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2-[4-(Maleimidylalanino)naphthalene 6-sulfonic acid (MIANS) irreversibly inactivates Na,K-ATPase in a time- and concentration-dependent manner. Inactivation is prevented by 3 mM ATP or low K+ (<1 mM); the protective effect K+ is reversed at higher concentrations. This biphasic effect was also observed with K+ congeners. In contrast, Na+ ions did not protect. MIANS inactivation disrupted high affinity ATP binding. Tryptic fragments of MIANS-labeled protein were analyzed by reversed phase high performance liquid chromatography. ATP clearly protected one major labeled peptide peak. This observation was confirmed by separation of tryptic peptides in SDS-polyacrylamide gel electrophoresis revealing a single fluorescently-labeled peptide of ~5 kDa. N-terminal amino acid sequencing identified the peptide (V545LGFCH...). This hydrophobic peptide contains only two Cys residues in all sodium pump α-subunit sequences and is found in the major cytoplasmic loop between M4 and M5, a region previously associated with ATP binding. Subsequent digestion of the tryptic peptide with V8 protease and N-terminal amino acid sequencing identified the modified residue as Cys577. The cation-dependent change in reactivity of Cys577 implies structural alterations in the ATP-binding domain following cation binding and occlusion in the intramembrane domain of Na,K-ATPase and expands our knowledge of the extent to which cation binding and occlusion are sensed in the ATP hydrolysis domain.

The Na,K-ATPase (EC 3.6.1.37) is a P2-type ATPase (1) that is responsible for the maintenance of the sodium and potassium ion gradients in most eukaryotic cells. The integral membrane protein is a heterodimeric enzyme comprised of an α-subunit and some specific amino acid residues that have been identified as being close to (or at) the ATP-binding region of the enzyme. To date, all of the residues identified lie in the major cytoplasmic loop of ~440 amino acids, between transmembrane helices 4 and 5. Recent evidence that bacterial overexpression of the M4M5 loop yields a peptide with the same nuclease phosphate binding specificity as the intact Na,K-ATPase lends further support to the notion that this segment supplies the ATP-binding residues in the intact sodium pump (7). The majority of the amino acids in the ATP-binding domain identified by direct labeling have been lysine residues, primarily because of the greater availability of lysine-selective reagents. These residues include Lys480, Lys487, Lys501, Lys589, Lys605, Lys618, Lys622, and Lys719 (for review see Refs. 2 and 3). In addition, cysteine-directed reagents have been used in a variety of transport systems, e.g. P-glycoprotein (8, 9) and lac permease (10). Such studies in the gastric H,K-ATPase (11) led to the identification of important extracytosolic cysteine residues unique to this member of the P-type ATPase family. The early use of cysteine-directed reagents in the study of Na,K-ATPase also led to important insights. The consequences of treating the enzyme with N-ethylmaleimide led to inhibition of the major phosphoenzyme transition (E1P E2P) and resulted in the formulation of the widely accepted Post-Albers scheme for the reaction pathway (12, 13). A previous kinetic study of the interactions of MIANS with the Na,K-ATPase reported effects of ATP in modulating the resulting inactivation of the enzyme (14). More recently, studies using fluorescent cysteine reagents on a sided preparation of Na,K-ATPase demonstrated that only two Cys residues (of the possible seven intramembrane cysteines) were exposed to the extracellular medium (15). These observations formed the basis of a site-directed mutagenesis approach to analyze the membrane topology of the Na,K-ATPase α-subunit (16).

In the present work, we provide evidence that MIANS inactivates Na,K-ATPase by its covalent attachment to Cys577, which results in the elimination of high affinity ATP binding. We also show that the reactivity of Cys577 is conformationally...
sensitive and that its reactivity alters in different enzyme-bound states. This indicates that structural changes in the ATP-binding domain are transmitted changes through the two transported K ions produces changes in the ATP-binding site structure.

