The M4M5 Cytoplasmic Loop of the Na,K-ATPase, Overexpressed in Escherichia coli, Binds Nucleoside Triphosphates with the Same Selectivity as the Intact Native Protein*

Craig Gatto, April X. Wang, and Jack H. Kaplan‡

From the Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, Oregon 97201-3098

Escherichia coli was used to overexpress the large cytoplasmic loop of the rat Na,K-ATPase. A 1260-base DNA segment encoding Lys354-Lys774 of the rat α1-subunit was constructed via polymerase chain reaction. The polymerase chain reaction product was successfully subcloned into the expression vector pET-28 (Novagen), which produces an N-terminal 6-histidine-tagged fusion protein. The pET-28 vector containing rat α-loop, i.e. pAN, was used to transform calcium-competent E. coli BL21(DE3) cells, and positive clones were selected by kanamycin resistance. Bacterial cultures were grown, and protein synthesis was induced with isopropyl β-D-thiogalactoside. Cells were harvested and lysed, revealing production of the His-tagged fusion protein (~46 kDa). The fusion protein was affinity-purified from other soluble cellular proteins via a Ni-NTA column, which routinely yielded ~20 mg of soluble His6-α-loop/L cell culture. The His6-α-loop retained significant native structure, as evidenced by the ability of ATP and ADP (but not AMP, CTP, GTP, or UTP) to protect against chemical modification by either fluorescein isothiocyanate or maleimidylalaninonaphthalene sulfonic acid. More specifically, circular dichroism spectroscopy was used to estimate the secondary structure of the His6 loop, revealing an ordered folding composed of 23% α-helix, 23% antiparallel β-sheet, 4% parallel β-sheet, 19% β-turn, and 32% random coil. The 6-histidine loop bound the fluorescent ATP analog trinitrophenyl-ATP with high affinity, as determined by measuring the fluorescence changes associated with binding. Affinities for ATP (~350 μM) and ADP (~550 μM) were determined by their ability to compete with and displace 2',3'-O-[2,4,6-trinitrophenyl]-ATP. These nucleotide affinities are similar to those observed for the E2 conformation of the intact Na,K-ATPase.

The Na,K-ATPase (3.6.1.37) is an integral membrane protein that is responsible for maintaining ion homeostasis in animal cells by mediating the active translocation of sodium and potassium ions against their electrochemical gradients across the plasma membrane. The ion gradients produced by the sodium pump are important for cell excitability and contractility, as well as for regulation of other intracellular ion and solute concentrations. The current model for the sodium pump cycle is conserved for other ion pumps, e.g. the plasma membrane and sarcoplasmic reticulum calcium pumps, the gastric hydrogen/potassium pump, and the Neurospora proton pump. These ion pumps make up the enzyme class known as the P-type ATPases. Although much information has been gathered about the kinetic mechanism of the sodium pump, there is still rather little detail known about the structure of the protein.

The sodium pump exists as a functional heterodimeric protein consisting of a catalytic α-subunit (~110 kDa) and a smaller, glycosylated β-subunit (~55 kDa). Both subunits have been cloned, and the primary structure has been determined in several isoforms from a variety of species (1). However, even after extensive study for several decades, information about the specific amino acids involved in formation of the binding sites for physiological ligands is rudimentary. Although the detailed topology of the α-subunit of the sodium pump is still the subject of investigation, an overall consensus on structure is emerging; it shows 10 transmembrane segments (3). All the residues implicated with ATP binding thus far have been localized to the major cytoplasmic loop, which is composed of about 430 amino acid residues between transmembrane segments M4 and M5 (2). Moreover, this loop contains four of the most highly conserved P-type ATPase sequences (3). Much of the information suggesting the involvement of various amino acid residues with pump function comes from chemical modification experiments. Such studies have identified a number of residues in the M4M5 loop thought to be involved in ATP binding. For example, Lys480, Lys501, Gly502, Asp710, Asp714 and Lys719 are all modified by a variety of chemical agents in the absence of ATP but not in its presence, suggesting a role in ATP binding (4). In subsequent studies, some of these residues were changed via mutagenesis with little or no effect on enzyme activity (1). However, measurements of overall enzyme activity only provide information on whether the mutated residue is absolutely essential for function, but they do not necessarily address whether the residue is involved directly in ATP binding. For example, mutation of Asp714 (i.e. the site of phosphorylation), not surprisingly, abolishes enzyme activity (5), but interestingly, mutation of this residue increases the ATP binding affinity (6). Consequently, to determine whether a residue is important for ATP binding one must directly measure ATP binding and characterize its affinity. The only approach that will identify ATP contact residues is to crystallize the protein in the presence of substrate. Unfortunately, crystallographic analysis of integral membrane proteins still remains difficult because generally applicable strategies for obtaining x-ray quality crystals of these molecules do not yet exist.

