Asp\textsuperscript{804} and Asp\textsuperscript{808} in the Transmembrane Domain of the Na,K-ATPase α Subunit Are Cation Coordinating Residues*

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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

The functional roles of Asp\textsuperscript{804} and Asp\textsuperscript{808}, located in the sixth transmembrane segment of the Na,K-ATPase α subunit, were examined. Nonconservative replacement of these residues yielded enzymes unable to support cell viability. Only the conservative substitution, Ala\textsuperscript{808} → Glu, was able to maintain the essential cation gradients (Van Huysse, J. W., Kuntzweiler, T. A., and Lingrel, J. B (1996) FEBS Lett. 389, 179–185). Asp\textsuperscript{804} and Asp\textsuperscript{808} were replaced by Ala, Asn, and Glu in the sheep α1 subunit and expressed in a mouse cell line where [\textsuperscript{3}H]ouabain binding was utilized to probe the exogenous proteins. All of the heterologous proteins were targeted into the plasma membrane, bound ouabain and nucleotides, and adopted E\textsubscript{1}Na, E\textsubscript{1}ATP, and E\textsubscript{2}P conformations. K\textsuperscript{+} competition of ouabain binding to sheep α1 and Asp\textsuperscript{808} → Glu enzymes displayed IC\textsubscript{50} values of 4.11 mM (n\textsubscript{Hill} = 1.4) and 23.8 mM (n\textsubscript{Hill} = 1.6), respectively. All other substituted proteins lacked this K\textsuperscript{+}-ouabain antagonism, e.g. 150 mM KCl did not inhibit ouabain binding. Na\textsuperscript{+} antagonized ouabain binding to all the expressed isoforms, however, the proteins carrying nonconservative substitutions displayed reduced Hill coefficients (n\textsubscript{Hill} 2.0) compared to the control (n\textsubscript{Hill} 2.8). Therefore, Asp\textsuperscript{804} and Asp\textsuperscript{808} of the Na,K-ATPase are required for normal Na\textsuperscript{+} and K\textsuperscript{+} transport, possibly coordinating these cations during transport.

The Na,K-ATPase\textsuperscript{1} is present in the plasma membrane of nearly all animal cells, where it is responsible for the maintenance of Na\textsuperscript{+} and K\textsuperscript{+} electrochemical gradients (1–3). The enzyme belongs to the P-type of ion transporting ATPases, sharing homology in the primary sequence, a common catalytic mechanism with others members of the group such as the sarcoplasmic reticulum (SR) Ca-ATPase and the gastric H,K-ATPase (4, 5). Gln, Lys, or Asp yielded functional enzymes with only modest activity. Mutagenesis studies showed that replacement of Glu\textsuperscript{327}, Glu\textsuperscript{327}, Asp\textsuperscript{804}, Asp\textsuperscript{808}, Asp\textsuperscript{926}, Glu\textsuperscript{953}, and Glu\textsuperscript{954} did not yield functional enzymes (6, 7). Previously, our laboratory demonstrated that the carboxylic side chains of Glu\textsuperscript{926}, Glu\textsuperscript{953}, and Glu\textsuperscript{954} are not involved in cation coordination. The ultimate goal of our current research efforts is to locate the cation binding sites within the structure of the Na,K-ATPase. It is clear that the cation occlusion sites reside in the transmembrane region of the α subunit (2, 6, 7), however, the specific residues that coordinate cations are still in part elusive. An early idea in the field was that carboxyl residues located within the membrane may be part of the occlusion cage, neutralizing the cation charged throughout the transport cycle.

Membrane topology models of the Na,K-ATPase based on hydropathy analysis indicate that seven carboxylic acid residues are located within the putative transmembrane segments: Glu\textsuperscript{327}, Glu\textsuperscript{327}, Asp\textsuperscript{804}, Asp\textsuperscript{808}, Asp\textsuperscript{926}, Glu\textsuperscript{953}, and Glu\textsuperscript{954} (10). Previously, our laboratory demonstrated that the carboxylic side chains of Glu\textsuperscript{926}, Glu\textsuperscript{953}, and Glu\textsuperscript{954} are not involved in cation coordination, since diverse substitutions of these amino acids (individually or simultaneously) lead to functional enzymes with no major alterations in cation affinities (17, 18). In contrast, Glu\textsuperscript{327} → Asp and Glu\textsuperscript{327} → Ala replacements inactivate the enzyme; whereas, Glu\textsuperscript{327} → Leu and Glu\textsuperscript{327} → Gln result in functional proteins (18, 19). Upon detailed examination of these substitutions, it appears that Glu\textsuperscript{327} plays a structural role, being important for stabilizing the E\textsubscript{5}(K\textsuperscript{+}) conformation (20). Glu\textsuperscript{779} was chemically modified using 4-(diazomethyl)-7-(diethylamino)coumarin (9, 10). This Na\textsuperscript{+} or K\textsuperscript{+} protective derivatization removed the cation binding capacities. Later mutagenesis studies showed that replacement of Glu\textsuperscript{779} by Ala, Glu, Lys, or Asp yielded functional enzymes with only modest changes in cation affinities (18, 21–23). Recently, we have found that although Glu\textsuperscript{779} is not a cation coordinating residue, it is involved in determining the voltage dependence of the enzyme, possibly by lining the putative access channel connecting the extracellular milieu with the ion binding sites (24).

