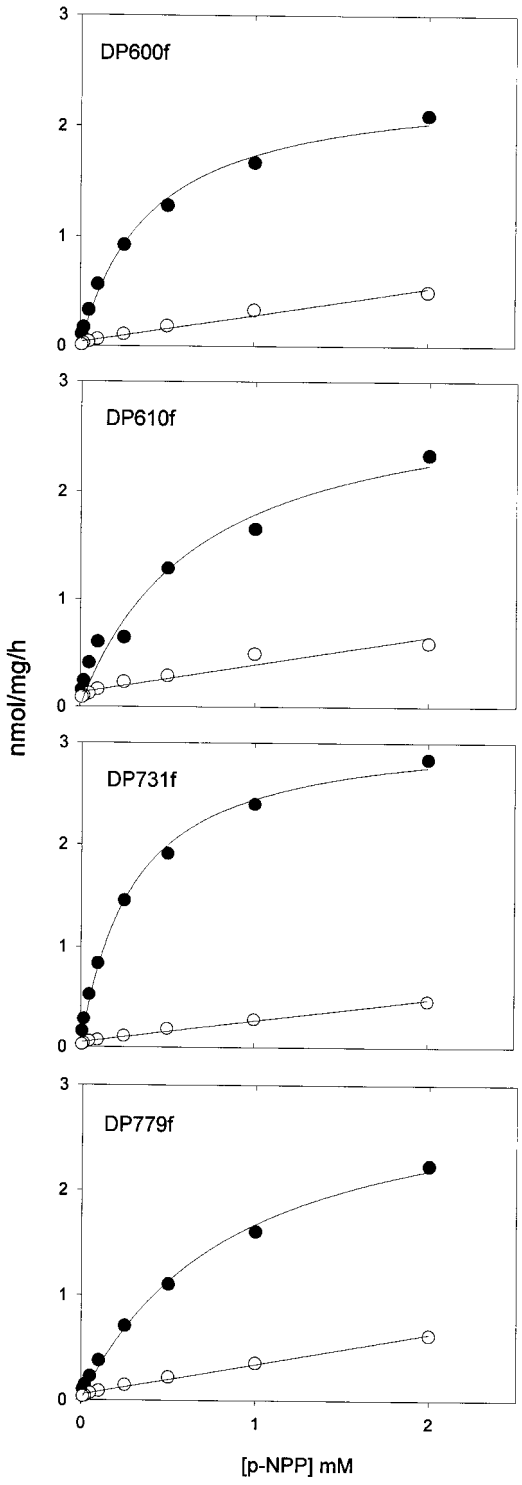

FIGURE 5 Hydrolysis of *p*-nitrophenylphosphate. One hundred to two hundred micrograms of each fusion protein was incubated in 25 mM HEPES/triethylamine (pH 7.4), 3 mM $MgCl_2$, 25 mM KCl, 0.2 mM Na_2EDTA, at the indicated concentrations of *p*-nitrophenylphosphate (pNPP) at 30°C for 24 h (●). Parallel reactions were incubated under identical conditions in the absence of protein (○). The reactions were stopped by the addition of 1 N NaOH, 0.1% (w/v) Triton X-100, and the amount of *p*-nitrophenol released was determined by absorbance at 410 nm.

p-nitrophenylphosphate by the fusion proteins did occur in the absence of K^+; however, inclusion of either Na^+ or K^+ usually increased the rate of hydrolysis by about twofold (data not shown). When *p*-nitrophenylphosphate hydrolysis by the fusion proteins was measured in the presence of 10 mM inorganic phosphate, the rate of hydrolysis in the presence or absence of fusion protein was the same. No hydrolysis of ATP by the fusion proteins was detected.

DISCUSSION

In the experiments described here, fusion proteins containing amino acids from the large cytoplasmic domain of the sheep Na,K-ATPase α_1-subunit and glutathione-*S*-transferase were expressed in *E. coli*. Photochemical labeling with [α-^{32}P]-2-N_3-ATP and protection by substrates and ligands of the Na,K-ATPase were used to determine which fusion proteins bind nucleotides. Because glutathione-*S*-transferase does not bind nucleotides and is not labeled by 2-N_3-ATP, the results indicate that the minimum structural unit of the Na,K-ATPase needed to bind nucleotides extends from near Ala^{345} to some residue no further along the polypeptide than Arg^{600}, or ~150–250 amino acids. The Na,K-ATPase nucleotide binding domain could be separated from glutathione transferase by proteolytic digestion, and both the fusion proteins and the isolated domains were labeled by 2-N_3-ATP. This result is consistent with the recent report that the cytosolic domain of Na,K-ATPase expressed with six histidine residues at the amino terminus binds TNP-ATP (Gatto et al., 1998). Thus the folding of the cytosolic domain of Na,K-ATPase does not appear to be affected by the presence of the glutathione-*S*-transferase.

Several ligands prevent labeling of both the fusion proteins and the expressed domains, demonstrating that the expressed cytoplasmic domain of Na,K-ATPase retains binding sites for nucleotides, Mg^{2+}, and *p*-nitrophenylphosphate. Both the K_d for 2-N_3-ATP (200–300 μM) and the K_i for Na_2ATP protection against photolabeling of the fusion proteins (2–11 mM), however, are higher than the K_d for ATP binding (0.1–1 μM; Hegyvary and Post, 1971) and the K_i for protection against photolabeling (0.4 mM; Table 1) of native Na,K-ATPase. These results most likely indicate that the structure of the ATP-binding site in the expressed domains is not exactly the same as the structure of this site in the native protein. Nevertheless, the difference in affinities between the expressed domains and the membrane-bound protein for the nucleotides is only ~10-fold. This corresponds to a difference in the Gibbs free energy of 1.36 kcal/mol, or less than the binding energy of one hydrogen bond. Thus the folding of the expressed domains in the region of the ATP binding site is likely to be very similar to that of the membrane-bound Na,K-ATPase.

The high K_i value for Na_2ATP interactions with the fusion proteins may indicate that the cytosolic domain is folded in an E_2-like conformation, to which ATP binds with a much lower affinity than to the E_1 conformation. The K_i

for Na_2ATP in these photolabeling experiments is similar to the estimated affinity of Na,K-ATPase for ATP at a low-affinity site characteristic of the E_2 conformation (Lauger, 1992). A K_i of ~10 mM has also been reported for the inhibition of TNP-ATP binding to the heterologously expressed large cytoplasmic domain of yeast H^+-ATPase by ATP (Capieaux et al., 1993). Consistent with the folding of the ATP binding domains in an E_2-like conformation is the high apparent affinity of both the membrane-bound Na,K-ATPase and the fusion proteins for *p*-nitrophenylphosphate.

The hydrolysis of *p*-nitrophenylphosphate by the fusion proteins provides the best evidence that the topological folding of the expressed domains is similar to the folding of this region of the native enzyme. As shown in Table 2, however, the rate of hydrolysis that is attributed to the fusion proteins is $\sim 10^5$-fold lower than the hydrolysis of *p*-nitrophenylphosphate by dog renal Na,K-ATPase (4×10^{-11} mol/mg/min versus 3.6×10^{-6} mols/mg/min; Tran and Farley, 1996). Despite this low activity, analysis of the reaction demonstrates similarities between the fusion protein-catalyzed reaction and the reaction catalyzed by the native enzyme. If the catalytic rates calculated from the measurement of *p*-nitrophenol at 15–24 h are treated as initial rates, then the data in Fig. 5 can be fit with the Michaelis-Menten equation. Values for K_M and V_{max} obtained from this analysis are shown in Table 2. For all of the fusion proteins, the K_M value for *p*-nitrophenylphosphate hydrolysis is 0.3–0.4 mM, which is slightly lower than the K_M value for membrane-bound Na,K-ATPase of ~1 mM (Tran and Farley, 1996). Like the membrane-bound Na,K-ATPase, the hydrolysis of *p*-nitrophenylphosphate by the fusion proteins requires Mg^{2+}, and when Mg^{2+} is not included in the reaction, *p*-nitrophenylphosphate hydrolysis is indistinguishable from the hydrolysis of the substrate in the absence of protein. The hydrolysis of *p*-nitrophenylphosphate by the fusion proteins in the presence of Mg^{2+} is also reduced to the level observed in the absence of protein when 10 mM inorganic phosphate is included in the reaction (data not shown).

The hydrolysis of *p*-nitrophenylphosphate occurs at comparable rates in all of the fusion proteins. This result indicates that Pro^{668}, which is labeled by 4-azido-2-nitrophenylphosphate (Tran and Farley, 1996) and which is not present in either DP600f and DP610f, is unlikely to participate in the mechanism of hydrolysis of this pseudosubstrate. The labeling of this residue by the photoprobe is most likely the consequence of accessibility of the probe to the amino acid.

TABLE 2 Hydrolysis of *p*-nitrophenylphosphate by glutathione-*S*-transferase fusion proteins containing the nucleotide binding domain of Na,K-ATPase*

Fusion protein#	V_{max}§ (± SEM) (nmol/mg/h)	K_M (± SEM) (mM)
DP600f (7)	2.4 ± 0.5	0.39 ± 0.06
DP610f (5)	3.0 ± 0.3	0.33 ± 0.04
DP731f (7)	2.4 ± 0.2	0.32 ± 0.08
DP779f (6)	1.9 ± 1.1	0.31 ± 0.11

**p*-Nitrophenylphosphate was hydrolyzed in 20 mM HEPES/triethylamine (pH 7.4), 3 mM $MgCl_2$, 25 mM KCl, 0.2 mM EDTA (free acid) at room temperature for 15–24 h, in the absence and the presence of 10 μM sodium orthovanadate.

#The number of experiments is shown in parentheses.

§Obtained from fit to data with the Michaelis-Menten equation after subtraction of *p*-nitrophenylphosphate hydrolysis in the absence of protein.

The absence of detectable ATP hydrolysis by fusion proteins containing the cytoplasmic domains of Na,K-ATPase, Ca^{2+}-ATPase (Moutin et al., 1994), or yeast H^+-ATPase (Capieaux et al., 1993) can also be understood if these expressed domains are folded in an E_2-like conformation, to which ATP binds with a low affinity. In the catalytic mechanism most commonly used to describe ATP-coupled ion transport by P-type ATPases (Lauger, 1992), hydrolysis of ATP to ADP and a phosphoenzyme intermediate occurs when the proteins are in the E_1 conformation. P_i is released from the phosphoprotein after an E_1P to E_2P conformational change, which does not appear to occur in the domains expressed in these experiments. The hydrolysis of *p*-nitrophenylphosphate by Na,K-ATPase, however, is thought to mimic the Mg^{2+}- and K^+-dependent hydrolysis of the E_2P phosphoenzyme. Although Mg^{2+} binds to most of the expressed domains with high affinity, both Na^+ and K^+ have only a small additional effect on the Mg^{2+}-dependent hydrolysis of *p*-nitrophenylphosphate by the fusion proteins (data not shown). Because the effects of Na^+ and K^+ are small, it is difficult to conclude whether binding sites for these monovalent cations exist on the cytoplasmic domain. Although ion binding sites are usually thought to exist within the membrane-embedded regions of the protein (Vasilets and Schwarz, 1993; Lingrel and Kuntzweiler, 1994; Andersen and Vilsen, 1995; Kuntzweiler et al., 1996), magnetic resonance data have shown that a monovalent cation binding site is located within 4 Å of a divalent cation site that is essential for ATP hydrolysis (Grisham et al., 1974). The requirement for Mg^{2+} in the hydrolysis of *p*-nitrophenylphosphate by the fusion proteins demonstrates that at least one divalent cation site is present in the expressed domains.

Because the fusion proteins and the isolated domains are soluble in aqueous buffers, they are potential candidates for structural analysis by magnetic resonance or x-ray diffraction methods. An important criterion for the application of these techniques, however, is that the population of expressed molecules be homogeneous. Photolabeling of the fusion proteins was used as an indicator of nucleotide binding to the expressed domains in these experiments, to enable us to quantify the extent of nucleotide binding by the domains. This information is very difficult to extract from fluorescence intensity measurements. We have previously shown that a preparation of DP779f binds ATP (Wang et al., 1991), TNP-ATP, and Cr-ATP (Farley et al., 1993), but we found that only 20–25% of the total number of expressed domains were binding ATP or CrATP in these preparations. By using the conditions for cell growth, cell disruption, and protein isolation described in this report, close to 100% of

the expressed domains appear to bind nucleotides. This conclusion, derived from photolabeling, has also been confirmed by quantifying the extent of binding of CrATP to the fusion proteins, by nuclear magnetic resonance (C. M. Grisham, Unversity of Virginia, personal communication). The results presented in this report, therefore, provide support for efforts to obtain high-resolution structural information about the ATP binding domain of Na,K-ATPase, using expression of the isolated domain in bacterial cells.

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