As an approach to achieving this aim, we describe the bac-
tential production and purification of a soluble polypeptide corresponding to the large cytoplasmic loop of the Na,K-ATPase. This soluble protein is able to bind ATP, as evidenced by the ability of ATP to prevent modification by both fluorescein 5'-isothiocyanate (FITC) and 2-(4'-maleimidylalanino)napthalene-6-sulfonic acid (MIANS), two fluorescent probes previously demonstrated to label the M4M5 loop of the full-length Na,K-ATPase in an ATP-protectable fashion. In addition, we used 2',3'-O-[2,4,6-trinitrophenyl]adenosine 5'-triphosphate (TNP-ATP) fluorescence to estimate, by competition with ATP, the ATP binding affinity to this segment of the sodium pump. A preliminary report of some of this work was presented at the Eighth International Meeting on the sodium pump (7).

**EXPERIMENTAL PROCEDURES**

Reagents, Media, and Bacterial Strains—NaCl, ATP, ADP, AMP, CTP, GTP, UTP, ethanol, ethidium bromide, bovine serum albumin, ultrapure urea, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and Tris base were purchased from Sigma. β-mercaptoethanol, SDS, ammonium persulfate, Coomassie Brilliant Blue R-250, DNA miniprep kit, and low molecular weight standards were from Bio-Rad. 4-(2-Aminothyl)-benzenesulfonil fluoride hydrochloride was from ICN. Acrylamide and bisacrylamide were from Sepragen. N-[2-(aminoethyl)-benzenesulfonyl fluoride hydrochloride was from Boehringer Mannheim. Rainbow gel electrophoresis standards were from Amersham Pharmacia Biotech. TNP-ATP, FITC, and MIANS were from Molecular Probes. Polyvinylidene difluoride electroblotting membrane was from Millipore. Tryp- tose, granulated agar, and yeast extract were from Difco. Agarose and restriction endonucleases were from Life Technologies, Inc. The pCR-Script cloning kit and Pfu DNA polymerase were from Stratagene. The pET-28 expression vector and Escherichia coli BL21(DE3) cells were from Novagen. Ampicillin and kanamycin were obtained from the University Hospital Pharmacy (Oregon Health Sciences University). E. coli DH5α cells were a generous gift from Dr. Linda Kenney (Molecular and Physical Biology, Oregon Health Sciences University). DNA miniprep kits, DNA gel extraction kits, IPTG, and Hisα antibody were from Qiagen. DNA sequencing was performed at the core facility at Oregon Health Sciences University.

**Construction of the Protein Expression Vector pAN—** The portion of the rat α1-subunit encoding the M4M5 cytoplasmic loop (Ly95-A94-Ly77+) was amplified via polymerase chain reaction (PCR) in the presence of 10 μM oligonucleotide primers (shown below), 1.2 μM of the four deoxyxynucleoside triphosphates, and 5 units of Pfu DNA polymerase in 50 μl of the manufacturer’s buffer. The template was pGEM-rc1α1a, a generous gift from Dr. Robert Mercer (Washington University, St. Louis, MO). Twenty-five PCR cycles (30 s at 94 °C, 1 min at 53 °C, and 2 min at 72 °C) were performed in a PTC-100 thermal cycler (MJ Research Inc.). Agarose gel electrophoresis of the PCR revealed a single DNA fragment of 1.2 kilobases.

**Column Chromatography—** The forward primer was as follows.

5'-GC ATG CAT ATG AAG AAC TGC CTG G-3'  

NdeI  

rat α1 loop

The reverse primer was as follows.

5'-GG GTG TAA GAA TTC TAT TTC TTC AAG TTA TC-3'  

EcoRI Stop  

rat α1 loop

After gel purification of the PCR product, a blunt end ligation was performed using the SfiI-digested pCR-Script plasmid according to the manufacturer’s protocol (Stratagene) (Fig. 1). (PCR-Script confers ampicillin resistance and contains a portion of the lacZ gene encoding the β-galactosidase gene product providing blue-white color selection of recombinant plasmids.) Calcium competent (8) E. coli DH5α cells were transformed with the ligation mixture, and positive colonies were selected on LBamp agar plates containing 50 μg/ml ampicillin and grown overnight in LBamp, and the DNA was isolated via minipreparation techniques (Bio-Rad and Qiagen DNA miniprep kits). Restriction endonuclease mapping revealed positive clones, which were subsequently sequenced to verify that no random mutations occurred during the PCR. The desired M4M5 loop was re-amplified from pGEM-rc1α1 using NdeI and EcoRI; these unique restriction sites were engineered into the oligonucleotide primers (see above). The 6-histidine fusion protein vector, pET-28 (Novagen), was also digested with EcoRI and NdeI. A ligation reaction was performed after gel purifying both DNA fragments at a ratio of 10:1 (insert to vector) using T4 DNA ligase (1 unit/10 ng of fragment). The cloned product, called pAN, was subsequently transformed into competent DH5α cells and selected by the kanamycin resistance conferred by pET-28. DH5α cells have a higher efficiency for transformation with ligation reactions than do BL21 cells. Therefore, we used DH5α cells for initial transformations and long term storage of vectors as glycerol stocks, whereas we preferred BL21(DE3) cells for protein expression.