Undoubtedly, Asp\textsuperscript{804} and Asp\textsuperscript{808} have been the most interesting and difficult carboxyl residues to characterize within the transmembrane domains. All substitutions introduced in these positions, except for the conservative replacement, Asp\textsuperscript{808} → Glu, have produced nonfunctional enzymes, i.e. enzymes unable to support cell growth (18, 21, 25–27). Although these studies have indicated that these two residues may be essential for enzyme function, the structural-functional role of the carboxylic side chains could not be discerned.

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† Recipient of National Institute of Health Fellowship HL08612.
‡ Recipient of Research Development Award for Minority Faculty HL03373 from the National Institute of Health. Current address: Worcester Polytechnic Institute, Dept. of Chemistry and Biochemistry, 100 Institute Rd., Worcester, MA 01609-2280.
§ To whom correspondence should be addressed. Tel.: 513-558-5324; Fax: 513-558-1190.

1 The abbreviations used are: Na,K-ATPase, (Na\textsuperscript{+} and K\textsuperscript{+})-dependent adenosine triphosphatase; E\textsubscript{1} and E\textsubscript{2}, two conformational states of the Na,K-ATPase; SR, sarcoplasmic reticulum; P\textsubscript{i}, inorganic phosphate.

2 Throughout this report the amino acid positions corresponding to the sheep α1 sequence will be used.
In the work presented here, we probed the roles of Asp\textsuperscript{804} and Asp\textsuperscript{808} by examining the expression, targeting, and functionality of the substituted forms of the Na,K-ATPase. We observed that both, Asp\textsuperscript{804} and Asp\textsuperscript{808}, are required for normal Na\textsuperscript{+} and K\textsuperscript{+} interactions with the protein, possibly coordinating these cations during transport by the Na,K-ATPase.

**EXPERIMENTAL PROCEDURES**

**General Methods—**Site-directed mutagenesis (28–30), establishment of stable 3T3 cell lines stably expressing substituted isoforms (20, 31, 32), isolation of crude plasma membranes (30, 33), ouabain binding to whole cells (20), and determination of [\textsuperscript{3}H]ouabain specific radioactivity (34) were all performed using previously described methods.

\[ \text{[\textsuperscript{3}H]Ouabain Binding to Crude Membranes—} \]

All ouabain binding studies were conducted under the following conditions: 5 mM MgCl\textsubscript{2}, 50 mM Tris-HCl, pH 7.4, 50–75 \mu M of membrane protein, and either 0.2 mM ATP or 5 mM P\textsubscript{i}, in a final volume of 0.5 ml incubated at 37°C for 6 h. To optimize the determinations of the different enzyme-ligand interactions, the concentration of [\textsuperscript{3}H]ouabain utilized was 20–50 nM, a concentration close to the ouabain \( K_D \) values calculated for wild type and each substituted protein (Table I). These \( K_D \) values were determined using unlabeled ouabain in a self-competition assay with [\textsuperscript{3}H]ouabain (35, 36). Na\textsuperscript{+} or K\textsuperscript{+} inhibition of [\textsuperscript{3}H]ouabain binding was characterized using at least 11 concentrations (including zero) of each ligand in duplicate. Data describing the competition of [\textsuperscript{3}H]ouabain with Na\textsuperscript{+} or K\textsuperscript{+} were fit to a similar four-parameter logistical function:

\[
\frac{B_{\text{max}} - B_{\text{min}}}{1 + \left( \frac{[\text{ligand}]}{IC_{50}} \right)^n} + B_{\text{min}}
\]

where \( B_{\text{max}} \) and \( B_{\text{min}} \) represent the maximum and minimum amounts of bound [\textsuperscript{3}H]ouabain, respectively. \( n \) represents the approximate number of ligands responsible for the inhibition/stimulation of [\textsuperscript{3}H]ouabain binding, a factor that is similar to a Hill coefficient. The IC\textsubscript{50} and AC\textsubscript{50} values are the concentration of ligand that produces 50% of the inhibition or activation, respectively. \( \times \) is the concentration of ligand, i.e. Na\textsuperscript{+}, K\textsuperscript{+}, or Mg\textsuperscript{2+}. All data analysis was done using the KaleidaGraph program by Abelbeck Software. A minimum of three curves in duplicate were performed to characterize each protein-ligand interaction using at least two separate clonal lines. The standard errors reported in the tables are asymptotic standard errors reported by the fitting programs. The errors shown within the error bars of the figures represent the range of replicate determinations for each ligand concentration.

