Ouabain Interactions with the H5-H6 Hairpin of the Na,K-ATPase
Reveal a Possible Inhibition Mechanism via the Cation Binding Domain*

Maria Palasis, Theresa A. Kuntzweiler, José M. Argüello, and Jerry B Lingrel

From the University of Cincinnati College of Medicine, Department of Molecular Genetics, Biochemistry and Microbiology, Cincinnati, Ohio 45267-0524

Cardiac glycosides such as ouabain and digitoxin specifically inhibit the Na,K-ATPase. Three new residues in the carboxyl half of the Na,K-ATPase, Phe-786, Leu-793 (PFLIF786IANIPL793PLGT797), and Phe-863 (FTYF863VIM) have been identified as ouabain sensitivity determinants using random mutagenesis. Polymerase chain reaction was utilized to randomly mutate the DNA sequence encoding the amino acids between Lys-691 and Lys-945 in the α subunit of the Na,K-ATPase. This region contains four transmembrane segments (H5, H6, H7, and H8) and the connecting extracellular and cytoplasmic loops. Different substitutions of these three residues resulted in proteins displaying 2-8-fold increases in the I50 of different cardiac glycosides for inhibition of the Na,K-ATPase activity. By locating these residues, in conjunction with Thr-797 (Feng, J., and Lingrel, J. B (1994) Biochemistry 33, 4218–4224), a new region of the protein containing the H5-H6 hairpin and the H7 transmembrane segment emerges as a major determinant of ouabain inhibition. Thus, a link between the cardiac glycoside binding site and the cation transport sites of the Na,K-ATPase transpires giving a structural base to the cation antagonism of ouabain binding. Furthermore, this link suggests a possible mechanism for cardiac glycoside inhibition of the Na,K-ATPase, such that ouabain binding to the implicated region blocks the movement of the H5 and H6 transmembrane domains which may be required for energy transduction and cation transport.

Na,K-ATPase is an integral membrane protein found in the cells of all higher eukaryotes and is responsible for translocating sodium and potassium ions across the cell membrane utilizing ATP as a driving force (1–4). The Na,K-ATPase is the pharmacological target for the cardiac glycoside class of drugs, such as digitoxin, which is used in the treatment of congestive heart failure and certain arrhythmias (5, 6). Defining the residues involved in the binding of these drugs will aid in our understanding of their inhibition mechanism.

Approaches to identify regions of the enzyme that interact with the cardiac glycosides have included both affinity labeling and molecular biology techniques. For example, binding of these drugs to the extracellular surface of the α subunit of the Na,K-ATPase was shown by probing the enzyme with photoactivatable cardiac glycoside analogues (7, 8). Chemical modification studies also showed that these drugs interact with both the N-terminal and C-terminal half of the protein (9, 10). Site-directed mutagenesis has been useful in identifying specific amino acid residues that are involved in cardiac glycoside binding (9–18). In these mutagenesis studies, specific amino acid substitutions were introduced into a ouabain-sensitive α isoform by site-directed mutagenesis, and the altered cDNAs were transfected into cells carrying an endogenous ouabain-sensitive α1 isoform. Amino acid substitutions that prevent inhibition by ouabain conferred ouabain resistance to the sensitive cells (11–21).

Since ouabain interacts with the extracellular portion of the Na,K-ATPase, initial studies concentrated on substituting amino acids in the extracellular loops of the enzyme (Fig. 1). Species-specific differences between the ouabain-sensitive sheep α1 and ouabain-resistant rat α1 subunits were observed in the first extracellular loop of the protein. When the border residues of this loop were substituted in the sheep α1 subunit, Gln-111 and Asn-122, with those amino acids naturally appearing in the ouabain-resistant rat α1 subunit, Arg and Asp, respectively, a subunit with ouabain-resistant binding properties equal to that of the rat α1 subunit was produced (11). Additional studies identified substitutions at Asp-121 of the first extracellular loop (12, 13) as well as Cys-104 and Tyr-108 of the first transmembrane region as conferring resistance to ouabain (15, 16). In agreement with these results, when the N-terminal portion of the Ca-ATPase was replaced with the corresponding region of the chicken Na,K-ATPase, the resulting chimeric protein exhibited Ca-ATPase activity that was sensitive to ouabain (22). However, a chimera carrying the N-terminal half of the ouabain-insensitive rat gastric H,K-ATPase and the C-terminal portion of the rat α1 subunit was also inhibited by ouabain implicating the C-terminal portion of the Na,K-ATPase in ouabain binding (23). This finding was supported by the identification of Thr-797 (18, 19) and Arg-880 (17) as required for ouabain binding.