**Overexpression of a 6-Histidine-tagged M4M5 Loop—** The constructed pAN expression vector was used to transform competent BL21(DE3) cells. The E. coli transformants were selected on LBamp (30 μg/ml) agar plates. A single colony was picked to grow overnight in 5 ml of LBamp, and this culture was subsequently used to inoculate 1 liter of LBamp containing a final concentration of 2% ethanol. After the culture grew to an OD600 of 0.8–1.0, 1 ml IPTG was added to induce the synthesis of protein from the lac promoter and grown further at 25 °C to an OD of ~2.0.

Cells were then collected and suspended in 30 ml of a lysis buffer containing 50 mM Tris, 100 mM NaCl, pH 8.0, with a hand-held homogenizer. A 10-μg quantity of lysozyme was added in the presence of 150 μM 4-(2-aminoethyl)benzenesulfonil fluoride hydrochloride (serine protease inhibitor), and the mixture was incubated on ice for 30 min, with occasional plunging of the homogenizer. After addition of 40 μg of deoxycholic acid, the suspension was heated to 37 °C with constant stirring. Once the suspension became viscous and difficult to stir, 200 μl of deoxyribonuclease (1 mg/ml) was added, and the mixture was incubated at 25 °C until the suspension was no longer viscous (~30 min). A final concentration of 1% Triton X-100 was added, and the cell lysate was incubated for an additional 30 min at 25 °C. The soluble fraction was separated from the membranous fractions by centrifugation at 12,000 × g for 45 min at 4 °C. Expression of the Hisα loop was verified by running a sample of the soluble cell lysate on a 10% Laemmli gel and electroblotting onto polyvinylidene difluoride membrane (10 μm CAPS, pH 11.0, 4.5 V). The polyvinylidene difluoride membrane was screened with a 1:1000 dilution of a 6-histidine antibody (Qiagen) revealing a protein (~46 kDa) from the lysate of IPTG-induced cells only.

**Purification of Hisα Loop via Nf2-Column Chromatography—** A 4-ml slurry of His-Bind resin (nitriloacetic acid-agarose, Novagen) was placed into a 5-ml column; after the resin settled, a bed volume of 2 ml remained. The column was washed with 10 bed volumes of sterile deionized water and then charged with nick by adding 5 bed volumes of 50 mM NiSO4. Unbound Nf2+ was washed away with 5 bed volumes of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). One-third of the soluble cell lysate (~10 ml) was applied to the Nf2+-column. The column was subsequently washed with 5 bed volumes of binding buffer to remove nonspecifically bound E. coli proteins. The desired Hisα loop protein was eluted from the Ni2+-column with a linear imidazole gradient from 50 to 400 mM in a buffer containing 500 mM NaCl and 20 mM Tris (pH 7.9). Twenty-five 1.5-ml fractions were collected (0.5–0.75 ml/min flow rate, gravity flow) over 45–60 min and analyzed for protein content by the method of Bradford (9). Protein-containing fractions were further analyzed via SDS-polyacrylamide gel electrophoresis (i.e. 10% Laemmli gel). The desired protein was routinely recovered over 5–6 fractions ranging from ~125 to 250 mM imidazole. The purity varied slightly between preparations; only preparations that were >95% pure (based upon densitometry of the Coomassie-stained peptide bands) were used for experimental measurements.