**RESULTS**

It has previously been shown that replacement of Asp\textsuperscript{804} with Ala, Leu, Asn, or Glu, and Asp\textsuperscript{808} with Ala, Leu, or Asn results in proteins that are unable to support cell growth when expressed in HeLa or COS cells (18, 21, 25, 27). Thus, these substituted proteins could not be characterized in these expression systems. We have circumvented this problem by making these substitutions in an isoform with a high affinity for ouabain (sheep \( \alpha 1 \), \( K_D = 10^{-9} \) M), and expressing these mutant proteins in a background with an endogenous isoform that has a low affinity for the drug (NIH 3T3 mouse cells, \( K_D = 10^{-3} \) M) (20, 35, 37). In this system ouabain binding at nanomolar concentrations of [\textsuperscript{3}H]ouabain was used to probe the exogenous protein without interference from the endogenous mouse Na,K-ATPase. Ouabain binding is an indication of the enzyme’s overall structural integrity and under appropriate conditions provides information on the enzyme localization (20, 27). Furthermore, the kinetics of ouabain binding depend on the different conformational states that the Na,K-ATPase can assume (38–41). Because the conformational status is determined by the ligands associated with the protein, ouabain binding can be used as an indirect sensor of such enzyme-ligand interactions (20, 35, 37).

The aspartic acid residues at positions 804 and 808 of the sheep \( \alpha 1 \) isoform were replaced by Ala, Asn, and Glu amino acids. Thus, conservative and nonconservative substitutions were examined, in addition to the replacement by asparagine which alters these carboxylate groups to amide side chains.

### Table I

| Mutants | Ouabain, \( K_D \) | Mg\textsuperscript{2+}, AC\textsubscript{50} | P\textsubscript{i}, AC\textsubscript{50} |
|---------|-----------------|----------------|----------------|
| WT sheep \( \alpha 1 \) | 1.2 ± 0.7 | 0.20 ± 0.01 | 0.022 ± 0.001 |
| Asp\textsuperscript{804} → Ala | 7.4 ± 1.6 | 1.14 ± 0.15 | 0.200 ± 0.041 |
| Asp\textsuperscript{804} → Glu | 13.5 ± 1.9 | 1.56 ± 0.14 | 0.291 ± 0.032 |
| Asp\textsuperscript{808} → Ala | 7.2 ± 2.1 | 0.97 ± 0.11 | 0.064 ± 0.003 |
| Asp\textsuperscript{808} → Glu | 13.0 ± 1.4 | 0.56 ± 0.04 | 0.114 ± 0.003 |
| Asp\textsuperscript{808} → Asn | 1.8 ± 0.2 | 0.42 ± 0.01 | 0.012 ± 0.002 |

The only substitution of this set which was previously shown to maintain ATPase activity at a level required for cell growth was Asp\textsuperscript{808} → Glu, a conservative substitution (27).

As a basic requirement for these experiments, the effects of these substitutions on the ouabain dissociation constants were analyzed via a self-competition assay (Table I). Although small changes in the ouabain dissociation constants were observed for Asp\textsuperscript{804} → Ala, Asp\textsuperscript{804} → Glu, Asp\textsuperscript{808} → Ala, and Asp\textsuperscript{808} → Glu, the protein-ouabain interaction was still detected with nanomolar amounts of [\textsuperscript{3}H]ouabain. These results are consistent with the concept that the substitutions do not lead to major structural disarray of the protein.

The initial observation that substitutions of Asp\textsuperscript{804} and Asp\textsuperscript{808} were unable to support cell growth could be due to a number of phenomena not directly related to specific alterations in the enzyme’s function. For example, improper folding or lack of targeting to the plasma membrane would not enable the mutant Na,K-ATPase to support cell viability. These problems in protein processing have been reported upon replacement of amino acids in the yeast H-ATPase (42, 43) or the cystic fibrosis transmembrane regulator protein (CFTR channel) (44). We have evaluated the capacity of the substituted enzymes to reach the plasma membrane by measuring ouabain binding to whole cells expressing the heterologous enzymes. In this experimental set-up only those enzymes with an intact extracellular structure and that reside in the plasma membrane can bind the impermeable drug present in the extracellular medium. Fig. 1 shows that all the enzymes carrying replacements at positions 804 and 808 were translated and targeted into the plasma membrane at levels similar to the wild type.