The complex nature of the protein–drug interaction is also suggested by binding kinetics that indicate that the interaction of the glycoside with the Na,K-ATPase is dependent on the conformation of the protein (25–27). For example, when the enzyme is phosphorylated in the catalytic cycle (E2P) it binds ouabain with high affinity (Kd ~ 1 × 10−9 M). However, when Na+ or K+ binds to the ATPase (E1(Na) or E1(K)), the conformation of the enzyme changes such that its affinity for ouabain is greatly reduced. From these binding characteristics, it can be deduced that the residues that coordinate the cardiac glycoside...
must be spatially rearranged upon conformational changes during the catalytic cycle (i.e. hidden, exposed, or moved with respect to one another and the drug). Thus, in spite of considerable studies, the three-dimensional organization of the cardiac glycoside binding site and the structural basis for the inhibition mechanism remain unknown.

Several reports from our laboratory and others (28–35) have recently identified the hairpin loop comprised of the fifth and sixth transmembrane helices as critical for cation binding and energy transduction. This region contains four residues, Ser-775, Glu-779, Asp-804, and Asp-808, most likely involved in cation coordination and transport (28, 30–32, 35). Furthermore, different experimental approaches suggest that this domain may move during the catalytic cycle following phosphorylation (29, 33, 34). Consequently, the interaction of ouabain with the H5-H6 segment would be critical in terms of the binding and inhibition mechanisms. One residue in this hairpin, Thr-797, was already shown to be involved in ouabain sensitivity (18, 19). Due to the importance of this domain in enzyme function, we have targeted this region in search of other residues that might be important for cardiac glycoside inhibition. While most of the substitutions that affect ouabain sensitivity have been identified using site-directed mutagenesis, Thr-797 (18) and Arg-880 (17) were produced by chemically treating the DNA to randomly introduce mutations (36). This type of mutagenesis alters particular codons more frequently than others due to the higher reactivity of certain bases with the mutagenic reagent. Recently, a new approach utilizing pothetin has been developed which in the mutating reagent. Recently, a new approach utilizing pothetin have been identified using site-directed mutagenesis—The 763-bp HindIII-BglII cassette (see above) was subcloned into the M13mp19 vector, and specific single substitutions were introduced by the method of Kunkel (40). Mutated cassettes were sequenced and then ligated back into the wild type sheep 1CDNA in the pCK4 expression vector. The pCK4 vectors were linearized with Scal and electroporated into HeLa cells (20).

Na,K-ATPase Activity Determination—Crude membranes from control and transfected HeLa cells were isolated using a NaI treatment (41) as described previously by Jellwell and Lingrel (42). The protein concentration of each preparation was determined by the method of Bradford (43). Na,K-ATPase activity in crude membrane preparation was quantitated as described previously (35) using the following assay medium: 0.5 mM EGTA, 130 mM NaCl, 20 mM KCl, 3 mM MgCl₂, 3 mM ATP, and 50 mM imidazole, pH 7.2 (20°C), 0.3 mg/ml bovine serum albumin, and 1–2 μg/ml membrane protein and varying concentrations of cardiac glycoside. The cardiac glycosides were dissolved in dimethyl sulfoxide in order to increase solubility. The final concentration of dimethyl sulfoxide in the ATPase assay media was 1%. The assay was performed at 37°C for 30 min and the released inorganic phosphate determined by the colorimetric method of Lanzetta et al. (44). The Na,K-ATPase activities at each inhibitor concentration were expressed as percentages of the total Na,K-ATPase activity, which was estimated as the difference in activity in presence of 0 and 10 μM ouabain. The total Na,K-ATPase activity in membrane preparations from the different substituted enzymes was between 10–15 μmol of Pi/mg-h. The ATPase data were fit to a four-parameter logistic function for inhibition: 

\[ V = \frac{V_{max} \cdot [I]^{n}}{V_{max} \cdot [I]^{n} + (X/I_{50})^{n} + V_{min}} \]

where \( V_{max} \) and \( n \) were fixed at 0 and 1, respectively, representing the minimum activity and the Hill coefficient. \( V_{max} \) is the maximum activity in the absence of inhibitor and \( X \) is the concentration of inhibitor. The \( I_{50} \) value is the concentration of ligand which produces 50% of the inhibition.

RESULTS

Random mutagenesis coupled with a ouabain selection system provides a powerful method for identifying residues that are involved in ouabain binding to the Na,K-ATPase. The random mutagenesis procedure used in this work is PCR-based which under appropriate conditions misincorporates nucleotide sequences into the amplified DNA in an unbiased manner. In principle, every nucleotide can be replaced by the other three. However, the concentration of at least four amino acid substitutions at each site in the primary sequence. However, due to the redundancy in the amino acid codons, it is not possible to convert each residue to every other amino acid. By using this approach, a pool of mutant cDNAs is introduced into cells, and the ouabain selection system screens for the mutants of interest. If an α subunit cDNA encoding a substitution in a residue critical for ouabain binding is expressed in sensitive HeLa cells, resistance to ouabain is conferred. Untransfected cells are not able to survive in the presence of 0.2 μM ouabain in the growth media since this level of drug inhibits the endogenous Na,K-ATPase activity.

The objective of random mutagenesis is to introduce saturating levels of substitutions into the cDNA, such that all residues are replaced by several if not all other amino acids. An indication that a specific fragment has been saturated can be deduced from the appearance of positive controls (i.e. substitutions that have been determined in the past to influence ouabain binding to the enzyme). The cassette of interest encodes amino acids 691–945 which includes two residues previously implicated in ouabain binding (Arg-880 and Thr-797). We also randomly mutated the DNA cassette which codes for amino acids 87–229, which contains six residues known to be involved in ouabain binding as additional positive controls (Fig. 1). As expected some of these residues were found in our PCR mutagenesis study (Table I). Statistical calculations can also provide a good

1 The abbreviations used are: PCR, polymerase chain reaction; I₅₀, concentration of cardiac glycoside which produces 50% of inhibition; DEAC, 4-(diazomethyl)-(7-dihydrine) coumarin; bp, base pair.
indication of the number of mutant plasmids that would represent more than 99% of all the possible single base substitutions (45); however, these calculations do not account for redundancy in the DNA code. Using a trinomial distribution, the number of DNAs required for saturation was estimated to be 55,000 for the 430-bp fragment encoding amino acid residues 86–229 and 80,000 for the 763-bp fragment encoding amino acid residues 691–945. The mutant plasmid pool for each of the cassettes used in this study is shown in Table I. This value demonstrates that saturation was likely achieved for the region of interest, namely Lys-691 to Lys-945. Another important parameter indicating the efficiency of this PCR mutagenesis approach is the average mutation rate. This is the number of single base substitutions found in 100 bp of mutagenized DNA. This value indicated that more than one base was substituted for each cassette mutated. Therefore, after the substitutions in the cassette were identified, site-directed mutagenesis was performed to isolate the amino acid replacement that conveyed the ouabain-resistant phenotype. Pools of mutated DNA were transfected into HeLa, and the ouabain-resistant clones were isolated. Six of these clones are listed in Table I. Immunoblot analysis of total protein isolated from ouabain-resistant clones verified that the transfected sheep α1cDNA was expressed (data not shown). The DNA from clones 1–6 was amplified by PCR and sequenced across the entire mutated cassette to determine the base changes responsible for the phenotype. Table I shows that each mutant cassette contained more than one amino acid change, either a new amino acid substitution, a known substitution, or a silent mutation. Some mutations were identified in conjunction with substitutions previously shown to affect ouabain binding (Table I, clones 1, 3, and 5). Since the mutation in the codon for Val-184 was silent and both Thr-781 → Ala and Thr-807 → Ala were previously shown not to affect ouabain sensitivity (19), the ouabain-resistant phenotype of clones 3 and 5 was obviously due to substitutions in residues previously identified. Due to the location of Ile-199 in the first cytoplasmic loop, the phenotype of clone 1 was thought to be due to the Asn-122 → Asp substitution. For those clones containing only new amino acid changes (clones 4 and 6), each amino acid substitution was made independently by site-directed mutagenesis to identify the mutation that was responsible for the phenotype. Three new individual amino acid substitutions were shown to confer ouabain resistance, Leu-793 → Pro, Phe-863 → Ile, and Phe-863 → Leu (Table I). No ouabain-resistant colonies were observed in cells transfected with substitutions Ile-742 → Leu, Ile-769 → Leu, or Gly-858 → Leu.

To explore the structure/function role of the side chains at position 793, 863, and 786, site-directed mutagenesis was used to convert these residues to others which vary in their chemical nature (Table II). In the case of Leu-793, the conservative substitution, Leu-793 → Ile, and the radical change, Leu-793 → Glu, did not result in ouabain-resistant cells. The nonconservative amino acid changes of Phe-786 and Phe-863 to aspartagine resulted in ouabain-resistant cells for the former but not the latter. On the other hand, the replacement of the aromatic side chains at position 786 and 863 with hydrophobic, linear side chains conferred ouabain resistance to cells. As expected, the substitutions that produced ouabain-resistant colonies reduced the affinity of the enzyme for the drug, as indicated by ouabain I50 values (Table II). Leu-793 → Pro was found to be the most highly resistant of the three original substitutions (24-fold) when compared with the human isoform encoded by the wild type HeLa cells.

Naturally occurring cardiac glycosides share the common basic structure shown in Fig. 2. They are characterized by a
Steroid ring system (5β,14β-androstane-3β,14-diol) which is the "lead" structure in cardiac glycosides (48). Both the lactone ring at position C17 and the sugar moiety at C3 increase the specificity and stability of the drug-enzyme complex (6). Although cardiac glycosides share the same basic structural features, it is predicted that the Na,K-ATPase contains different binding pockets that interact with specific moieties of the cardiac glycosides in a complex manner (6), as demonstrated by Askew and Lingrel (20).

To test for putative interactions between the newly identified residues and a specific moiety of the cardiac glycoside, inactivation of the mutant enzyme activities by a series of cardiac glycosides was analyzed. The results of these studies are shown in Fig. 3 and summarized in Tables III and IV. All the substitutions increased the I50 values of each drug tested (Table III). For example, the substitution Phe-863 → Leu increases the I50 values of all the drugs 4–8-fold compared with wild type. On the contrary, substitutions of Leu-793 affect the I50 values for each of the cardiac glycosides to a different degree (2–48-fold). The largest change in I50 values was observed with the Leu-793 substitutions and the inhibition by ouabagenin (28–48-fold increase). Fig. 3, A and B, shows the differential effects of the substitutions at Leu-793, compared with those at Phe-786 and Phe-863, on the inhibition patterns of ouabain and ouabagenin. The large ouabagenin I50 values observed for the Leu-793 substitutions increase the ouabagenin/ouabain ratios suggesting that the sugar moiety of ouabain stabilizes the mutant-drug interactions. However, this trend was not observed between digitoxigenin and digitoxin which also vary only in the sugar moiety. Unlike Leu-793, the substitutions in Phe-786 decrease the ratios of the I50 values for the paired drugs indicating that the sugar moiety is less important for cardiac glycoside binding to these mutants.

### DISCUSSION

Identification of Ouabain Sensitivity—The cardiac glycoside binding site of the Na,K-ATPase has been the target of numerous site-directed mutagenesis studies (11–20, 24). Based on the extracellular action of the drug, these studies have focused on residues in the extracellular or transmembrane domains which vary in a species-specific manner (i.e. rat α1 versus sheep α1) or that possess side chains which could potentially interact with the drug moieties (i.e. H-bond). Although these studies have been fruitful, the tedious nature of changing one residue at a time and the researchers' bias to locate extracellular interaction sites limits these investigations. The PCR-random mutagenesis procedure utilized in this work is unique in that substitutions are introduced throughout the entire sequence of the Na,K-ATPase without bias to the nature of the side or the location of the residue in the membrane topology of the protein. All possible changes are transfected into cells, and the ouabain selection scheme is the only limiting force.