**EXPERIMENTAL PROCEDURES**

**Materials**—Triethylamine and phosphoric acid were purchased from Aldrich. [3H] ADP and rainbow high and low molecular weight markers were from Amersham. Endo-glu C proteinase (V8 protease) was from Boehringer Biochemicals. Acrylamide, ammonium persulfate, Coomassie Brilliant Blue R-250, TEMED, low molecular weight standards, and SDS were purchased from Bio-Rad. Ammonium molybdate, hydrochloric acid, and sodium phosphate were from Fisher. Acetone, triethylamine, cupric sulfate, potassium chloride, sucrose, and urea were from Mallinckrodt. Polyvinylidene difluoride electroblotting membrane was from Millipore. MIANS was from Molecular Probes. Dog kidneys were from Pelfreeze. Trifluoroacetic acid and aminoacyl 8 reagent were from Pierce. Ammonium bicarbonate, ascorbic acid, β-mercaptoethanol, EDTA, EGTA, Folin and Ciocalteu’s Phenol Reagent, imidazole, iodoacetamide, 10% in the reaction mixture and 0.025% in the assay medium. Enzyme activity was determined by the method of Lowry et al. (18). Proteolytic digestion was performed in 25 mM ammium bicarbonate with V8 protease (1:15, w/w, protease:protein) for 18 h at room temperature. Protein fragments were precipitated with 10% TCA (pH approximately 2.0). The enzyme was pelleted at 4°C for 40 min. The pellet was then washed twice with 2 ml of H2O. Labeled protein was eluted from the membrane with H2O, and the amount of protein recovered was determined by the method of Lowry et al. (18). Reverse Phase HPLC Separation of Soluble Peptide Fragments—Soluble peptide fragments from trypsin digestion were separated by linear gradient elution using a Vydac C18 (0.45 × 25 mm) reverse phase column. The gradient was generated with a Beckman System Gold 126 HPLC Dual Pump System Module. For the first purification, pump A delivered 100% H2O, 0.1% trifluoroacetic acid, pH 2, and pump B delivered 50% acetonitrile, 20% H2O, 0.1% trifluoroacetic acid, pH 2. The flow rate was 0.75 ml/min, and the eluent was monitored with a System Gold 168 photodiode array detector. Fractions were collected in 2-min intervals and those containing the peptide fragments of interest were concentrated with a Savant Automatic AES 1000 Speed Vac System. The volume was brought up to 500 μl with H2O and further purified on a shallower gradient with the same HPLC system and solvents (pH increased to 6.0 with triethylamine) as described above.

**Separation of Labeled Peptide Fragments on Tricine Gels**—Soluble peptide fragments were precipitated with 6 volumes of acetone at −20°C for 16 h. Membrane-associated peptide fragments were resuspended in imidazole/EDTA buffer, and one volume of 10% SDS was added to solubilize the proteins. These solubilized membrane proteins were precipitated by the addition of 10 volumes of methanol at −20°C for 16 h. The precipitated peptides were pelleted at 5,000 × g and redissolved in sample buffer. Peptides were separated on a 16.5% Tricine gel according to Schagger and von Jagow (21). After electrophoresis protein fragments were transferred to polyvinylidene difluoride membrane (Millipore) by electroblotting in 10 mM CAPS 10% methanol, pH 9.5 for 1.5 h. Fluorescently labeled bands were sent for N-terminal amino acid sequencing and total amino acids analysis (Dr. Jan Pohl, Microchemistry Facility, Emory University, Atlanta, GA).

**Stoichiometry of ADP Binding—**ADP binding was determined according to the method of Robinson (23) with slight modifications, in a medium containing 30 mM HEPES, 0.1 mM EDTA, 5 mM NaCl, and 0.5 mM MgCl2 (pH 7.5). A control reaction was incubated without MIANS. Na,K-ATPase activity was determined as described previously. The labeled enzyme was labeled with MIANS at 37°C for at least 70% inactivation (in the absence of substrates), and the reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of soybean-trypsin inhibitor (1:7, w/w, trypsin:inhibitor), and the solution was separated from the membrane bound fraction by centrifugation at 436,000 × g.