In order to test the capacity of these proteins to undergo basic conformational transitions, we analyzed the protein-ouabain interactions under conditions which stimulate formation of different mechanistic intermediates. For example, enzyme phosphorylation by P\textsubscript{i} stimulates the formation of E\textsubscript{2}P, the intermediate with the highest ouabain affinity (40). The affinity of the Na,K-ATPase for ouabain is also increased by phosphorylation of P\textsubscript{i} (E\textsubscript{2}P(Na)) or by the binding of a nucleotide (E\textsubscript{2}ATP) (37, 41). Fig. 2 shows that both conditions, Mg-P\textsubscript{i} or Mg-ATP (absence of Na\textsuperscript{+}), increased the binding of ouabain to the substituted enzymes in a fashion similar to the wild type Na,K-ATPase. This suggests that the substitutions do not abolish the enzyme phosphorylation by P\textsubscript{i}, nucleotide binding, or the required flexibility to adopt basic conformations (E\textsubscript{2}P, E\textsubscript{2}ATP).

The ability of the mutant proteins to form the E\textsubscript{2}P intermediate was examined by observing the stimulation of ouabain binding by Mg\textsuperscript{2+} and P\textsubscript{i}. The AC\textsubscript{50} values describing the Mg\textsuperscript{2+} interactions in the presence of 5 mM P\textsubscript{i} and the values describing the P\textsubscript{i} interactions in the presence of 5 mM Mg\textsuperscript{2+} are shown in Table I. Both the Ala and Glu substitutions of Asp\textsuperscript{804} and Asp\textsuperscript{808} appear to reduce the stability of the E\textsubscript{2}P complex as suggested by the higher amount of ligand required to stimulate ouabain binding. In all of the experiments which follow, saturating concentrations (5 mM) of these ligands were used to...
maximally stimulate the amount of labeled protein.

Na⁺ and K⁺ prevent ouabain binding in the presence of inorganic phosphate in a pseudo-competitive and partially-competitive fashion, respectively (35, 40). We took advantage of this phenomenon to assess the interaction of cations with the Na,K-ATPase lacking carboxyl side chains at positions 804 or 808. The wild type sheep curve of Fig. 3A shows the K⁺-induced reduction of ouabain binding, characteristic of high affinity K⁺ binding to the Na,K-ATPase (see “Experimental Procedures”). These values were normalized such that the amount bound in the presence of Mg,P₃ was 100%.

The effects of replacing Asp808 are presented in Fig. 4. Similar to the replacements of Asp804, both Asp808 → Ala and Asp808 → Asn substitutions abolished the antagonistic effects of K⁺ on ouabain binding (Fig. 4A). Only at high KCl concentrations were small reductions of ouabain binding observed. These effects were determined to be a result of nonspecific ionic strength changes, since similar curves were obtained with choline chloride (data not shown). As expected, K⁺ interactions with the Asp808→Glu demonstrated an IC₅₀ value of 23.8 ± 1.9 mM which is 6-fold higher than that found with the wild type protein (IC₅₀ = 4.1 ± 0.4 mM) (Table II). This result is

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**Table II**

| Mutants     | K⁺ IC₅₀ (nHill) | Na⁺ IC₅₀ (nHill) |
|-------------|----------------|-----------------|
| WT sheep α₁ | 4.1 ± 0.4 (n = 1.4) | 35.5 ± 0.9 (n = 2.8) |
| Asp⁸⁰⁴→Ala  | No K⁺ antagonism | 21.5 ± 2.5 (n = 2.0) |
| Asp⁸⁰⁴→Glu   | No K⁺ antagonism | 75.7 ± 3.1 (n = 2.4) |
| Asp⁸⁰⁴→Asn   | No K⁺ antagonism | 41.6 ± 1.5 (n = 2.0) |
| Asp⁸⁰⁸→Ala  | No K⁺ antagonism | 27.1 ± 4.2 (n = 2.0) |
| Asp⁸⁰⁸→Glu   | 23.8 ± 1.9 (n = 1.6) | 23.2 ± 1.1 (n = 2.4) |
| Asp⁸⁰⁸→Asn   | No K⁺ antagonism | 21.5 ± 1.2 (n = 1.6) |

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**Fig. 1.** Ouabain binding to NIH 3T3 cells expressing Asp⁸⁰⁴ and Asp⁸⁰⁸ substituted sheep α₁ subunits. Amount of [³H]ouabain bound to intact 3T3 cells untransfected (3T3), transfected with a wild type sheep α₁ cDNA (WT Sheep), or transfected with a cDNA encoding a single amino acid replacement of either Asp⁸⁰⁴ or Asp⁸⁰⁸. The conditions used were similar to those described previously by Kuntzweiler and co-workers (20). An example of the amount of nonspecific binding associated with these cell lines is shown in the last column (NS-WT) where 20 μM unlabeled ouabain was added to compete with the [³H]ouabain for binding to the extracellular surface.

**Fig. 2.** