Site-directed Chemical Labeling of Extracellular Loops in a Membrane Protein

THE TOPOLOGY OF THE Na,K-ATPase α-SUBUNIT

We have mapped the membrane topology of the renal Na,K-ATPase α-subunit by using a combination of introduced cysteine mutants and surface labeling with a membrane impermeable Cys-directed reagent, N-biotinylaminoethyl methanethiosulfonate. To begin our investigation, two cysteine residues (Cys911 and Cys964) in the wild-type α-subunit were substituted to create a background mutant devoid of exposed cysteines (Lutsenko, S., Daoud, S., and Kaplan, J. H. (1997) J. Biol. Chem. 272, 5249–5255). Into this background construct were then introduced single cysteines in each of the five putative extracellular loops (P118C, T309C, L793C, L876C, and M973C) and the resulting α-subunit mutants were co-expressed with the β-subunit in baculovirus-infected insect cells. All of our expressed Na,K-ATPase mutants were functionally active. Their ATPase, phosphorylation, and ouabain binding activities were measured, and the turnover of the phosphoenzyme intermediate was close to the wild-type enzyme, suggesting that they are folded properly in the infected cells. Incubation of the insect cells with the cysteine-selective reagent revealed essentially no labeling of the α-subunit of the background construct and labeling of all five mutants with single cysteine residues in putative extracellular loops. Two additional mutants, V969C and L976C, were created to further define the M9M10 loop. The lack of labeling for these two mutants showed that although Met997 is apparently exposed, Val960 and Leu976 are not, demonstrating that this method may also be utilized to define membrane aqueous boundaries of membrane proteins. Our labeling studies are consistent with a specific 10-transmembrane segment model of the Na,K-ATPase α-subunit. This strategy utilized only functional Na,K-ATPase mutants to establish the membrane topology of the entire α-subunit, in contrast to most previously applied methods.

Na,K-ATPase (sodium pump) is an integral membrane protein that is present in most animal cells. The enzyme consists of two subunits, a large catalytic α-subunit (about 110 kDa) and a glycosylated β-subunit (∼55 kDa); both subunits are required for enzymatic activity (1–3). Na,K-ATPase utilizes the energy derived from ATP hydrolysis to transport Na⁺ and K⁺ ions across plasma membranes against their electrochemical gradients and is a member of the P₄-ATPase family (4). The ion gradients generated by the sodium pump are important for regulating a variety of physiological functions such as cell excitability, contractility, and secondary active transport.

Several isoforms of the α- and β-subunits have been cloned from various species, and the primary sequences have been described (5). The secondary structural information on the protein, on the other hand, remains controversial despite extensive investigation; such information is essential for understanding structure/function relationships of the Na,K-ATPase. Based on hydrophathy analysis (6), protease accessibility (7, 8), and immunochemical studies (9, 10), the amino-terminal third of the α-subunit appears to contain four membrane-spanning regions. These regions are followed by a large cytoplasmic loop that has been identified as the ATP-binding domain. Chemical reagents that inactivate the Na,K-ATPase (and abolish high affinity ATP binding) in an ATP protectable manner all modify amino acid residues in this loop (reviewed in Ref. 5). This loop has recently been overexpressed in Escherichia coli and exhibits the same nucleotide-binding specificities as native Na,K-ATPase (11). Studies on the carboxyl-terminal third of the α-subunit have produced conflicting results (7–10, 12–15). Recent data using epitope accessibility claim to establish four transmembrane segments in the carboxyl-terminal region and have a total of eight transmembrane segments (12), whereas several other studies claim to establish that there are 10 transmembrane segments (13–15).