In the PCR mutagenesis of the DNA cassette which codes for Lys-691-Lys-945, three new residues were identified, Leu-793, Leu-793, and...
Phe-786, and Phe-863 as ouabain sensitivity determinants. These residues are located in the extracellular half of the protein either in transmembrane domains (Phe-786, Phe-863) or in the short extracellular loop between H5 and H6 (Leu-793) (Fig. 1). The hydrophobic characteristics of the naturally occurring side chains at these positions clearly demonstrate the power of using an unbiased approach to identify drug-interaction sites as they would not necessarily be targeted in site-directed mutagenesis studies. It is interesting to note that the cytoplasmic residues in this region were also altered by the PCR mutagenesis of this cassette, however, no cytoplasmic substitutions resulted in ouabain-resistant colonies. This observation is consistent with the extracellular inhibitory action of the drug. In the analysis of our results, it is necessary to keep in mind that the ouabain selection scheme and the required function of the Na,K-ATPase for cell viability limit the observable mutations. Amino acid substitutions that alter the ouabain selectivity only slightly will not survive the presence of 0.2 μM ouabain in the growth media. In addition, any mutation that largely inhibits enzyme activity will not survive the selection process. Nonetheless, based on the saturating level of mutagenesis induced by the PCR conditions utilized (Table I), it is unlikely that any other residues that greatly affect ouabain binding exist in this cassette.

Leu-793 is located in the extracellular loop (NIPL793PLGT) between the fifth and sixth transmembrane domains. As expected when Leu-793 was conservatively substituted with isoleucine, no ouabain-resistant colonies resulted (Table II). In contrast, the substitutions with large polar residues (Leu-793 → Asn and Leu-793 → Lys) yielded ouabain-resistant enzymes. This is consistent with the nonpolar characteristics of this leucine contributing to the ouabain-sensitivity of Na,K-ATPase. However, if a negative charge was introduced at position 793 (Leu-793 → Glu), no ouabain-resistant colonies formed. We speculate that this substitution might disrupt the overall activity of the enzyme. Based on the need for side chain length and on the fact that Leu-793 → Pro was resistant, it is reasonable to postulate that the residue at position 793 is structurally important for maintaining a hairpin loop such that this residue stabilizes the strain induced by the two adjacent proline residues (Fig. 1). Previous site-directed mutagenesis studies of Thr-797 demonstrated that a polar group at this position was required for ouabain sensitivity (19). Replacement of this Thr-797 with a hydrophobic residue (Val or Ala) decreased the enzyme's affinity for ouabain 60-80-fold, the largest change observed for a single mutant. It is possible that when the amino acid at position 793 is nonpolar and the hairpin loop is maintained, the hydroxyl moiety of Thr-797 is exposed and can interact with ouabain to inhibit the Na,K-ATPase activity. In contrast, when the hairpin loop between H5 and H6 is disrupted by a polar residue at position 793, the hydroxyl of Thr-797 is less accessible to interactions with ouabain.

Phe-786 is located in the fifth transmembrane segment of the α subunit of the Na,K-ATPase. When this residue was replaced with either a nonaromatic residue (Phe-786 → Ile) or a nonaromatic, polar residue (Phe-786 → Gin), ouabain-resistant colonies were formed. This observation suggests that the aromatic nature of Phe-786 is required for ouabain sensitivity. Phe-786 is in close proximity to Leu-793 and Thr-797 and may act in conjunction with these residues to bind the cardiac glycoside and inhibit the Na,K-ATPase. Upon modeling of the H5 transmembrane segment as a helical wheel (47, 48), this phenylalanine residue lies on a polar face of the helix which is believed to compose one surface of the cation path. In addition, two residues also on the polar face, but in the cytoplasmic half of this helix, are known to be important for cation transport (Glu-779 and Ser-775) (28–32, 35). Interestingly, when the same analysis is applied to H6, Thr-797 is also located on the polar face of the helix together with Asp-804 and Asp-808, two carboxyl residues very likely involved in cation coordination (28).

Phe-863 is located in the seventh transmembrane domain of the Na,K-ATPase. When Phe-863 was replaced with a nonaromatic, hydrophobic residue (Phe-863 → Leu), ouabain resistance was conferred to HeLa cells. However, unlike Phe-786, replacement of Phe-863 with a polar residue (Phe-863 → Arg) did not confer resistance. Similar to Phe-786, the aromatic nature of this side chain at position 863 is not essential for overall ATPase activity. Arg-880 has previously been identified as a residue involved in ouabain sensitivity and is located in the extracellular loop between H7 and H8 (17). Both Arg-880 → Pro and Phe-863 → Leu exhibit similar changes in ouabain sensitivity, 7.9- and 5.7-fold decreases, respectively, possibly indicating that these two substitutions disrupt a common protein-drug interaction.

