Identification of Two Conformationally Sensitive Cysteine Residues at the Extracellular Surface of the Na,K-ATPase α-Subunit

Svetlana Lutsenko, Sylvia Daoud, and Jack H. Kaplan‡

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

Na,K-ATPase in right-side-out oriented vesicles was stabilized in different conformations, and the location of intramembrane Cys residues of the α-subunit was assessed with membrane-permeable and membrane-impermeable Cys-directed reagents. In the presence of Mg2+ and P,

\[
\text{Cys}^{904}
\]

was the most accessible for both membrane-impermeable 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (or stilbene disulfonate maleimide, SDSM) and membrane-permeable 7-diethylamino-3-(4'-maleimidyl)-4-methylcoumarin (CPM). In the presence of K+, \text{Cys}^{964} was modified only by hydrophobic CPM, indicating that the environment around \text{Cys}^{964} was different in these two conformations. \text{Cys}^{964} seems to mark the extracellular border of transmembrane segment M9. \text{Cys}^{911} in transmembrane segment M8 showed similar behavior; however, it was not so readily modified.

Complete modification of \text{Cys}^{904} and \text{Cys}^{911} causes only partial (about 50%) inactivation of both ATPase activity and Rb+ (or K+) occlusion, indicating that the effect on cation occlusion is indirect and not within the occlusion cavity. The ATP binding capacity remains unaltered by the modifications.

Treatment of the K+-stabilized post-tryptic preparation of purified Na,K-ATPase revealed labeling of several cysteines by CPM, none of which were labeled with SDSM. Removal of K+ ions from the preparation, which we have previously shown is accompanied by release of the M5M6 hairpin to the supernatant (1), causes changes in the organization of the C-terminal 21-kDa fragment. In particular \text{Cys}^{903} in M10 became labeled by both CPM and SDSM, pointing to a tight association between the C terminal and the M5M6 hairpin of the α-subunit.

The Na,K-ATPase is a P2-ATPase (2) which is responsible for the cellular homeostasis of Na+ and K+ ions. The Na,K-ATPase or sodium pump couples the active extrusion of 3 Na+ ions and uptake of 2 K+ ions to the hydrolysis of the terminal phosphate bond of ATP. The primary structures of both α-subunit (112 kDa) and the glycoprotein β-subunit (55 kDa) have been determined for several isoforms and evidence has been accumulated to define more accurately the overall membrane topology and location of the specific residues involved in the binding of the various physiological ligands (3, 4). However, the spatial organization of the sodium pump is far from being understood. The precise lengths and arrangements of the transmembrane segments of the α- and β-subunits as well as any dynamic changes in their mutual orientation and their membrane disposition occurring during active transport are still largely unknown.

We have shown recently that binding of different physiological ligands to the Na,K-ATPase in right-side-out vesicles causes changes at the extracellular portion of the enzyme (5). In particular phosphorylation of the α-subunit results in changes in interactions between the α- and the β-subunit, which can be monitored by proteolytic digestion of the extracellular portion of the β-subunit (5). According to the generally accepted 10-transmembrane segment topological model of the α-subunit, several cysteine residues are predicted to be located close to the extracellular portion of the membrane (see Fig. 1) and hence might participate in ligand-induced structural rearrangements. However, this model is largely untested in its important details, and the positions of the membrane-aqueous boundaries are unknown.

In this article we describe the use of two cysteine-directed reagents (maleimides) to address some of those questions. We have exploited the properties of these reagents to provide evidence on the accessibility of α-subunit cysteines to the extracellular medium and investigated whether or not their locations are altered in various sodium pump conformations. One of these reagents, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM)1 by virtue of its high hydrophobicity can readily access superficial cysteine residues and those buried within the membranes, while the other, 4-acetamido-4'-maleimidylstilbene-2'-2'-disulfonic acid, disodium salt (or stilbene disulfonate maleimide, SDSM) is hydrophilic and membrane-impermeable and thus limited to the surface residues exposed to the aqueous compartment to which SDSM is added.

We provide evidence for the aqueous exposure of only a small number (two) of the total membrane-associated cysteine residues of the α-subunit and show that their location alters in different sodium pump conformations. Evidence is also provided for a tight interaction between the C-terminal transmembrane segment (probably M10) and the M5M6 hairpin which was recently shown to be intimately involved in cation transport and energy transduction (1, 6).

EXPERIMENTAL PROCEDURES

SDSM and CPM were obtained from Molecular Probes; inhibitor of serine proteases 4-(2-aminoethyl)benzenesulfonfonyl fluoride (AEBSF) was purchased through ICN Biomedicals Inc.

1 The abbreviations used are: CPM, 7-diethylamino-3-(4'-maleimidyl)-4-methylcoumarin; SDSM, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, or stilbene disulfonate maleimide; AEBSF, 4-2-aminoethylbenzenesulfonfonyl fluoride; RSOV, right-side-out vesicle; Tricine, \(N\)-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; CAPS, 3-(cyclohexylamino)propanesulfonic acid; BIPM, \(N\)-(p-(2-benzimidazolyl)-phenyl)maleimide.
Mobile Cys Residues in Na,K-ATPase α-Subunit

Na,K-ATPase was purified from dog kidney according to Jørgensen (7) with the average specific activity of 22–30 μmol of P/mg of protein per min, which was assayed according to Brotherson et al. (8). Membrane protein concentrations were determined using the method of Lowry et al. (9) with bovine serum albumin as a standard.

Microsomal Vesicles—The crude microsomal fraction was prepared from dog kidney outer medulla according to Jørgensen (7), and vesicle preparation was obtained according to Forbush (10). The fraction of sealed right-side-out vesicles (RSOVs) was isolated on a two-step gradient of sodium diatrizoate (16%/5%; 17 ml/5 ml) in 25 mM histidine, 1 mM EDTA, 250 mM sucrose buffer, pH 7.2 (buffer A). Average activity of Na,K-ATPase obtained in these preparations varied between 8 and 10 μmol of P/mg of protein-min, and 80–90% of the vesicles were sealed. The ratio of sealed versus open vesicles was determined by an increase in observed ATPase activity following a mild treatment with SDS as described by Forbush (10). To ensure that the Na,K-ATPase molecules in open vesicles were not functional, microsomal vesicles were equilibrated overnight at 2 mg/ml protein concentration with 25 mM imidazole, 1 mM EDTA, 250 mM sucrose buffer, pH 7.2 (buffer A). Activity of Na,K-ATPase was purified from dog kidney according to Jørgensen (7) with the average specific activity of 22–30 μmol of P/mg of protein per min, which was assayed according to Brotherson et al. (8).

Labeling with Cys-directed Reagents and Identification of Modified Residues—The RSOVs were modified with 0.7–3.5 mM SDS for 30 min at 37 °C in the dark in buffer B, containing either 5 mM MgPi or 10 mM KCl. SDS was always dissolved in H2O just prior to modification and never contained impurities that did not affect the conclusions drawn from our data. 3.5 mM SDS was required to get the same level of inactivation. In some batches of SDSM (up to 1 μM) were required to get the same level of inactivation. In cases where there was little or no inactivation, there was no effect on CPM labeling. Thus we believe that SDSM contained impurities that did not affect the conclusions drawn from our data. A23187 (hemimagnesium salt) was then added to 10 mM for 30 min at room temperature. During this procedure all the Na,K-ATPase molecules in the sealed RSOVs remain intact, while the sodium pump in nonsealed vesicles is completely digested. Trypsin inhibitor (10:1, w/w, respect to trypsin) and/or protease inhibitor AEBSF (up to 1 mM) was added to stop digestion, and samples were incubated for 15 min at room temperature. The vesicles were pelleted at 33,000 rpm for 45 min at 4 °C (Beckman, TLA-100.2), and the pellet was washed twice with the corresponding buffers, containing 1 mM AEBSF. For modification with Cys-directed reagents, pellets were resuspended in corresponding buffer to a protein concentration of 1 mg/ml.