**FITC and MIANS Modification Experiments—** An aliquot (10–20 μg) of the purified dog kidney Na,K-ATPase or the purified rat liver Na,K-ATPase was incubated with 5 μM FITC or 50 μM MIANS at 4 °C for 20 min. The reaction was quenched with 1% SDS prior to the chemical modification reactions were stopped by the

1 The abbreviations used are: FITC, fluorescein 5'-isothiocyanate; TNP-ATP, 2',3'-O-[2,4,6-trinitrophenyl]adenosine 5'-triphosphate; MIANS, 2-(4'-maleimidylalanino)napthalene-6-sulfonic acid; IPTG, isopropyl β-D-thiogalactoside; MOPS, morpholinepropanesulfonic acid; LB, Luria broth; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Hisα loop, 6-histidine-tagged M4M5 cytoplasmic loop from the rat Na,K-ATPase α1-subunit; CAPS, 3-(cyclohexylamino)propanesulfonic acid; STPS, 4-acetamido-4'-isothiocyanostilbene-2',2'-disulfonic acid.}

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addition of 40 μl of Laemmli sample buffer containing (at a 1:1.1 ratio) 10% SDS, 8 M urea, and 0.1 M Tris (pH 8.8) and a 5% final concentration of β-mercaptoethanol. The entire 60-μl sample volume was loaded and run on a 12% SDS-polyacrylamide gel according to the method of Laemmli (10). FITC-labeled bands were visualized under UV illumination (366 nm), and then proteins were fixed and stained with 0.1% Coomassie Brilliant Blue R-250 in 10% acetic acid.

**Nucleotide Competition Assay**—TNP-ATP binding to the His6 loop was performed essentially as described by Moczydlowski and Fortes (18) for TNP-ATP binding to Na,K-ATPase with minor changes. Briefly, fluorescence changes were measured in quartz cuvettes on a model PTI-QM1 (Photon Technology International, Monmouth Junction, NJ) steady state fluorometer. The excitation wavelength was 410 nm (5 nm width), and the emission wavelength was 545 nm (2 nm width). Aliquots of a 1 mM TNP-ATP stock solution were titrated into a 1 ml solution of 50 mM MOPS (pH 7.5) containing 100 mM TNP-ATP and 10 mM His6 loop. The same TNP-ATP additions were made into buffer only. The difference between the protein-containing and protein-free solution revealed the fluorescence changes associated with the nucleotide-in-competition. Titrations were performed in the presence of either 5 mM ATP or 5 mM AMP, ADP, or AMP. Moreover, when the FITC incubation was performed in the presence of either 5 mM ATP or 5 mM AMP, only ATP (not AMP) prevented FITC labeling by FITC. In addition, when the FITC incubation was performed in the presence of 5 mM ATP or 5 mM AMP, the His6 loop was labeled by FITC. In summary, when the FITC incubation was performed in the presence of either 5 mM ATP or 5 mM AMP, only ATP prevented FITC labeling by FITC. In summary, when the FITC incubation was performed in the presence of either 5 mM ATP or 5 mM AMP, only ATP prevented FITC labeling by FITC. In summary, when the FITC incubation was performed in the presence of either 5 mM ATP or 5 mM AMP, only ATP prevented FITC labeling by FITC. In summary, when the FITC incubation was performed in the presence of either 5 mM ATP or 5 mM AMP, only ATP prevented FITC labeling by FITC.

**Circular Dichroism Measurements**—CD spectra were taken on a JASCO J-500 A spectrophotometer. Measurements were made using a 0.1-mm path length cell (Helma) at a constant temperature of 20 °C. Data were collected on an IBM/PC-XT using the IF-2 interface; software was provided by Jasco. Spectra and buffer baselines were the average of 10 scans, each recorded at 0.1 nm intervals, using a scanning rate of 5 nm/min and a 2-s time constant. The protein concentration was determined by amino acid analysis and was approximately 2.0 mg/ml. Before spectral deconvolution for secondary structure analysis, the buffer baseline was subtracted and the resulting spectrum was smoothed using the program provided by Jasco. The secondary structure of the His6 loop was computed using the singular-value and variable-selection methods of Compton et al. (11).

**RESULTS**

Our goal was to characterize the isolated ATP binding domain of the Na,K-ATPase. As a means to this end, we developed a method to produce large quantities of a soluble peptide corresponding to the cytoplasmic loop between transmembrane segments M4 and M5 (i.e. from Lys354 to Lys774; rat α1 subunit).

**Overexpression and Purification of the His6 α-Loop Fusion Protein**—The plasmid pAN was constructed by inserting the cDNA encoding the rat α1 M4M5 cytoplasmic loop into pET-28 multiple cloning site at the ndel (5') and the EcoRI (3') restriction site locations downstream from the histidine coding sequence (Fig. 1). pAN was used to transform a BL21(DE3) E. coli strain. For induction of gene expression, cells were grown in 1 liter of LBkan medium containing a final ethanol concentration of 2%, and fusion protein production was induced with IPTG. (The presence of ethanol significantly increased the amount of fusion protein in the soluble fraction.) This process routinely yielded significant production of the His6 loop fusion protein (molecular mass, ~46 kDa) with approximately 40% associated with the soluble fraction (Fig. 2). The His6 loop was purified via a Ni-NTA affinity column (~20–25 mg/liter; Fig. 2).