To summarize, we have identified three new residues in the Na,K-ATPase that are important for cardiac glycoside inhibition. In doing so, we have implicated three new domains of the protein that interact with the drug (H5, H7, and extracellular...
The lactone moiety was altered. Although the sugar moiety was unchanged whether the sugar moiety, steroid moiety, or effect of the steroid compounds on the mutant enzyme activity the ligand which interacts with the carboxyl-terminal half of glycosides were examined to identify the structural moiety of inhibitors of wildtype and mutant receptors for structurally variant cardiac glycosides was altered. Although the sugar moiety, steroid moiety, or lactone moiety was unchanged whether the sugar moiety, steroid moiety, or the ligand which interacts with the carboxyl-terminal half of the Na,K-ATPase (Fig. 2 and Table III). The main inhibitory effect of the steroid compounds on the mutant enzyme activity was unchanged whether the sugar moiety, steroid moiety, or the lactone moiety was altered. Although the sugar moiety appeared to be important in the inhibitory properties of ouabain versus ouabainogen in association with substitutions in Leu-793, this trend was not mimicked by the paired drugs digitoxin versus digitoxigenin (vary only in sugar moiety) (Fig. 2). Thus, it appears that the inhibitory action of cardiac glycosides on Na,K-ATPase activity may be so complex that single residue changes cannot eliminate or consistently alter the inhibitory ratios of the various paired drugs.

Models for Ouabain Inhibition—Since the identification of the species-specific variations in residues that convey ouabain sensitivity within the H1-H2 hairpin loop (11, 12), this domain of the Na,K-ATPase has been referred to as the “ouabain binding domain.” Unfortunately, the mechanism of inhibition associated with this drug-receptor site has remained a mystery. In this study we observed that 3-4-fold increases in the $I_{50}$ value for ouabain when single substitutions were made in residues located in the C-terminal half of the protein. These increases in the $I_{50}$ value for ouabain are similar to those found upon individually mutating the border residues of the H1-H2 extracellular loop; the $I_{50}$ value for ouabain increases approximately 12.5-fold after the substitutions Glu-111 → Asp and Asn-122 → Asp are individually introduced into the enzyme (11, 12). Interestingly, the largest change in ouabain sensitivity induced by a single amino acid substitution was observed when Thr-797 was substituted with a hydrophobic residue (18, 19). Thr-797 → Val demonstrated a 79-fold increase and Thr-797 → Ala displayed a 66-fold increase in the $I_{50}$ value for ouabain (19). Thus, it appears that both the H1-H2 and the H5-H6 hairpins contribute equally to the inhibitory action of cardiac glycosides. Unlike the H1-H2 region, mechanisms for ouabain inhibition of the Na,K-ATPase emerge with the identification of Phe-786, Leu-793, Thr-797, and Phe-863 as ouabain sensitivity determinants due to the importance of the H5-H6 and H7-H8 hairpin loops in transporting cations (28–35) (see below).

Several investigations have recently identified the H5-H6 hairpin loop as a crucial domain for cation binding and energy transduction. First, site-directed mutagenesis of Ser-775 (35), Asp-808, and Asp-8042 has shown that these residues may coordinate K⁺ ions as they are translocated across the plasma membrane. In addition, chemical modification studies have shown that Glu-779 is protected from 4-(diazomethyl)-7-(diethylamino)coumarin (DEAC) labeling by Na⁺ and K⁺ (29). Thus, the polar faces of the fifth and sixth transmembrane helices are composed of several residues that coordinate K⁺ and form the putative pore through which ions are translocated by the Na,K-ATPase. Second, several studies have suggested that this H5-H6 domain is important in energy transduction as a conformationally flexible region which moves during the catalytic cycle. For example, substitutions of Glu-779, Ser-775, and Asp-808 resulted in proteins that possess unstable phospho-enzymeprotein intermediates indicative of a conformational role for this region (31, 35). In addition, modification of Glu-779 by DEAC is greatly increased upon phosphorylation of the protein, suggesting that this residue is exposed to different degrees throughout the catalytic cycle (29). Further evidence for a conformational role of the H5-H6 hairpin involves proteolytic digestion studies done in the presence of various substrates (33, 34). These studies have demonstrated that two cleavage sites bordering this domain are protected similarly by cations and ouabain but are exposed upon phosphorylation of the protein (33, 34). Moreover, this H5-H6 hairpin loop remains embedded in the membrane upon digestion in the presence of ouabain or cations but is released into the soluble fraction in the absence of these ligands indicative of the cations or ouabain interactions restricting the free movement of this domain (34). Thus, it appears that the H5-H6 hairpin loop plays a role in the binding of cations (and ouabain) and moves during the catalytic cycle in a substrate-dependent manner.