Rb⁺ Oclusion—Rb⁺ occlusion was measured essentially as described previously (11). Briefly, Na,K-ATPase in the RSOVs or post-tryptic membrane preparation was resuspended in buffer B, containing 2 mM RbCl for post-tryptic preparation or 250 mM sucrose for vesicle preparation. 86Rb was added to 1.8–2.0 × 10⁶ cpm per sample of 25–30 μg of protein, and Rb⁺ occlusion was measured using ion-exchange columns as described in Shani et al. (11). Rb⁺ occlusion was operationally defined as the %Rb remaining bound to the membrane preparation after passage through the ion-exchange column.

ATP Binding—To measure ATP binding by Na,K-ATPase in the right-side-out vesicles, a vesicle preparation in 25 mM imidazole, 1 mM EDTA, 250 mM sucrose buffer, pH 7.5 (protein concentration 4–5 mg/ml) was treated with SDS (0.1 mg of SDS/mg of protein) at room temperature for 5 min. Then samples were diluted 5–20 times with the same buffer, and membranes were pelleted at 33,000 rpm (Beckman, TLA-100.2) for 30–45 min. Pellets were resuspended in the buffer B, and high affinity ADP binding was measured as described by Robinson (12).

Position of the methionines in the C-terminal part of the α-subunit are marked by rectangles. The arrow indicates the N terminus of the C-terminal 21-kDa segment.
concentrated once more to the minimal volume. Then for trypic digestion protein was diluted with 0.1 M ammonium bicarbonate, 1 M urea (pH 7.8) to get a protein concentration of about 0.5–1 mg/ml, and digested with trypsin (1:10, w/v, ratio of trypsin to protein) for 5–6 h at 37 °C. Acetone (5–8 volumes) was added to precipitate peptides and to remove excess of lipids, and samples were kept at -20 °C overnight. Precipitated peptides were collected by centrifugation at 33,000 rpm for 15 min at 4 °C (Beckman, TL-100); the pellet was dried in air for 10–15 min. Peptides were dissolved in sample buffer containing 2 M urea, 3.3% SDS, 33 mM Tris, 33 mM Tricine, and separated on Tricine gel (12).

For cleavage with CNBr, α-subunit, eluted from the gel as described above, was dissolved in 70% formic acid to get a protein concentration of 1 mg/ml and cleaved with CNBr (0.3 mg/mg of protein) under nitrogen overnight in the dark. The excess of acid was removed by evaporation on a SpeedVac, then water was added, and evaporation was repeated twice. Peptides were dissolved in sample buffer, containing 2 M urea, 3.3% SDS, 33 mM Tris, 33 mM Tricine, and separated on Tricine gel (12).

After electrophoresis, protein fragments were transferred onto polyvinylidene difluoride membrane by electroblotting in 10 mM CAPS, 10% methanol, pH 11, and sequenced.

Proteolytic Digestion—Proteolytic digestion of purified Na,K-ATPase was done as described previously (5, 14). Briefly, Na,K-ATPase at 1.5 mg/ml was suspended in buffer B containing 5 mM MgCl2, 5 mM Trismano, 25 mM RbCl. After incubation of the samples at room temperature for 30 min, tosylphenylalanyl chloromethyl ketone-trypsin (1:20, w/v, with respect to Na,K-ATPase) was added, and tubes were transferred to a water bath and incubated at 37 °C for 1 h. Soybean trypsin inhibitor was added (5–7:1, w/v, with respect to trypsin) to stop digestion, and samples were incubated an additional 10 min at 37 °C. The samples were then diluted with 1 ml of buffer B, containing 2 mM RbCl, and pellets, membranes were collected by centrifugation at 353,000 × g for 30 min (Beckman, TLA-100.2), at 4 °C. The membranes were homogenized in the latter buffer, and centrifugation was repeated twice. Pellets were suspended in buffer B containing 2 mM RbCl.