**FITC Labeling of the His6 Loop**—FITC is a fluorescent amine-reactive molecule that labels Lys901 in the purified Na,K-ATPase; this reaction is prevented by the simultaneous presence of ATP (12, 13). In this study, we used ATP protection against FITC labeling as a tool to demonstrate that ATP binds to His6 loop (Fig. 3A). It is clear that after incubation with 5 μM FITC, both the purified Na,K-ATPase and the His6 loop were labeled by FITC. Moreover, when the FITC incubation was performed in the presence of either 5 mM ATP or 5 mM AMP, only ATP (not AMP) prevented FITC modification of both the intact purified sodium pump and the His6 loop (Fig. 3A).

To further investigate the nucleotide specificity of the His6 loop, we tested the ability of several nucleotides to protect against FITC modification (Fig. 3B). Purified His6 loop was incubated with 5 μM FITC in the presence of 5 mM ATP, ADP, AMP, GTP, and UTP. It is clear that both ATP and ADP protected against FITC, whereas AMP, CTP, GTP, and UTP were unable to prevent FITC modification. These data are in agreement with the nucleotide specificity demonstrated for the purified Na,K-ATPase, which has been shown to bind ATP and ADP with high affinity (14). Therefore, it appears that the His6 loop retains the structural parameters that confer nucleotide specificity in the intact enzyme.

**Structural Analysis of the His6 Loop**—The structural integ-
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Grown overnight and used to inoculate 1L of LB kan. Protein synthesis was induced with 1 mM IPTG. Stds, molecular mass standards (kD); Uninduced, 100 µl of uninduced whole cell growth pelleted and resuspended in Laemmli sample buffer; Induced, 100 µl of induced whole cell growth pelleted and resuspended in Laemmli sample buffer; Pellet, aliquot of pelleted fraction after cell lysis; Supernatant, aliquot of soluble fraction after being applied to Ni²⁺-NTA column; Eluate, His₆ fusion protein eluted from the Ni²⁺-NTA column with imidazole.

The peptide (Fig. 4). Similar results were observed when we denatured purified dog Na,K-ATPase with 1% SDS (Fig. 4). These results are not due to an anomalous effect of SDS on the FITC reaction; FITC was also unable to modify the His₆ loop after denaturation with 6 M guanidine-HCl or by repeated freezing and thawing (data not shown).

More directly, the overall secondary structure of the His₆ loop fusion protein was estimated by CD spectroscopy. As shown in Fig. 5, the resultant spectrum, which summarizes the mean residue molar ellipticity as a function of wavelength, exhibits a maximum at 188 nm and two minima, one at 206 nm and the other at 221 nm. The spectrum is the average of 10 scans, the general shape of which did not change. Analysis of the CD spectrum of the His₆ loop, calculated using the singular-value decomposition method (11), predicts secondary structural elements distributed approximately as 23% α-helix, 23% antiparallel β-sheet, 4% parallel β-sheet, 19% β-turn, and 32% random coil. The total of the fractions in this method is not constrained to be 100%, but rather should lie between 95 and 105%. The observation that the components sum to close to 100% indicates that the fusion protein is highly structured as assessed by this technique.

MIANS Labeling of the His₆ Loop—Recently, the fluorescent sulfhydryl reagent MIANS was shown to inactivate the dog kidney Na,K-ATPase by specifically labeling a cysteine residue in the M4M5 loop of the Na,K-ATPase (15). Both MIANS labeling and enzyme inactivation were prevented by the simultaneous presence of ATP.² In this study, we tested whether ATP was able to protect against MIANS modification of the His₆ loop. Indeed, ATP, but not AMP prevented MIANS labeling of the bacterially produced His₆ loop (Fig. 6). However, unlike FITC, MIANS does label the denatured peptide, but ATP no longer protects against its modification (Fig. 6). The simplest explanation for a lack of ATP protection is that there is no longer significant structure to bind ATP.

TNP-ATP Binding to the His₆ Loop and Competition with Nucleotides—The interactions of the His₆ loop and TNP-ATP were studied. This fluorescent ATP analog has been shown to bind with high affinity to the native Na,K-ATPase, as well as to other members of the P-type ATPase family (17–19). TNP-ATP is a useful fluorescent probe for studying the nucleotide binding

² S. J. Thorneewell, J. P. Holden, and J. H. Kaplan, manuscript in preparation.
We have produced a soluble polypeptide by bacterial overexpression that is identical in sequence with the central loop of the rat a1-subunit. We have shown that the peptide binds ATP and ADP, but not other nucleoside triphosphates, with the same specificity as native Na,K-ATPase. This highly ordered peptide also shows labeling and protection reactions characteristic of the intact Na,K-ATPase.