Most methods used until now to establish membrane topology of the Na,K-ATPase suffer from inherent limitations. Several methods employ proteolytic digestion in sealed vesicles or with purified protein (7, 15). It is not clear in such studies whether after the initial protease cleavages that the protein retains what can be considered as its native structure. In more recent cleavage methods, based on metal-ion catalyzed cleavages (15), assumptions are made that the cleavage event occurs close to the metal binding sites, and the locations of the bound metals are also unknown. Indeed with this and other cleavage approaches, interpretations based on minor fragmentation products need further confirmation by other methods. In the use of epitope accessibility, assumptions must be made about the effects of permeabilizing detergents, i.e. that such detergent treatments do not alter protein structure (9, 10, 16). These methods are particularly prone to provide misleading information with a protein such as the Na,K-ATPase, where abundant evidence exists demonstrating the mobility or flexibility of its carboxyl-terminal regions. In other methods originally introduced in studies of prokaryotic membrane protein topology, the amino-terminal domain truncations are attached to reporter
proteins to determine the topogenetic properties of the fusion region (13, 14). However, because the complete structure of the protein of interest is not used and functional tests are not then available, it is assumed that the membrane orientation of the truncations faithfully reflects their orientation in the complete proteins.

In this paper, we describe a different approach to determine the correct number of transmembrane-spanning regions of the Na,K-ATPase α-subunit. This method uses data obtained from functional Na,K-ATPase molecules in their native states. Two cysteine residues (Cys\(^{911}\) and Cys\(^{964}\)) in the α-subunit are changed into serine or alanine residues; the resulting membrane topology of the α-subunit can be probed by surface labeling with membrane impermeable sulphydryl reagents. The Na,K-ATPase mutants are heterologously expressed in insect cells using a baculovirus expression system. We are able to characterize and perform labeling experiments exclusively on expressed proteins, because the insect cells (sf9 and High Five cells) contain no detectable amount of endogenous Na,K-ATPase activity (3, 18). Our results are consistent with a specific 10-transmembrane segment topological model of the Na,K-ATPase α-subunit. A preliminary report of some of our findings has been published previously (19).

EXPERIMENTAL PROCEDURES

A full-length cDNA clone encoding the wild-type sheep Na,K-ATPase α- and β-subunits was a gift of Dr. Elmer Price (University of Missouri, Columbia). The Bac-To-Bac\textsuperscript{TM} baculovirus expression system was obtained from Life Technologies, Inc.

Plasmids and Construction of Mutant—Site-directed mutagenesis of the α-subunit was carried out in the pST100 vector via the polymerase chain reaction overlap extension mutagenesis method (20, 21) using primers listed in Table I. The plasmid pST100 was constructed by subcloning the wild-type sheep α-subunit cDNA into the multiple cloning site of pUC8-2 vector (Novagen) as a NotI and Sse8387I fragment; two silent mutations were then introduced into the α-subunit cDNA to facilitate cassette mutagenesis (see Fig. 1A). Using the C911S/C964A mutant as the background construct, cysteine residues listed in Table I were introduced in the α-subunit individually.

Recombinant baculovirus was produced by following the Bac-To-Bac\textsuperscript{TM} system protocols provided by the manufacturer. Briefly, the donor plasmid pFASTBACDUALαop (Fig. 1B) was constructed by subcloning the wild-type β- and α-subunit cDNA into the multiple cloning sites I and II of the pFASTBACDUAL vector, respectively. The β-subunit cDNA was introduced into the vector as an EcoRI/SpeI fragment and the α-subunit cDNA as a SnaBI/SfiI fragment. To construct donor plasmids containing the cysteine mutations, the wild-type α-subunit cDNA in the pFASTBACDUALαop was replaced with the α mutants using AflII and StuI. DH10BAC cells were transformed with pFASTBACDUALαop vectors to obtain recombinant baculovirus shuttle vectors (bacmids), which were used to transfect insect cells to generate recombinant baculoviruses. The genomic DNA of the recombinant baculoviruses were isolated by using the Easy-DNA Kit (Invitrogen) and were sequenced to ensure the appropriate cysteine mutations in the α-subunits.