**V8 Protease Digestion of Na,K-ATPase—**MIANS-labeled α-subunit was isolated from a 7.5% acrylamide gel and eluted from the gel slice with 5 mM Tris-HCl, 0.05% SDS for a minimum of 18 h at room temperature. The protein was dialyzed against a Condition 30 (Amicon) and washed twice with 2 ml of H2O. Labeled protein was eluted from the membrane with H2O, and the amount of protein recovered was determined by the method of Lowry et al. (18). Reverse Phase HPLC Separation of Soluble Peptide Fragments—Soluble peptide fragments from trypsin digestion were separated by linear gradient elution using a Vydac C18 (0.45 × 25 mm) reverse phase column. The gradient was generated with a Beckman System Gold 126 HPLC Dual Pump System Module. For the first purification, pump A delivered 100% H2O, 0.1% trifluoroacetic acid, pH 2, and pump B delivered 50% acetonitrile, 20% H2O, 0.1% trifluoroacetic acid, pH 2. The flow rate was 0.75 ml/min, and the eluent was monitored with a System Gold 168 photodiode array detector. Fractions were collected in 2-min intervals and those containing the peptide fragments of interest were concentrated with a Savant Automatic AES 1000 Speed Vac System. The volume was brought up to 500 μl with H2O and further purified on a shallower gradient with the same HPLC system and solvents (pH increased to 6.0 with triethylamine) as described above.

**Separation of Labeled Peptide Fragments on Tricine Gels**—Soluble peptide fragments were precipitated with 6 volumes of acetone at −20°C for 16 h. Membrane-associated peptide fragments were resuspended in imidazole/EDTA buffer, and one volume of 10% SDS was added to solubilize the proteins. These solubilized membrane proteins were precipitated by the addition of 10 volumes of methanol at −20°C for 16 h. The precipitated peptides were pelleted at 5,000 × g and redissolved in sample buffer. Peptides were separated on a 16.5% Tricine gel according to Schagger and von Jagow (21). After electrophoresis protein fragments were transferred to polyvinylidene difluoride membrane (Millipore) by electroblotting in 10 mM CAPS 10% methanol, pH 9.5 for 1.5 h. Fluorescently labeled bands were sent for N-terminal amino acid sequencing and total amino acids analysis (Dr. Jan Pohl, Microchemistry Facility, Emory University, Atlanta, GA).

**Stoichiometry of ADP Binding—**ADP binding was determined according to the method of Robinson (23) with slight modifications, in a medium containing 30 mM HEPES, 0.1 mM EDTA, 5 mM NaCl, and 0.5 mM MgCl2 (pH 7.5). A control reaction was incubated without MIANS. Na,K-ATPase activity was determined as described previously. The labeled enzyme was divided into two aliquots, and the protein was pelleted at 436,000 × g for 30 min at 4°C. One aliquot was resuspended in 200 μl of buffer A (30 mM HEPES, 0.1 mM EDTA, 5 mM NaCl), and the other was resuspended in 200 μl buffer A with 10 μM ATP. The samples were incubated at room temperature for 30 min and then on ice for 1 min. [3H]ADP was added, and the samples were incubated on ice for 30 min. Protein was pelleted at 436,000 × g for 5 min, and the supernatant was removed. Ice-cold buffer A (0.5 ml) was added to the pellet and immediately removed. Protein was resuspended in 0.4 ml of 0.4 mM NaOH. The amount of [3H]ADP bound to the enzyme (200 μl) was determined in a scintillation counter with EcoLite scintillation fluid. The amount of protein was determined in triplicate by the method of Lowry using bovine serum albumin as a standard.