Chemical modification experiments (4, 2) of the Na,K-ATPase, as well as site-directed mutagenesis studies (1), suggest that several amino acids located between the fourth and fifth putative transmembrane segments participate in the coordination of ATP. However, in some instances, the data generated from these two methods appear to be contradictory. For example, labeling Lys501 with a number of reagents (e.g., FITC, SITS, and N-(2-nitro-4-isothiocyanophenyl)-imidazole) completely inactivates the enzyme; the prior binding of ATP prevents both modification and inactivation, consistent with Lys501 playing a role in ATP binding. However, when Lys501 was changed to methionine via site-directed mutagenesis (21), the enzyme retained activity, demonstrating that Lys501 is not essential for enzyme activity. How could Lys501 participate in ATP binding and yet not be required for enzyme activity? It is possible that Lys501 is one of several residues that form the

nucleotides is evidenced by a decrease in fluorescence intensity upon the release of TNP-ATP from the His6 loop. A plot of [nucleotide] versus percentage of change in fluorescence from a typical experiment is shown in Fig. 9. The apparent $K_d$ values for ATP and ADP were $-350 \mu M$ and $-550 \mu M$, respectively (Table I). AMP showed no saturable binding in the concentration range examined. For each nucleotide, the binding experiments were performed on three separate preparations of His6 loop with comparable results.

**DISCUSSION**

We have produced a soluble polypeptide by bacterial overexpression that is identical in sequence with the central loop of the rat a1-subunit. We have shown that the peptide binds ATP and ADP, but not other nucleoside triphosphates, with the same specificity as native Na,K-ATPase. This highly ordered peptide also shows labeling and protection reactions characteristic of the intact Na,K-ATPase.

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ATP binding pocket. Loss of a single contact residue might result in a lower ATP affinity, but not in a loss of ATP binding. Therefore, in the presence of saturating ATP, Na,K-ATPase activity might remain normal. However, attaching a chemical reagent to a contact residue or a nearby residue not only removes that residue from coordination but also occupies space in the vicinity of that residue; thus, modification of Lys501 may prevent ATP binding via steric factors. Consequently, it is becoming readily apparent that a detailed three-dimensional structure is needed to conclusively identify the contact sites for ATP and to adequately describe its binding pocket.

To approach such experiments, the major cytoplasmic domain between M4 and M5 of the Na,K-ATPase was overexpressed in E. coli. Similar approaches have been employed by other laboratories to isolate the ATP binding domains of the yeast proton pump (22), the sarcoplasmic reticulum calcium pump (23), the sodium pump (24), and the cystic fibrosis transmembrane conductance regulator (25). The cDNA from the rat α1 subunit, encoding 420 amino acids from Lysα354–Lysα751, was cloned into a fusion protein vector (His6 tag, pET-28) and transformed into E. coli. This method routinely yields approximately 20 mg of pure soluble peptide per liter of cell culture. We demonstrated that this expressed and purified peptide 1) retains an ordered structure, 2) can be labeled by both FITC and MIANS, 3) binds both ATP and ADP, and 4) binds the fluorescent ATP analog, TNP-ATP. Experiments are currently under way to crystalize the His6 loop.

**Structural Analysis of the His6 Loop**—Detailed structural topology of the sodium pump awaits three-dimensional x-ray crystallographic analysis. However, some assessments have been made that suggest an α-subunit structure with 10 transmembrane segments. Also, all the residues associated with ATP binding have been localized to the major cytoplasmic loop between transmembrane segments M4 and M5 (2). Furthermore, the residues thus far implicated in nucleotide binding among all the members of the P-type ATPase family have been assigned to this cytosolic domain. Structural models of the nucleotide binding pocket of P-type ATPases have been proposed based upon sequence homology with different kinases (e.g. adenylyl kinase and phosphoglycerate kinase; see Ref. 26). According to these models, the large cytoplasmic loop is divided into three domains: a phosphorylation domain, a nucleotide binding domain, and a central domain. We calculated the α-helix and β-sheet content of the nucleotide binding domain portion (Arg263–Ile365) of the sheep Na,K-ATPase cytoplasmic loop from the data previously reported (26). According to their predictions using the method of Chou and Fasman (27), we obtained a composition of about 35% α-helix and 22.5% β-sheet. These values agree reasonably well with our current findings of 23% α-helix and 27% β-sheet for the secondary structure of the bacterially expressed M4M5 loop. The difference in α-helical content may suggest that the phosphorylation domain and the central domain (included in our analysis and not in that of Taylor and Green (26)) have significantly less helical structure than does the nucleotide binding domain. Alternatively, it may simply reflect the differences in secondary structure analysis (this study) and primary structure comparison (26) or a less than completely retained structure of our His6 loop compared with native intact Na,K-ATPase.