Cells and Viral Infections—High Five cells were maintained at 27 °C in 250-ml suspension cultures and were split every 3 days with fresh medium containing: 1 M NaCl, 5 mM MgCl\(_2\), 3.6 mM ATP, 60 mM imidazole, 10 mM sodium azide, pH 7.2) and 100 μl of cell membrane preparations containing 8 μg of protein. The protein concentration was measured by the method of Lowry et al. (23) using bovine serum albumin as a standard. The assay mixture was incubated at 37 °C for 30 min, and the phosphate release was determined as reported by Brotherus et al. (24). The Na,K-ATPase activity was the difference between the ATP hydrolysis measured in the presence and absence of 83 μM ouabain.

Phosphorylation with \(^{32}\)P-ATP—This procedure was carried out essentially as described (25) in a 50-μl medium containing: 1 mM NaCl, 5 mM MgCl\(_2\), 50 mM Tris-HCl, pH 7.6, and 50 μg of membrane protein. The reaction was initiated by the addition of ATP (γ-32P)-ATP, NEN Life Science Products, and 7.27 μM Tris-ATP) on ice for 60 s, and the phosphorylation was quenched with 750 μl of ice-cold 5% (v/v) trichloroacetic acid containing 0.5 mM Tris-ATP and 1.5 mM Tris-phosphate. The samples were filtered through Millipore filters (pore size 0.45 μm), washed three times with 2 ml of quenching buffer, and counted in a scintillation counter. Phosphorylation of the Na,K-ATPase enzyme was

![FIG. 1. A, restriction enzyme map of the α-subunit. Unique restriction sites in the α-subunit cDNA were used to facilitate cassette mutagenesis. The Ndel and MluI sites were introduced in this work as silent mutations. B, diagram of the donor plasmid pFastBacDualαβ. The α-subunit cDNA was subcloned under the p10 promoter, and the β-subunit cDNA was subcloned under the polyhedrin promoter. Both promoters are baculovirus-specific and are expressed at the very late stage (>24 h) of insect cell infection.](http://www.jbc.org/doi/10.1074/jbc.M110.188494/supplemental/M110188494.S1)
calculated from the difference between $^{32}$P phosphate incorporation in the medium above and that measured in a medium containing 1 mM KCl instead of NaCl.

Equilibrium $[^3H]$Ouabain Binding—Ouabain binding of the expressed proteins was measured as described (26) with the following modifications. Insect cell membrane protein (100 μg) was incubated at 37 °C for 1 h in 50 μl of incubation buffer (3 mM MgSO₄, 1 mM NaTris·VO₄, 1 mM EGTA, 10 mM MOPS·Tris, pH 7.2) containing 3.78 μM $[^3H]$Ouabain (NEN Life Science Products) and 1.55 μM ouabain and then was placed on ice for 30 min. The reaction mixtures were filtered through Millipore filters (pore size 0.45 μm) and the $[^3H]$Ouabain recovery was determined. When the 2-mercaptoethanol quench was omitted, the outcome of the labeling reactions was unaltered (data not shown). Cells were harvested, and the cell pellet was resuspended in 200 μl of incubation buffer. The CHAPS mixture was diluted to 1% CHAPS in the incubation buffer and 10% methanol, and immunostained with peroxidase-linked streptavidin (Amersham Pharmacia Biotech). An identical nitrocellularly exposed cysteine was introduced into the wild-type $\alpha$-subunit sites were introduced into the wild-type $\alpha$-subunit cDNA as silent mutations to facilitate cassette mutagenesis.