**RESULTS**

**Kinetic Characterization of MIANS Inactivation and the Relationship of Sodium Pump Conformation**—Treatment of purified enzyme with MIANS at 37°C resulted in a reduction in the Na,K-ATPase activity that was dependent on the concentration of MIANS present (Fig. 1A) and the length of incubation at 37°C (Fig. 1B). The time dependence of MIANS inactivation is not a single exponential, suggesting that the inactivation of one class of cysteine residues and labeled cysteine residues with MIANS treatment in continuous presence and continuous absence of E2C, with MIANS treatment and continuous presence of labeled cysteine residues with MIANS. In conclusion, we believe that the fast initial inactivation reaction is at a single site and that prolonged exposure to MIANS results in indiscriminate labeling of several different cysteines (see Figs. 3B and 6 below). Consequently, subsequent labeling conditions were optimized (e.g., shorter times and
lower MIANS concentrations) to determine the specific site of initial inactivation of the Na,K-ATPase.

The presence of enzyme ligands in the reaction mixture significantly altered the extent of Na,K-ATPase inactivation (Fig. 2A). It seemed that Na\(^{+}\) ions facilitated inactivation, whereas the presence of ATP or K\(^{+}\) ions protected the enzyme from inactivation (Fig. 2A). The presence of Mg\(^{2+}\) and phosphate ions did not protect the enzyme from inactivation. These results indicate that inactivation of Na,K-ATPase by MIANS is dependent upon the enzyme conformation, i.e. whether E1 or E2, phosphorylated or not, and the cations bound. Labeled sodium pump protein from the experiments shown in Fig. 2A was subjected to SDS-polyacrylamide gel electrophoresis, and MIANS labeling was visualized under UV light (Fig. 2B). The \(\beta\)-subunit was not labeled by MIANS. The fluorescence intensity of \(\alpha\)-subunit labeled in the presence of ligands was proportional to the level of enzyme inactivation observed. No fluorescence was observed in the control reaction lacking MIANS (lane 1). In the presence of ATP (lane 5) and to a lesser extent K\(^{+}\) (lane 3), the amount of labeling by MIANS was reduced as compared with enzyme labeled in the absence of ligands (lane 2) or in the presence of Na\(^{+}\) (lane 4) or Mg\(^{2+}\) (lane 6).

The data in Fig. 2A suggested that Na\(^{+}\) and K\(^{+}\) ions have different effects on the enzyme reactivity toward MIANS. This appears to be in contrast with previous reports suggesting that all monovalent cations (Na\(^{+}\), K\(^{+}\), and choline) increase the reaction rate of MIANS with the Na,K-ATPase (14). However, Gupta and Lane (14) measured the MIANS fluorescence changes associated with enzyme modification and not enzyme inactivation as performed in our study. Consequently, their measurements would detect all molecules of MIANS that bind to the Na,K-ATPase, whereas our measurements only detect covalent modifications that result in enzyme inactivation. However, it is clear that a low level of binding can occur without significant inactivation (Fig. 2B). It is likely that these experimental differences are the reason for the different interpretations of the effects of cations on MIANS modification.

The protective effect seen with 10 mM K\(^{+}\) (Fig. 2A) appeared to vary slightly between experiments. Therefore, we measured MIANS inactivation in the presence of varying concentrations of several monovalent cations. These data clearly show that the presence of Na\(^{+}\) ions increased enzyme inactivation by MIANS, at either low (<1 mM) or high (>10 mM) concentrations (Fig. 3). In contrast, low concentrations of K\(^{+}\) congeners (<1 mM K\(^{+}\), Rb\(^{+}\), or Cs\(^{+}\)) protected against MIANS inactivation. However, at higher concentrations of K\(^{+}\) and its congeners (>25 mM), this effect was reversed and enzyme inactivation was not prevented. This biphasic effect was previously observed with di-
hydro-4,4'-DIDS (5, 24), 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonate (25), and fluorescein isothiocyanate (6), compounds that also inactivate Na,K-ATPase in an ATP-protectable manner and whose sites of action have been localized to the ATP-binding loop (i.e. K501). It is likely that the variability we had seen with MIANS labeling experiments in the presence of 10 mM K+ was because this concentration falls between [K+] that protect well versus [K+] that do not. It is interesting that the differences between K+ congeners seen with fluorescein isothiocyanate and H2DIDS labeling at K501 (6, 24) were much less pronounced among the K+ congeners protecting Cys(277) from MIANS (Fig. 3A, inset). The differing distances between Lys(501) and Cys(277) from the cation-binding sites may be the reason for this different sensitivity to monovalent cations. A clear explanation for this phenomenon awaits a high resolution structure of the Na,K-ATPase.