**Nucleotide Protection against Chemical Modification of the His6 Loop**—For almost two decades, it has been known that FITC irreversibly inhibits the Na,K-ATPase in an ATP-protective manner. In the presence of saturating ATP, Na,K-ATPase activity might remain normal. However, attaching a chemical reagent to a contact residue or a nearby residue not only removes that residue from coordination but also occupies space in the vicinity of that residue; thus, modification of Lysα501 may prevent ATP binding via steric factors. Consequently, it is becoming readily apparent that a detailed three-dimensional structure is needed to conclusively identify the contact sites for ATP and to adequately describe its binding pocket.

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To approach such experiments, the major cytoplasmic domain between M4 and M5 of the Na,K-ATPase was overexpressed in E. coli. Similar approaches have been employed by other laboratories to isolate the ATP binding domains of the yeast proton pump (22), the sarcoplasmic reticulum calcium pump (23), the sodium pump (24), and the cystic fibrosis transmembrane conductance regulator (25). The cDNA from the rat α1 subunit, encoding 420 amino acids from Lysα354–Lysα751, was cloned into a fusion protein vector (His6 tag, pET-28) and transformed into E. coli. This method routinely yields approximately 20 mg of pure soluble peptide per liter of cell culture. We demonstrated that this expressed and purified peptide 1) retains an ordered structure, 2) can be labeled by both FITC and MIANS, 3) binds both ATP and ADP, and 4) binds the fluorescent ATP analog, TNP-ATP. Experiments are currently under way to crystalize the His6 loop.
able manner (28). The site of FITC modification was identified as Lys\textsuperscript{501} (12, 13). Considerable evidence suggests that Lys \textsuperscript{501} resides in the ATP binding site of the sodium pump; this evidence includes the following: 1) ATP prevents modification of Lys\textsuperscript{501} (28), 2) the reactivity of Lys\textsuperscript{501} is sensitive to cation binding in a way similar to the effect that cation occupancy has on direct ATP binding (29–31; 15), and 3) an equivalent lysine residue exists in a conserved sequence among most members of the P-type II ATPase family (3).

In a fashion similar to the intact Na,K-ATPase, ATP and ADP (but not AMP) protect against FITC modification of our purified His\textsubscript{6} loop (Fig. 3). Moreover, nucleotides that do not bind with high affinity to the native enzyme also do not protect the His\textsubscript{6} loop from FITC modification (Fig. 3). These findings demonstrate that the His\textsubscript{6} loop has folded sufficiently to allow formation of a nucleotide binding pocket that shows selectivity. Indeed, denaturing the His\textsubscript{6} loop prior to FITC treatment results in no FITC labeling; the same finding was observed with the native Na,K-ATPase (Fig. 4). Thus, it appears that the selective reactivity of Lys\textsuperscript{501} toward FITC is a product of the special environment in the folded central loop. The appropriate folding of this ATP binding loop generates a highly reactive lysine at position 501. This is in contrast with the behavior of MIANS and Cys\textsuperscript{577} (see below).

We have observed that ATP, but not AMP, can also protect the loop against modification by the sulphydryl reagent MIANS (Fig. 6). Recently, MIANS has been shown to modify a specific cysteine residue in the large cytoplasmic loop of purified Na,K-ATPase; ATP protects against MIANS modification. Proteolytic digestion and N-terminal amino acid sequencing has identified the MIANS-modified residue as either Cys\textsuperscript{549} or Cys\textsuperscript{577}. ATP protection against modification at Lys\textsuperscript{501} and Cys\textsuperscript{577} (or Cys\textsuperscript{549}) suggests that a compact folding of the loop may bring the distant segments of the peptide together. It is interesting, however, that after denaturation of this loop, modification still occurs with MIANS, but such modification is no longer affected by the simultaneous presence of ATP.

**Nucleotide Binding Affinity**—The sodium pump and most other P-type ATPases have a complex ATP dependence; a high affinity ATP effect and a low affinity ATP effect. In the sodium pump, phosphorylation of E\(_1\) to E\(_{1-P}\) requires Na, Mg and ATP. The \(K_m\) value for Na,ATPase (\(K_m\) for ATP \#1 \(\mu M\)) agrees well with the measured ATP binding affinity (32, 33). At low ATP concentrations, the rate of hydrolysis is slow and limited by the release of potassium (34). However, higher ATP concentrations (\(K_m(\text{ATP}) \approx 100 \mu M\)) facilitate the deocclusion of potassium. This low affinity effect is seen as the \(K_m\) for ATP under (Na + K)-ATPase conditions. ATP activation of the sodium pump, with both high and low apparent affinities, has been interpreted as being due to either two distinct ATP sites or to a single ATP binding region that alters its affinity in different pump conformations.