| Mutations | Reactive external loop | PCR primers (5'-3') | Subcloning sites |
|-----------|------------------------|---------------------|-----------------|
| Silent    |                        | ggtacctgcctcgcaggaatgaggatagagaaaaactgg | DrdIII/NeoI |
| NdeI site |                        | gctgacctgcctcgcaggaatgaggatagagaaaaactgg | BspHI/Sacl |
| MluI site |                        | gctgacctgcctcgcaggaatgaggatagagaaaaactgg | BspHI/MluI |
| Null mutants |                    | cgtccgctccctcggtgattgggtgccc | MluI/BsrBI |
| C911A     |                        | ggtacctggactcgcaggaatgaggatagagaaaaactgg | XhoI/XbaI |
| C911S     |                        | ggtacctgcctcgcaggaatgaggatagagaaaaactgg | XbaI/NeIa |
| C964A     |                        | gttccccctgctctacccgcttgattgggtgccc | MluI/SacI |
| Cys mutants |                    | ggcctgacttggtcagcatctgcaagacgcgtaggaattcgtcttcc | MluI/SacI |
| P118C     | M1M2                   | ccaagtcgctcacagaggaatgaggatagagaaaaactgg | XhoI/XbaI |
| T309C     | M3M4                   | cttcttcgctcggatcgcgtcttcgattgggtgccc | XbaI/NeIa |
| L793C     | M5M6                   | gattatattatgcaacacattcatccctctctctctggagacc | MluI/BspHI |
| L876C     | M7M8                   | cttccctctatctcatctcatctcgctggattgggtgccc | BspHI/MluI |
| V969C     | M9M10                  | gccctgacttggtgactcgattgcagggactcatccctc | MluISacI |
| M973C     | M9M10                  | ggtctgctctcgcctcgccttcctgattgggtgccc | MluISacI |
| L976C     | M9M10                  | aggtcatctccctgcaaacaccttcc | MluISacI |

To further define the M9M10 extracellular loop, V969C and L976C mutants were constructed. The signal intensity levels on the blots were analyzed by the computer software NIH Image.

RESULTS

Expression and Enzymatic Characterization of Cysteine Mutants—Using the C911S/C964A construct as the null background, a panel of Na,K-ATPase $\alpha$ mutants was generated. Each mutant contained one cysteine residue in each of the putative extracellular loops (Table I) and was co-expressed with the wild-type sheep $\beta$-subunit using infection with baculovirus. The expressed plasma membrane proteins were purified by a five-step sucrose gradient centrifugation and characterized to confirm functional integrity. As shown in Table II, the mutants displayed specific activities in the range of 0.1–0.6 μmol Pi · mg · min⁻¹, had equal ouabain-binding and phosphorylation levels, which ranged from 10–100 pmol · mg⁻¹ · min⁻¹ protein, and the turnover number, based on these specific activities and the ligand-binding levels, were all between 7000 and 10,000 min⁻¹. In other words, all the expressed mutants showed normal functional activity.

Labeling of Cysteine Mutants—High Five cells expressing the Na,K-ATPase $\alpha$-subunit mutants were incubated with MTSEA-biotin so that a biotin group would be introduced into the $\alpha$-subunits that contain extracellularly exposed cysteines. After the MTSEA-biotin treatment, the mutant proteins were immunoprecipitated and resolved by SDS-polyacrylamide gel electrophoresis, and the protein biotinylation levels were determined on a blot using peroxidase-linked streptavidin and chemiluminescence. An identical blot was stained with anti-α1 antibody to determine the amount of α-subunit immunoprecipitated. Fig. 2A shows the typical α-subunit biotinylation patterns for the expressed proteins, and Fig. 2B shows the amount of α-subunit on the blot. A plot of the specific activity of the mutants against their respective α-subunit intensities on the blot is shown in Fig. 3A. A strong correlation between the two variables is found in this plot, demonstrating that the difference in the specific activity is because of the difference in the expression level. The very low level (if any) of labeling of the null mutant can be readily seen by visual examination of the blot. To allow for the different expression levels of the mutants, the ratio of the α-subunit biotinylation level to the α-subunit intensity level on the blot was calculated for each mutant. Fig. 3B shows such ratios normalized against that of the null construct. The relative biotinylation signals for the P118C, T309C, L793C, and L876C, and M973C mutants are 4–12 times stronger than that of the null mutant. The ratios demonstrate the significantly greater access of the cysteine residues in these constructs than in the null mutant. To further define the M9M10 extracellular loop, V969C and L976C mutants were constructed. The α-subunit biotinylation and intensity levels for these and the M973C mutant are shown in Fig. 4, A and B, respectively. Good-ex

The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; MTSEA-biotin, N-biotinylaminoethyl methanethiosulfonate; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio-1-propanesulfonate; CAPS, 3-cyclohexylamino)propanesulfonic acid.
Expression levels for the three mutants in the M9M10 regions are observed (Fig. 4B); however, the labeling data of the V969C and L976C mutants showed that these two residues are not accessible from the extracellular medium (Fig. 4A).