Tris Form of Post-tryptic Preparation with the MSM6 Fragment Released from the Membrane—Post-tryptic membrane preparations (usually about 100 μg/sample) obtained in the presence of Rb+ as described above were diluted with 1 ml of buffer B, containing 2 mM Rb+, and sedimented at 375,000 × g for 30 min at 4 °C (Beckman, TL-100.2). Pellets were resuspended in 100 μl of ice-cold buffer B, containing either 10 mM Tris-HCl (to obtain release of the M5M6 fragment) or 10 mM Rb+ (to retain the MSM6 fragment in the membrane). AEBSF was added to all buffers to a final concentration of 1 mM. The resuspended pellets were incubated at 37 °C for 10 min, then placed on ice, and an equal volume of ice-cold buffer B, containing 10 mM Rb+, was added to each tube. Membrane-bound fragments were collected by centrifugation at 375,000 × g for 30 min, at 4 °C (Beckman, TL-100.2). Pelleted membrane-bound fragments were resuspended in corresponding buffer for further labeling with Cys-directed reagents. In all of the present studies where either AEBSF or the peptide trypsin inhibitor was used and a comparison with our previously published data (1, 5) showed that the use of AEBSF did not affect subsequent labeling of protein-SH residues by CPM.

Labeling of Post-tryptic Fragments with SDSM and or CPM—Labeling was done as described above for vesicles. To isolate the labeled C-terminal 21-kDa fragment, membrane-associated peptides were separated by 12% Laemmli gel, and bands were cut and eluted with either H2O or 5 mM Tris-HCl, 0.05% SDS, pH 8.0. The eluted 21-kDa peptide was concentrated through Centricon-10 to the minimal volume, diluted with 70% formic acid to get a protein concentration of 1 mg/ml, and cleaved with CNBr (0.3 mg/mg protein) under nitrogen overnight in the dark (15). The excess of acid was removed by evaporation on a SpeedVac, then water was added, and evaporation was repeated twice. Peptides were resuspended in Tricine sample buffer (see above) and separated on Tricine gels (13). After electrophoresis, protein fragments were transferred onto polyvinylidene difluoride membranes by electroblotting in 10 mM CAPS, 10% methanol, pH 11, and sequenced or immunostained with anti-KETYY antibody.

RESULTS

Extracellular and Membrane-buried Cys Residues Can Be Distinguished by a Two-step Chemical Modification—In order to identify cysteine residues located at the extracellular portion of the α-subunit of the sodium pump and to determine whether these residues change orientation during the pump cycle, Na,K-ATPase in the right-side-out vesicles was stabilized in two different conformations (in the presence of MgP2, to produce the phosphorylated form E1P or in the presence of Rb+ to stabilize the eation-occluded form E1Kc and then treated with the hydrophilic membrane-impermeable reagent SDSM.

Modification of phosphorylated Na,K-ATPase with 1 mM reagent results in partial (about 50%) inactivation of ATPase activity. A second treatment of the Na,K-ATPase with the same concentration of SDSM did not result in greater inactivation, indicating that modification was complete. Measurements of high affinity ADP binding demonstrated that the ATP site is not affected, while Rb+ occlusion was diminished by about 40%. The simultaneous presence of 10 mM Rb+ completely protected enzyme against inactivation with SDSM.

When another Cys-directed reagent, CPM, was used, treatment with 1 mM CPM led to 50% inactivation, which was unaffected by the presence of Rb+. These observations suggest that the membrane-permeable reagent CPM has access to and reacts with a different set of Cys residues than the membrane-impermeable SDSM. Furthermore, the location of water-exposed and lipid-exposed cysteines might be revealed by differential treatment with these two reagents.

Since CPM is highly fluorescent following attachment to a cysteine and SDSM is only slightly fluorescent, prior labeling of the Na,K-ATPase α-subunit with SDSM should decrease the labeling due to CPM, if a subset of the CPM-reactive cysteines are also SDSM-reactive. Fig. 2 illustrates that, in fact, when
two preparations of the Na,K-ATPase in RSOVs were modified either with CPM only (Fig. 2, lane 1) or first with 1 mM SDSM and then with 1 mM CPM (lane 2), labeling of the α-subunit was different. SDSM decreases labeling with CPM (and slightly changes mobility of the α-subunit, which became more diffuse), indicating that there are cysteines exposed at the extracellular surface of the α-subunit of the sodium pump. However, protection by SDSM against CPM is only partial, indicating that most of the Cys residues, modified with CPM, are membrane-buried and not accessible to the hydrophilic, membrane-impermeable SDSM.