Fig. 3B shows the extent of MIANS incorporation into the α-subunit in the presence of varying [KCl]. The amount of MIANS modification follows the biphasic pattern seen with activity (Fig. 2A), although there is some MIANS incorporation into the α-subunit under conditions in which enzyme activity is fully protected, suggesting that some functionally noncritical cysteine residues are modified by MIANS. This labeling that is not associated with inactivation could explain the differences between a previous study (14) and this report (mentioned above).

**Evidence Suggesting That Inactivation by MIANS Results from Labeling the Nucleotide-binding Site—Modification of sodium pump by MIANS resulted in a loss of Na,K-ATPase activity that was prevented by preincubation with 3 mM Tris-ATP (Fig. 2A). A closer look at the concentration dependence of ATP protection against MIANS inactivation showed that preincubation of sodium pump protein with concentrations of ATP greater than 250 μM resulted in the same level of protection observed with 3 mM Tris-ATP in previous experiments (Fig. 4). The apparent Ki for ATP protection was 34 ± 6.3 μM. Because reversible ATP binding is protecting against covalent MIANS modification and because the status of the low affinity ATP site in the absence of K+ is unclear, it is difficult to identify with confidence this ATP effect as the familiar high or low affinity binding to E1 or E2, respectively.

ATP protection against inactivation suggests that MIANS binds to a residue in the ATP site. To further test this hypothesis we directly measured high affinity nucleotide binding (Fig. 5). Purified Na,K-ATPase (100 μg) was labeled with MIANS in the presence and absence of 3 mM ATP or in the presence of 10 mM Na+. Aliquots of enzyme from each condition were used to determine Na,K-ATPase activity (Fig. 5, gray bars) and [3H]ADP binding (Fig. 5, black bars). The maximal enzyme activity and nucleotide binding capacity were determined in the nonlabeled controls. MIANS-inactivated enzyme does not bind ADP. However, enzyme protected against MIANS inactivation does bind ADP. The amount of ADP bound per mg of protein appears to be proportional to the amount of enzyme activity remaining. Thus it appears that the loss of high affinity ADP (i.e. ATP binding) correlates well with the loss of activity. It seems reasonable to conclude that the loss of ATPase activity is a consequence of the loss of high affinity binding that is
necessary for sodium-activated enzyme phosphorylation and ATPase activity.

Purification of the MIANS-labeled Peptide Fragment—Purified Na,K-ATPase (0.1 mg/ml) was preincubated in the presence of varying concentrations of ATP for 10 min at room temperature prior to the addition of 100–200 μM MIANS. The reaction was incubated at 37 °C for 30 min, after which the reaction was stopped by the addition of 50 mM β-mercaptoethanol, and Na,K-ATPase activity was assayed as described under “Experimental Procedures.” Open circles, activity after MIANS modification in the presence of the corresponding ATP concentration; open squares, represent the enzyme activity without MIANS treatment (points are plotted at 3200 μM ATP for visual purposes only, not to indicate a preincubation with ATP). The open circles were fit to the following equation: Activity = Pmax*[ATP]/(K1⁄2 + [ATP]); where Pmax indicates maximal protection elicited, [ATP] indicates concentration of ATP, and K1⁄2 indicates the concentration of ATP required to achieve Pmax/2. Data are triplicate values from a single experiment that was representative of five. The extent of MIANS inactivation in the absence of ATP varied between experiments (from 54 to 87%), but this did not significantly change the apparent K1⁄2 values.

Purification of the MIANS-labeled Peptide Fragment—Purified Na,K-ATPase (~1 mg) was labeled with MIANS in the presence and absence of 3 mM ATP and digested with trypsin (see “Experimental Procedures”). The soluble tryptic fragments were separated from the membrane-associated fragments via centrifugation and all of the MIANS labeling (i.e. fluorescence) was associated with the soluble fraction. Separation of the soluble tryptic peptides was performed by reverse phase HPLC on a Vydac C18 column using isocratic elution with 0.1% trifluoroacetic acid (10 min) followed by a three-stage linear gradient (0.1% trifluoroacetic acid to 80% acetonitrile/0.1% trifluoroacetic acid of 3%/min (5 min), 1%/min (15 min), and 0.7%/min (100 min). Absorances at 215 nm and 320 nm are shown for sodium pump labeled in the absence (Fig. 6A) and presence (Fig. 6B) of 3 mM Tris-ATP. The complex elution profiles of tryptic peptides monitored at 215 nm (top panels) were essentially identical, demonstrating that the digestion and chromatography were reproducible and not affected by MIANS modification. The chromatograms at 320 nm (bottom panels) represent MIANS-labeled peptides.

Although several peptides contain the MIANS probe, the peptide distribution pattern from enzyme labeled in the presence or absence of ATP differed by a single MIANS peak, clearly visible at ~30 min in the 320 nm chromatogram (Fig. 6B, cf. A and B, bottom panels). An overlay of the two chromatograms at 215 nm (not shown) also revealed the absence of this peak (at ~30 min) in the ATP-protected sample. The elution stage containing the ATP-protectable peak was refined to achieve better separation of the MIANS-labeled peptide. The pH of the elution buffers was altered to 6.0 with ethylamine and a linear gradient (H2O to 80% acetonitrile) was run at 0.33%/min (150 min). The MIANS-labeled peptide eluted as a
single sharp peak at $\sim$98 min (data not shown).

An alternative method employed to isolate the MIANS-labeled tryptic fragment was separation via polyacrylamide gel electrophoresis on a 16.5% Tricine gel (Fig. 7) (21). As was the case with the HPLC experiments, no MIANS fluorescence was associated with the membrane-associated insoluble peptide fraction. However, a single fluorescent band was observed from soluble peptides labeled in the absence of ATP (lane 3), whereas the soluble peptides from the sample protected by ATP (lane 2) contained an equivalent band with much less intensity.

**Amino Acid Sequencing of the MIANS-labeled Peptide and Identification of Labeled Residue**—Under normal conditions maleimides are selective for cysteine residues. The MIANS-labeled fragment ($\sim$5 kDa), which was identified from the Tricine gel (Fig. 7), was sent for N-terminal amino acid sequencing. Sequence data from several experiments revealed a relatively hydrophobic peptide beginning at Val$^{545}$. The shortest peptide fragment that would be produced by trypsin would begin at Val$^{545}$ and continue through Arg$^{589}$. This peptide is 45 amino acid residues in length and in most Na,K-ATPase $\alpha$-subunits contains two cysteine residues, Cys$^{549}$ and Cys$^{577}$, as potential targets for MIANS attachment.