**DISCUSSION**

We have established the membrane topology of the Na,K-ATPase α-subunit by testing the accessibility of cysteine residues introduced in the predicted extracellular loops. Our method rejects all models consisting of less than 10 transmembrane segments and confirms a specific 10 transmembrane segment model (Fig. 5). Our strategy is based on the earlier observations that Cys911 and Cys964 are the only two cysteine residues exposed to nonpenetrating cysteine-specific reagents in the extracellular medium (17). In the current study, the C911S and C964A substitutions were made to construct an α-subunit background and the lack of labeling of this mutant confirmed the removal of all exposed sulfhydryl groups (Fig. 2), which supports the conclusion of our earlier labeling studies (17). Cysteine was then introduced at residues 118, 309, 793, 876, 969, 973, or 976 of the background construct in each of seven mutants and probed with a membrane impermeable cysteine-directed reagent.

Table II

| Characteristics of Na,K-ATPases isolated from dog kidney inner medulla and from membrane preparations of baculovirus-infected High Five cells |
|---------------------------------------------------------------|
| Dog kidney enzyme was obtained as described (39,40). Crude membrane preparations from baculovirus-infected High Five cells were fractionated on a five-step sucrose gradient (22), and the resulting plasma membrane fractions were collected for activity assays. The specific activity (ouabain-sensitive ATPase activity) and ligand binding properties of the mutants were determined as described under “Experimental Procedures.” The turnover number, which is the turnover rate of phosphoenzyme/minute, was calculated by taking the ratio of specific activity over phosphorylation number. Each value below represents the mean of triplicate determinations of specific activity and ligand binding from at least three different membrane preparations and has a standard error of less than 10%. |

| Specific activity | Ligand binding | Turnover number |
|------------------|----------------|-----------------|
|                  | Ouabain        | Phosphorylation |                 |
| Dog Kidney       |                |                |                 |
| High Five Expressed: |                |                |                 |
| wild-type        | 178            | 18             | 6357            |
| C911A, C964A     | 163            | 16             | 10138           |
| C911S, C964A     | 452            | 72             | 6278            |
| P118C (M1M2)     | 108            | 11             | 9818            |
| T309C (M3M4)     | 128            | 16             | 8000            |
| L793C (M5M6)     | 108            | 12             | 9000            |
| L576C (M7M8)     | 122            | 17             | 7176            |
| V969C (M9M10)    | 617            | 36             | 7174            |
| M973C (M9M10)    | 403            | 36             | 6907            |
| L976C (M9M10)    | 300            | 41             | 7317            |

* The Na,K-ATPase activity for the plasma membrane preparations of noninfected High Five insect cells is 0.33 nmol P_i · mg protein · min⁻¹.
of the entire α-subunit. It should also be pointed out that this strategy contains both positive and negative controls. Our previous work established the accessibility of Cys811 (in M8) and Cys964 (in M9) from the extracellular compartment in studies of native canine renal Na,K-ATPase (17). This provides a positive control for our heterologously expressed wild-type enzyme (Fig. 2, lane 1). The removal of these two cysteine residues provides a negative control where little or no labeling from the extracellular medium is observed (Fig. 2, lane 2). All of our mutants are functionally active. The turnover number for the phosphoenzyme intermediates of the mutants is close to the value for the wild-type phosphoenzyme heterologously expressed in High Five cell membranes (Table II). This suggests that their processing and folding is not affected by the substitutions. In contrast, previous topology studies employed α-subunit mutants that were less well characterized (due to endogenous Na,K-ATPase activity) (9, 10) or nonfunctional (12, 13), when assumptions about the “normal” orientations of the expressed proteins were made. This was particularly relevant to approaches that use carboxyl-terminal truncations and reporter groups (such as glycosylation status) to establish topology. If truncations are used, usually functional activity is lost so that no reliable test can be made to demonstrate that the truncated polypeptide is processed or folded in the same way as the intact native protein.