In order to identify those Cys residues located at the extracellular surface of the sodium pump, α-subunits modified with CPM or with SDSM/CPM in the presence of MgP_i were separated on a 7.5% Laemmli gel, eluted from the gel (see “Experimental Procedures”), and digested with trypsin. The trypsin digests were then resolved using a 16% Tricine gel, and the SDSM-modified peptides were identified after examining the fluorescence emission of the bands (Fig. 3). Several peptides show fluorescence due to CPM modification, which is unaffected by prior treatment with SDSM, while for two bands (4 and 2.5 kDa) fluorescence was decreased as a result of pretreatment with SDSM (Fig. 3).

N-terminal amino acid sequence analysis of the latter peptides identified the 4-kDa band as a segment of the α-subunit beginning at 935NSVFQQGM . . . (Table I). Mobility of this peptide agrees well with expected molecular weight for tryptic digestion, which includes a single Cys residue Cys964 (see Fig. 1). Sequence analysis of the 4-kDa fragment revealed that this band corresponds to the segment Lys943–Met972 (Table I). This region is a part of putative transmembrane segment M9 and includes a single Cys residue, Cys964 (Fig. 1). Sequence analysis of the 4-kDa fragment SINAEEVVV ... corresponds to the segment of the α-subunit, which begins at Ser973 and includes the first cytoplasmic loop and transmembrane hairpin M8M9. There are a number of cysteine residues in this peptide. The 5-kDa peptide begins with a NDHKLSL sequence and corresponds to the fragment Lys905–Met930 in the M8 transmembrane segment as the sites of SDSM modification. Fragments, which were labeled equally well with CPM independently of pretreatment with SDSM, were also sequenced. Two homogeneous peptides were identified after cyanogen bromide cleavage (see Table I). Those are 14-kDa (upper band) and 5-kDa peptides (upper band) in Fig. 4. N-terminal sequence of the 14-kDa fragment SINAEVVV . . . corresponds to the segment of the α-subunit, which begins at Ser172 and includes the first cytoplasmic loop and transmembrane hairpin M3M4. There are a number of cysteine residues in this peptide. The 5-kDa peptide begins with a NDHKLSL sequence and corresponds to the segment Lys842–Met872 of the Na,K-ATPase, labeled with CPM (left lane) or first with SDSM and then with CPM (right lane) as in Fig. 2. Differentially labeled peptides are marked by arrows, letters indicate their N-terminal amino acid sequence.

| Band          | Sequence                  |
|---------------|---------------------------|
| 4-kDa         | 935NSVFQQGM . . .          |
| 2.5-kDa       | 905KIVEFT . . .            |
| 973MYPLKP . . |                           |
| 5-kDa (upper) | 943KNKILIF . . .           |
| 3-kDa (upper) | 35NDHKLSL . . .            |
| 5-kDa (lower) | Mixture of more than two peptides |
| 11-kDa band   | 853IQALGG . . .            |
| 14-kDa (upper)| 605VTGDHPI . . .           |
| 11-kDa band   | 157VPQQALV . . .           |
| 14-kDa (lower)| 172SINAEEVVV . . .         |

**Fig. 4. Electrophoretic separation of the peptides, obtained after cyanogen bromide cleavage of the α-subunit of the Na,K-ATPase, labeled with CPM (left lane) or first with SDSM and then with CPM (right lane) as in Fig. 2.** Differentially labeled peptides are marked by arrows, letters indicate their N-terminal amino acid sequence.
directly involved in coordination with the K$^+$-permeable reagent (not shown). If the residues were stored in the presence of K$^+$, the intracellular border of the membrane. To do so we used the post-tryptic membrane preparation of Na,K-ATPase obtained by extensive trypsin digestion of sodium pump in the presence of K$^+$ (14). These post-tryptic membranes, obtained, and stored in the presence of K$^+$, retain a Rb$^+$ occlusion capacity, which is similar to the K$^+$ occlusion capacity of the native enzyme. Labeling of the post-tryptic membrane preparations with 1 mM CPM demonstrates that prior incubation with SDS does not significantly diminish the CPM fluorescence of any of these membrane-associated fragments, indicating that, in the presence of occluded K$^+$, none of the 11 cysteines is aqueous-exposed and/or accessible to SDSM (not shown).