The protein sequence reported for dog Na,K-ATPase $\alpha$-subunit is the translation from a cDNA clone (GenBank™ accession number L42173; Ref. 26). The amino acid translation in the region of the MIANS-labeled peptide begins V$^{545}$LG-FR$^{549}$HL, whereas all other cloned Na,K-ATPase $\alpha$-subunits contain a cysteine residue at position 549 (or its equivalent, analyzed using BLAST; Ref. 27). If the reported sequence is correct for dog $\alpha$-subunit, our target residue must be Cys$^{577}$; however, if the reported sequence is in error and residue 549 is in fact cysteine and not arginine, our target could be either Cys$^{549}$ or Cys$^{577}$. Indeed, this appeared to be the case because in all of our sequencing attempts we never had a clear amino...
acid signal in the fifth cycle even when the signals in the fourth (Phe548) and sixth (His550) cycle were strong. The fact that amino acid sequencing of the MIANS-labeled peptide did not detect arginine at position 549 is an indication that the protein sequence obtained from translation of the cDNA sequence is incorrect and dog, like all other known Na,K-ATPases, contains a Cys at position 549. Unfortunately, this left unresolved whether MIANS was tethered to Cys549 or Cys577, because free cysteine residues are not identified well in Edman N-terminal amino acid sequencing. Consequently, we digested MIANS-labeled Na,K-ATPase α-subunit with protease V8 under conditions that facilitated cleavage after glutamate because there were three glutamate residues between Cys549 and Cys577. After V8 digestion, the peptide fragments were run on a 16.5% Tricine gel, and a single fluorescent band was observed and sent for sequencing. The peptide was identified as G561FQFDV..., confirming that MIANS was covalently attached to Cys577.

**DISCUSSION**

In this work we have provided evidence that Cys577 in the α-subunit of canine renal Na,K-ATPase is selectively modified by MIANS. Modification of this cysteine results in inactivation of the enzyme and loss of ATP binding. The presence of ATP during treatment with MIANS prevents both inactivation and labeling of the enzyme. These data provide evidence for the presence of Cys577 in the ATP-binding pocket of Na,K-ATPase. In addition, the reactivity of this residue changes when different cations are bound providing information about the nature of the conformational changes taking place during active cation transport.

The central cytoplasmic loop of the Na,K-ATPase α-subunit has been associated with ATP binding and phosphorylation of the pump during the reaction cycle. Several specific amino acids presumed to be in the ATP-binding pocket have been identified by chemical modification studies (for review see Ref. 2). However, this is the first report identifying Cys577; interestingly, it is not close in the primary sequence to any of the previously identified amino acids (e.g. Lys501, Lys480, and Asp369) and defines a new region of the M4M5 loop involved in ATP binding. This peptide has previously been thought to be closely associated with the membrane, because of its hydrophobic nature and as a result of chemical modification studies with lipophilic reagents. For example, this peptide was labeled with hydrophobic reagents such as 3-(trifluoromethyl)-3-m-[125I]iodophenyl-diazirine, [3H]adamantanyldiazirine (29), and 1-tritospiro-[adamantane-4,3'-diazirine] (30). However, in con-
证据一致说明 MIANS 标记的 ATP 酶的 ATP 结合位点 cysteine of Na,K-ATPase

Evidence Consistent with MIANS Labeling the ATP-binding Domain—The observation that the target for inactivation by MIANS, Cys577, is in the central loop and can be protected against reaction with MIANS by the presence of ATP suggests that this residue is in the ATP-binding domain. In addition, the inactivation of the enzyme that results from modification is due to a loss of high affinity ATP binding (Fig. 5). However, it is not clear yet whether Cys577 is a contact site for ATP binding in the nucleotide-binding domain or whether MIANS exerts an inhibitory effect by virtue of its bulk or by alteration of the tertiary structure of this region following modification. It is likely that answers to these questions will only become available when the Na,K-ATPase, or the cytoplasmic loop containing the ATP-binding domain, is crystallized in the presence of substrate. Recent studies suggest that this latter approach may be a productive strategy and a Hisg 46-kDa peptide corresponding to the M4M5 loop has recently been shown to exhibit ATP-protectable labeling by MIANS (7).

There is also much suggestive evidence that Cys577 is in the ATP-binding domain. For example, it has been reported previously that several aromatic isothiocyanates, which modify Lys501 in the central loop of the α-subunit, react much more readily when the enzyme is in the E1Na form than in the E2(K2) form. These include fluorescein isothiocyanate (6), N-(2-nitro-4-isothiocyanophenyl)-imidazol (4), and DIDS (5, 24). This increase in reactivity upon sodium binding is another example where occupancy of the cation-binding region of the protein, believed to be contained within the intramembrane segments, affects the properties of the ATP-binding domain in the cytoplasm. However, since all of these compounds react with Lys501, it was unclear whether these cation-induced changes: 1) significantly altered the nucleotide-binding pocket, thus allowing easier access to the site, or 2) were relatively small and localized to the region close to Lys501. In the present work, we found that Na+ ions also increased the reactivity of Cys577 toward MIANS. Thus, it appears that cation binding effects are not limited to Lys501 but rather are transmitted a considerable distance along the primary sequence. It is interesting that Cys577 has been postulated to be close to Lys501 in the tertiary structure (see above).