The amino-terminal third of the Na,K-ATPase α-subunit has been proposed to contain four membrane-spanning regions (7–10), and the accessibility profiles of the P118C and T309C mutants confirmed the locations of Pro118 and Thr309 in the respective M1M2 and M3M4 extracellular loops (Figs. 2 and 3). The region around the M1M2 extracellular loop has been identified as a primary determinant of ouabain-binding affinity in several studies (28–31). We found that the P118C substitution did not greatly alter the ouabain sensitivity of the mutant (Table II), suggesting that the amino acid residue at this position does not make direct contact with the cardiac glycoside. Furthermore, because the substitution replaces a proline residue, it is unlikely that the precise structure of the M1M2 loop in the region is critical for the ouabain binding interaction.

There has not been a clear consensus in the numbers of membrane-spanning domains in the carboxyl-terminal third of the Na,K-ATPase α-subunit. Indeed the carboxyl-terminal topology of the α-subunit has been the source of most debate. This is undoubtedly because of the extra mobility and flexibility of the region compared with the amino-terminal region, so that methods that involve disruptive procedures (such as proteolysis and peptide bond cleavage) are prone to generate artificial observations. We began our investigation in this region by introducing a cysteine at residue 793, which has been predicted to be a part of the M5M6 external loop (7, 14, 17). Previous studies have shown that the M5M6 hairpin, following proteolysis and removal of K⁺ ions (at 37 °C), is released from the membrane (32) to the extracellular space (33). Such release was not observed in the presence of K⁺ ions, suggesting that the M5M6 hairpin in the intact protein is dynamic (possibility moving in a way which is perpendicular to the plane of the membrane) during the conformational shifts of the Na,K-ATPase catalytic cycle. The present study has identified Leu793 as one of the residues that are exposed to the extracellular phase in the native protein (Figs. 2 and 3). This provides the first evidence for the extracellular location between the loops M5 and M6 in the native Na,K-ATPase. Studies using a cysteine-specific inhibitor, omeprazole, have previously provided evidence for such a configuration in the gastric H,K-ATPase (34).

Our demonstration of the exposure of Leu793 at the outside surface enabled us to distinguish between two 10 transmembrane models recently proposed for the α-subunit. In one study using carboxyl-terminal truncations of the α-subunit expressed in yeast (14), the authors place Ala789 close to the cytoplasmic side and Met809 in the extracellular loop between M5 and M6. Their results are compared with another 10 membrane-spanning model proposed earlier by Karlsh et al. (7), which placed Ala789 close to the extracellular part of M5. Our results support the latter model, because we find Leu793 exposed in the extracellular loop, which would place Ala789 close to the extracellular phase in M5.

Lemas et al. (35) have shown that a 26-sequence peptide (Asn889–Ala914) in the predicted M7M8 extracellular loop interacts directly with an external fragment of the β-subunit. It should be pointed out that their study utilized a co-immunoprecipitation protocol, and the functional importance of the association was not determined. Our labeling result with the L76FC mutant provides evidence for the extracellular location of this loop in the intact protein (Figs. 2 and 3). It is interesting to note that previous immunochemical labeling studies using an antibody against the Trp887–Arg904 sequence have
produced conflicting results. In one study, the antibody detected Na,K-ATPase from the extracellular domain after prolonged incubation (36). In that study three new models were suggested for the transmembrane organization of the Na,K-ATPase α-subunit, consisting of either 8 or 10 transmembrane segments. All three of these models (models A, B, and C in Ref. 36) are rejected by our observations of the extracellular localization of Leu976 and Leu977. In another study, the anti-Trp887 antibody failed to label Na,K-ATPase without cell permeabilization by detergent (37). An explanation that might account for the discrepancy would be that this region of the M7M8 loop is not freely accessible but rather associates with the β-subunit in the extracellular space. Prolonged incubation with the antibody or treatment with detergent disrupted the α/β interaction, thus enabled the binding of the antibody.