Removal of Rb$^+$ or K$^+$ from this post-tryptic preparation is accompanied by release of the M5M6 transmembrane hairpin from the membrane and loss of Rb$^+$ occlusion capacity (1). We noticed that under these conditions the C-terminal 21-kDa fragment became more exposed to CPM labeling, indicating that residues, which were not accessible to CPM in the presence of K$^+$, became exposed after the M5M6 hairpin was released from the membrane (1). In order to identify the Cys residues in the C-terminal 21-kDa fragment affected by the release of the M5M6 fragment from the membrane, we examined the fluorescence of the 21-kDa fragments following treatment with CPM under a variety of conditions. These were (i) the Rb$^+$-bound form (M5M6 present, Rb$^+$ occlusion normal); (ii) the Tris form (M5M6 released, Rb$^+$ occlusion lost); (iii) Tris form pretreated with SDSM before CPM labeling; and (iv) Tris form in the presence of SDS (all the cysteine residues are available for modification). The 21-kDa fragments were eluted and the fluorescence of the peptides measured (Fig. 5). There are 4 cysteine residues in the 21-kDa fragment and only a fraction of these are modified when the fragment is not denatured with SDS (Fig. 5). In the presence of Rb$^+$, fluorescent labeling with CPM is unaffected by prior SDSM modification. In the Tris form (i.e. M5M6 fragment released, no ion occlusion), CPM modification is greater, and this is prevented by prior treatment with SDSM. Together these data show that, when Rb$^+$ (and consequently M5M6) is removed, some cysteine residues not only become more accessible to CPM, but also that a fraction of these are relocated outside of the membrane (accessible to SDSM). Such reorganization and associated increase of CPM labeling occurs only in the C-terminal 21-kDa fragment, while CPM labeling of Cys residues in the M1M2 and M3M4 transmembrane hairpins remain essentially unchanged (not shown). This suggests that Cys residues in M1M2 and M3M4 segments may not be involved in direct interactions with the M5M6 hairpin.

In order to identify the mobile cysteine residues in the 21-kDa fragment, it was labeled with CPM in membrane-bound form in Rb$^+$ and Tris forms, isolated using a 12% Laemmli gel (see “Experimental Procedures”) and cleaved with CNBr. The location of three methionine residues in this C-terminal fragment (beginning at Asn$^{583}$) predicts the appearance of three Cys-containing fragments following CNBr cleavage with molecular masses of approximately 11, 6, and 3.5 kDa (see Fig. 1). Following CNBr cleavage of the 21-kDa fragment, the mixture was resolved in a Tricine gel, and the fluorescence of the resulting peptides was compared. It can be seen from Fig. 6 that the predicted 3.5- and 6-kDa CNBr products were obtained and were CPM-labeled, while the 11-kDa fragment was poorly labeled in either Tris and Rb$^+$ form.

The 3.5-kDa band is equally well labeled in both Rb$^+$ and Tris forms. Based on its apparent molecular mass and the location of methionine residues in the 21-kDa fragment, this highly fluorescent peptide corresponds to the M9 segment, and the high level of CPM fluorescence agrees well with the ease of labeling of Cys$^{964}$ seen in our experiments using right-side-out vesicles (described above). The band with an apparent mass of
6 kDa was the only band in which fluorescence was greater in the Tris form (i.e. when Rb\(^+\) was removed and M5M6 fragment released); see Fig. 6, right lane. This band would be predicted to stretch from Tyr\(^{974}\) to Tyr\(^{1016}\), i.e. to the C terminus of the α-subunit. In order to confirm this and ensure that the 11-kDa fragment (which we have not seen modified) can be obtained after CNBr cleavage, the 21-kDa band was labeled in the presence of SDS and cleaved with CNBr. After electrophoresis and blotting, the obtained peptides were examined under UV (Fig. 7, lane 1) and immunostained with antibody directed against KETYY, an epitope at the very C terminus of the Na,K-ATPase. This residue is exposed to the aqueous phase when the Rb\(^+\) occlusion capacity is lost and M5M6 leaves the membrane.