Using the fluorescent ATP analog, TNP-ATP, we were able to estimate the ATP affinity of the His\textsubscript{6} loop. In experiments designed to determine the His\textsubscript{6} loop affinity for TNP-ATP, we discovered that there were two components to the binding of this ATP analog, a saturable and nonsaturable component. Hellen and Pratap (20) reported a similar two-component binding curve for TNP-ATP binding to the native Na,K-ATPase. Subtracting the nonspecific binding from the data, revealed a specific saturable component with an apparent \(K_d\) of \(\approx 1 \mu M\) for TNP-ATP (Fig. 8B; Table I). This value is about an order of magnitude higher than the Na,K-ATPase binding affinity for TNP-ATP (\(-0.5 \mu M\) at \(25^\circ\)C; Ref. 17), i.e. it appears to represent a low affinity site.

An affinity of 300–400 \(\mu M\) was found for ATP by competition
and displacement of TNP-ATP (Table I). This value is in reasonable agreement with the low ATP affinity for the $E_2(K_2)$ state of the native Na,K-ATPase associated with potassium deocclusion. In addition, the expressed domain has a similar affinity ($K_m$ 500–600 mM; Table I) for ADP. This is consistent with the observation that in the low affinity $E_2$-state of the sodium pump, ADP has been shown to facilitate potassium deocclusion and transport (16, 35–36). AMP concentrations up to 3 mM were not sufficient to displace TNP-ATP (Fig. 9C), and concentrations of up to 5 mM were unable to protect against FITC modification (Fig. 3).

Recently, the nucleotide binding domains of the sarcoplasmic reticulum calcium pump (23), the yeast proton pump (22), and the sodium pump (24) have been expressed in *E. coli*. In the sodium pump-expressed domain, ATP binding was demonstrated by measuring MgATP protection against photolabeling with 2-N$_2$-ATP$^{32}$; however, Mg alone appeared to protect as well as MgATP (24). All our experiments were performed in the absence of magnesium; thus, the effects are solely due to the nucleotides themselves. ATP binding to expressed loops from the proton pump and sarcoplasmic reticulum calcium pump was estimated by ATP competition of TNP-ATP, as in this study. The affinities for TNP-ATP binding to the calcium pump and proton pump domains were 2 and 6 mM, respectively, similar to our value of ~3 mM for the sodium pump domain. In contrast, the ATP affinity reported for the proton pump domain was ~3 mM (22), significantly different from that reported for the calcium pump domain (~200 mM; Ref. 23) and here for the sodium pump domain (~300 mM; Table I). The considerably lower affinity for the proton pump ATP binding domain may be due to an inherent property of the domain or possibly because the proton pump construct was a glutathione-S-transferase fusion protein, whereas the calcium pump and sodium pump constructs were both His-tagged proteins. Indeed, we were unable to successfully measure the ATP affinity for the glutathione S-transferase version of our construct even though we were able to demonstrate that ATP protected against FITC modification. It turned out that ATP (5 mM) also protected against FITC labeling of glutathione S-transferase alone.$^3$

It appears, then, that expression of the isolated central loops of these P-type ATPases produces a protein that is able to selectively bind ATP (or ADP) but with an affinity close to that seen in $E_2$ forms. Because the isolated loop has considerable secondary structure, it seems reasonable to suppose that the high affinity binding form is generated by interactions with other parts of the ATPase (probably the cation binding domains). One way of modeling these changes in the ATP binding site would be to suppose that there are two states or forms, an R (relaxed) form and a T (tense) form, the R form having lower affinity for ATP and the T form, higher affinity (~1 mM). The changes in structure of the intact protein that we identify as $E_1$ (high sodium affinity and high ATP affinity) and $E_2$ (high potassium affinity and low ATP affinity) are mirrored by changes in T and R forms, respectively, in the ATP binding loop. It is interactions between the ATP binding domain in the loop and other segments of the protein that hold the loop in the tense form, which has a high affinity for ATP. In isolation, the loop is not constrained by these interactions, and a relaxed (R) form exists with low substrate affinity.

The present study of ATP binding to the purified M4M5 loop of the Na,K-ATPase provides a basis for future mutagenesis studies of the residues thought to play a role in ATP binding. Furthermore, the ability to obtain large quantities of pure soluble protein makes this method valuable for detailed structural analyses of the wild-type and mutant ATP binding domains of P-type ATPases.

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