Furthermore, MIANS modification of Cys577 showed a biphasic dependence on K+ ion concentration. That is, at low concentrations, the presence of K+ ions protect against modification, whereas at high K+ ion concentrations the effect is reversed and Cys577 retains its reactivity. A similar observation was first reported for DIDS inactivation of the Na,K-ATPase (24) and later with several other arylisothiocyanates reacting with Lys501 (6). It seems that this biphasic effect has relevance to the normal functioning of the pump as the modification by ATP site probes (i.e. MIANS, DIDS, and fluorescein isothiocyanate) was similar in the presence of K+ congeners such as Rb+ and Cs+ (this work and Refs. 5 and 6). Although several complex models can be proposed to explain this behavior, we believe the simplest model is that the enzyme conformation of a single K+ ion bound, E2(K+), differs from that with two K+ ions bound E2(K+2) (for details see Ref. 6). This difference involves either changes in the environment near Cys577 and Lys501 or a change in the overall ATP site accessibility. Nonetheless, Cys577 (like Lys501) is conformationally mobile, and its reactivity toward MIANS reflects this flexibility.

Previous work on the Na,K-ATPase with MIANS focused on the rate of reaction of Cys residues without identifying the modified residues which caused inactivation (14). These investigators did not observe cation-selective effects in the total labeling but like us saw effects of ATP, which reduced the site and extent of reaction of protein cysteines under somewhat different conditions (e.g. protein/reagent ratios, temperature, and ionic conditions; cf. Ref. 14 and this work).

Domain Interactions and the Mechanism of Cation Pumping—The active transport of Na+ and K+ ions involves coupling...
the hydrolysis of ATP to the transmembrane movement of these ions. These separate but coupled functions seem to occur at spatially separate but linked regions of the protein. To understand the mechanism of active cation transport, it is necessary to understand how these separate protein regions interact with each other. Recent work from chemical modification studies or site-directed mutagenesis of the Na,K-ATPase has identified the M5M6 region as being intimately involved in cation binding and occlusion (38–40). In particular, there are four residues, Ser^{775} (38), Glu^{779} (38, 39, 41, 42), Asp^{804}, and Asp^{808} (43), that appear to be involved in cation transport. Furthermore, different experimental approaches suggest that this M5M6 hairpin may move during the catalytic cycle and play an active role in the cation translocation process in both Na,K-ATPase (44) and the H,K-ATPase (45). The recent suggestion that ouabain may interact with the extracellular loop between M5 and M6 is consistent with both ouabain inhibition and K+ antagonism of ouabain binding (46).

In a similar way to the identification of the M5M6 hairpin in cation binding, chemical modification studies and expression of the central loop of the α-subunit have suggested that most of the ATP-binding residues are in the large cytoplasmic loop between M4 and M5 (7, 47). The chemical modification results presented in this paper identify yet another residue that appears to be in a location intimately involved with ATP binding. Indeed, the data show that the environment around Cys^{577} changes when either K+ or Na+ ions are bound in the cation-binding domain. Other evidence has also suggested that the position of several residues or segments with respect to the membrane-aqueous interface change as the enzyme assumes its different conformations (32). It is becoming more evident that these cation-induced structural changes are the basis for the long known ATP affinity differences that exist when the enzyme exchanges Na+ for K+ at its binding sites (13). Therefore, it seems that modification with agents such as MIANS can begin to reveal the regions of the ATP-binding domain that undergo spatial rearrangements during such conformational transitions.

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