**DISCUSSION**

In the present work we have shown that cysteine residue Cys\(^{964}\) is located at the extracellular surface of the α-subunit of Na,K-ATPase. This residue is exposed to the aqueous phase in a phosphorylated form (E\(_2\)P) of the sodium pump, and it can be readily modified with a number of Cys-directed reagents. Cys\(^{964}\) seems to be at the aqueous membrane boundary of the M9 segment since on K\(^+\) occlusion (E\(_2\)(K\(_2\))) the environment around Cys\(^{964}\) becomes more hydrophobic, and it is no longer accessible to water-soluble hydrophilic reagents.

Similar behavior was observed for a cysteine residue in transmembrane segment M8, indicating that this cysteine is also close to the membrane-aqueous border, and its location is also conformationally dependent. We assume that this cysteine is Cys\(^{911}\) since another residue Cys\(^{930}\) in the M8 segment is close to Lys\(^{924}\) and Ser\(^{928}\), which have previously been localized to the cytoplasmic portion of the α-subunit (16–19). Unlike Cys\(^{964}\), residue 911 is not well modified with CPM, suggesting that steric hindrance may preclude its reactivity. It is quite possible that involvement of this segment of the α-subunit in the interaction with the β-subunit (20) is responsible for such decrease in the reactivity. We find this possibility particularly appealing since such conformational mobility of M8 agrees well with the conformational transitions in the β-subunit we observed earlier (5).

Previous studies on labeling of the purified Na,K-ATPase with BIPM (a hydrophobic-SH directed chromophore) showed little, if any, effect of modification of Cys\(^{964}\) on Na,K-ATPase activity (21). This supports our conclusion that, although the accessibility/reactivity of Cys\(^{964}\) is altered by K\(^+\) occlusion, the residue is probably not essentially involved in the K\(^+\) occlusion cavity. Our observation that Cys\(^{964}\) and Cys\(^{911}\) have different reactivities in different enzyme conformations is reminiscent of the finding that the fluorescence emission of BIPM-labeled Cys\(^{964}\) changes, depending on Na,K-ATPase conformation (22). Although not critical for cation binding and occlusion, both cysteine residues 964 and 911 are involved in movements perpendicular to the membrane surface, which are likely coupled to rearrangements within the membrane of the M5M6 hairpin (see below). It is also interesting that modification of Cys\(^{964}\) with hydrophobic reagent (BIPM) probably does not affect relocation of Cys\(^{964}\) during the ATPase cycle, while attachment of a negatively charged hydrophobic moiety (SDSM) results in a significant effect on Rb\(^+\) (K\(^+\)) occlusion (a loss of about 40%). This loss in Rb\(^+\) occlusion probably results from a destabilization of the E\(_2\)(K\(_2\)) form when SDSM is attached to Cys\(^{964}\) and Cys\(^{911}\).

Our results also provide new suggestive evidence for interactions between specific segments of the α-subunit of the Na,K-ATPase. It seems likely that the M9M10 hairpin in the C-terminal portion of the α-subunit is tightly associated with segments which have been recently identified with cation occlusion and energy coupling functions. We recently showed, by chemical modification, that the M5M6 segment is directly involved in cation occlusion (6). Furthermore, when the K\(^+\)-stabilized post-tryptic membrane preparation is incubated in the absence of K\(^+\), the loss of occlusion capacity is due to the spontaneous loss of the M5M6 hairpin to the supernatant phase (1). In the present work we demonstrate that, when the M5M6 segment is released from the membrane, the Cys\(^{983}\) residue in the C-terminal transmembrane segment M10 becomes exposed to the aqueous environment. Prior to loss of the M5M6 segment this residue is not accessible to SDSM.

Our observation that Cys\(^{983}\) in M10 has an increased susceptibility to modification with SDSM following release of M5M6 from the membrane indicates a tight interaction between M5M6 and M10. The M5M6 hairpin is a rather hydrophilic and proline-rich transmembrane hairpin and stabilization via interactions with M10 may play an important role in the intramolecular interactions with the membrane-bound domain of the α-subunit. This structural role can have important functional implications. Currently available information from chemical modification studies and site-directed mutagenesis do not provide strong evidence for direct role of this segment in the formation of the cation-translocation pathway, but the possibility cannot yet be eliminated. Our data led us to speculate that M9M10 plays an important role stabilizing M5M6 segment in the membrane, rather than directly providing groups for cation coordination. Several earlier reports support this conclusion. While chemical modification of Glu residue in M10 transmembrane segment abolishes ATPase activity and K\(^+\) occlusion (23), site-directed replacement of the same residue does not have a significant effect on functional properties of the Na,K-ATPase (24), leading to suggestion that introduction of a bulky group in this region was a primary cause of the loss of K\(^+\) binding after chemical modification (24).