By identifying Phe-786, Phe-863, and Leu-793 of the H5 and H7 transmembrane domains and the H5-H6 extracellular loop, together with Thr-797 in H6, as ouabain sensitivity determinants, we have identified a structural link between the cardiac glycoside binding site, the cation transport sites, and the ATP binding domain. This link reveals two possible mechanisms for cardiac glycoside inhibition of Na,K-ATPase transport activity. The interaction of ouabain with the H5-H6 and the H7-H8 transmembrane domains of the Na,K-ATPase may inhibit the enzyme by sterically blocking the cation access channel (49–50) or by stabilizing an intermediate conformation of the protein, effectively locking the movement of the H5-H6 transmembrane domains. Both of these mechanisms are supported by the allosteric effects of cations on ouabain binding (25–27). In the wild type sheep α1 protein the hydrophobic nature of the residues at positions 793, 786, and 863 allows cations to bind to the carboxyl- and hydroxyl-containing residues located in the cytoplasmic half of the membrane (Asp-804, Asp-808, Glu-779, and Ser-775). If ouabain interacts directly with these residues, especially Phe-786 and Thr-797 which appear to be facing the ion channel, the drug might sterically block the cations from reaching the charged/hydroxyl residues in the cation site. However, since an ATPase molecule with ouabain bound can still bind and occlude two K⁺ or Na⁺ ions (51), this simple steric inhibition model is unlikely. Alternatively, Leu-793, Phe-786, Thr-797, and Phe-863 may be important for maintaining flexibility of the protein in the H5-H6 transmembrane region. Thus, ouabain binding to the H5-H6 extracellular loop would inhibit the enzyme by directly locking the movement of the transmembrane domains that is required for cation translocation.

Linking ouabain binding to the H5-H6 hairpin also suggests a possible explanation for the higher affinity of the phosphorylated intermediate for ouabain. It is known that the phosphorylated form of Na,K-ATPase binds ouabain with higher affinity (1 × 10⁻⁹ M, with Mg⁺⁺ and Pₐ) compared with the unphosphorylated form (2 × 10⁻⁸ M, with Mg⁺⁺ alone) (25, 26). This change in affinity indicates that the binding site for ouabain changes upon phosphorylation. The movement of the H5-H6 loop following phosphorylation (29, 30, 33, 34) supports the idea that the residues interacting with ouabain in this domain move and confer the higher affinity for ouabain characteristic of the phosphorylated state.

Based in the central role of the C terminus in ouabain inhibition, what is the role of the H1-H2 region? We hypothesize that the H1-H2 region specifically recognizes cardiac glycosides. However, the cardiac glycoside inhibits the enzyme.
through interactions with the H5-H6 and H7-H8 regions. This theory is consistent with the species-specific variations in the H1-H2 region, which convey the ouabain binding characteristics of the pump and by the conservative nature in several P-type ATPases of the residues in the H5-H6 and H7 domains which are required for cation transport (sequences reviewed in Ref. 47). In this sense, chimeric proteins made between the N terminus of a ouabain-sensitive protein and the C terminus of different cation pumps appear to be sensitive to ouabain inhibition (22, 23). It is interesting to note that the H,K-ATPase inhibitors, omeprazole and pantoprazole, chemically modify Cys-813, the residue, analogous to Thr-797, and Cys-892, located four residues away from Arg-896 which corresponds to Arg-880 in the sheep α1 Na,K-ATPase (52, 53). This suggests that the inhibition of P-type ATPases by pharmacological drugs may have a common mechanism.

Conclusion—By utilizing PCR-random mutagenesis we have located three residues in the carboxyl half of the Na,K-ATPase that are essential for ouabain inhibition, Leu-793, Phe-786, and Phe-863. We propose that through these amino acids along with Thr-797, ouabain binds to the H5-H6 hairpin loop and inhibits cation transport probably by immobilizing these transmembrane domains. In the future, by examining the ouabain binding properties of the mutant proteins in which the cation binding sites are substituted, we may be able to further understand the exact mechanism for ouabain inhibition of the Na,K-ATPase.

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