In a recent article, Møller et al. (16) carried out a very thorough study of the membrane topology of the sarcoplasmic reticulum Ca-ATPase. Their work employed a combination of sequence-specific antibodies and proteolysis methods, and they emphasized the extreme caution needed when using detergents or similar treatments for probing intravesicular locations with the antibodies. The most striking conclusion of their studies was that the extracellular loop between M7 and M8 in the previously accepted model was proposed to be cytosolic. Two different models were then suggested that might account for the cytosolic location of this loop: (i) M7 does not completely protrude across the membrane but folds back to the cytosol from within the membrane or (ii) the existence of an additional membrane return after M7 but before the usual M8 segment. Our data provide clear evidence for the extracellular localization of a residue (Leu976) immediately after M7 and for the extracellular accessibility of Cys911 close to the beginning of M8. This establishes that the region Lys876-Cys911 may be accessed from the extracellular space. These data contradict the conclusion of Møller et al. (16) or else there is a basic difference between the membrane topology of the Na,K-ATPase α-subunit and the Ca-ATPase. Furthermore, Møller et al. (16) raised the possibility that there may be a “plasticity” in the membrane location of the M8 segment in the Ca-ATPase. They discuss earlier work on the Na,K-ATPase (8, 17, 32) to suggest the importance of carboxyl-terminal plasticity in ion pumping by P-type ATPases. On the contrary, our current observation that Met973 is exposed at the extracellular surface, as well as by our earlier suggestion about the membrane location of a segment beginning at Met973 based on tryptic digestion studies of membrane-associated peptides (8). Similar conclusions, that the α-subunit contained eight transmembrane segments, were also reached by Canfield et al. (10) based on their epitope addition studies showing that residues beyond 978 were all intracellular, whereas residue 953 was extracellular. Their study also found that there were no transmembrane segments between 832 (cytoplasmic) and 895 (cytoplasmic), which is at odds with our finding of the extracellular locations of residues Leu876 and Met973. It seems likely that all eight transmembrane segment models can now be disregarded.

The V969C and L976C mutants were generated to further define the M9M10 loop, and our data showed that these two residues are not freely accessible from the extracellular medium (Fig. 4). These observations establish the very small size of this extracellular loop (it probably only consists of residues Ala970-Pro975) and show that the M9 and M10 helices must be closely apposed. The relative lack of accessibility of V969C and L976C compared with M973C demonstrates that our approach is capable of distinguishing accessibility of residues separated by only three or four residues and may be useful in establishing the locations of the membrane interface in the protein structure. The location of these important membrane-interface regions is difficult to predict from limited structural knowledge and has proven to be difficult to define experimentally using other methods.

Our previous study on the labeling of purified renal Na,K-ATPase with 4-acetamino-4′-maleimidylstilbene-2,2′-disulfonic acid (a charged and nonpenetrating maleimide) and 7-diethyl-amino-3-(4′-maleimidyl)-4-methylcoumarin (a penetrating maleimide) showed that Cys964 is accessible from the extracellular space when the enzyme is in a phosphorylated form (17). Upon dephosphorylation and K+ occlusion, however, Cys964 is no longer exposed to the aqueous phase, suggesting the dynamic nature of the M9M10 loop region. It is quite possible that the positions of Val969 and Leu977 are also sensitive to the protein conformation and that their accessibility alters as the different ligands bind and dissociate. The present studies utilized labeling in intact cells where the protein is turning over and control of protein conformation is limited. Nevertheless, the M973C mutant is consistently labeled, and in future studies where labeling will be performed in isolated membranes, it will be possible to test the issues of conformation-dependent mobility of specific residues.

In summary, we have utilized a Cys substitution strategy to establish the membrane topology of the α-subunit of the Na,K-ATPase. The α-subunit has 10 transmembrane segments. Our approaches can be utilized to test whether or not the accessibility of a particular residue close to the membrane aqueous interface alters during the reactive cycle.

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