It is clear that the M9M10 hairpin undergoes changes in its location in the membrane as different physiological ligands of the sodium pump are bound to the α-subunit. Such changes in the M9M10 segment are probably coupled to changes in the transmembrane location of the M5M6 segment. The extent to which such structural changes in M9M10 and M5M6 within the membrane during pump turnover are transmitted to some or all of the other α-subunit transmembrane segments remains to be determined. Piston-like movements of M5M6 can be associated with similar movements of, say M9 and M10, or alter-
natively, changes in depths of M5M6 and M9 and M10 in the membrane may result from coupled rotations (or spiral movements) between these tightly associated transmembrane helices.

REFERENCES
1. Lutsenko S., Anderko, R., and Kaplan J. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7936–7940
2. Lutsenko, S., and Kaplan, J. H. (1995) Biochemistry 34, 15607–15613
3. Lingrel, J. B. and Kuntzweiler, T. (1994) J. Biol. Chem. 269, 19569–19662
4. Pedemonte, C. H., and Kaplan, J. H. (1990) Am. J. Physiol. 258, C1–C23
5. Lutsenko, S., and Kaplan, J. H. (1994) J. Biol. Chem. 269, 4555–4564
6. Arguello, J. M., and Kaplan, J. H. (1994) J. Biol. Chem. 269, 6892–6899
7. Jorgensen, P. L. (1975) Biochim. Biophys. Acta 401, 399–415
8. Broderius, J. B., Muller, J. V., and Jorgensen, P. L. (1981) Biochem. Biophys. Res. Commun. 100, 146–154
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
10. Forbush, B., III (1982) J. Biol. Chem. 257, 12678–12684
11. Shani, M., Goldsleger, R., and Karlish, S. J. D. (1987) Biochim. Biophys. Acta 904, 13–21
12. Robinson, J. D. (1980) J. Bioenerg. Biomembr. 12, 165–176
13. Schagge, H., and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379
14. Capasso, J. M., Hoving, S., Tal, D. M., Goldsleger, R., and Karlish, S. J. D. (1992) J. Biol. Chem. 267, 1150–1158
15. Morrison, J. R., Fidge, N. H., and Grego, B. (1990) Anal. Biochem. 186, 145–152
16. Anderberg, S. J. (1995) Biochemistry 34, 9508–9516
17. Feschenko, M. S., and Sweadner, K. J. (1994) J. Biol. Chem. 269, 30436–30444
18. Fisone, G., Cheng, S. X.-J., Nairn, A. C., Czernik, A. J., Hemmings, H. C., Jr., Hoog, J.-O., Bertotto, A. M., Kaiser, R., Bergman, T., Jornvall, H., Aperia, A., and Greengard, P. (1994) J. Biol. Chem. 269, 9568–9573
19. Beguin, P., Begga, A. T., Chibalin, A. V. Burgener-Kairuz, P., Jaisser, F., Mathews, P. M., Rossier, B. C., Coteoehia, S., and Geering, K. (1994) J. Biol. Chem. 269, 24437–24445
20. Lemas, M. V., Hamrick, M., Takeyasu, K., and Fambrough, D. M. (1994) J. Biol. Chem. 269, 8255–8259
21. Nagai, M., Taniguchi, K., Kangawa, K., Matsuo, H., Nakamura, S., and Iida S. (1986) J. Biol. Chem. 261, 15197–15202
22. Taniguchi, K., Suzuki, K., and Iida, S. (1985) J. Biol. Chem. 257, 10659–10667
23. Godshleger, R., Tal, D. M., Moorman, J., Stein, W. D., and Karlish, S. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 89, 6911–6915
24. Van Huyse, J. W., and Lingrel, J. B (1993) Cell. Mol. Biol. Res. 39, 497–503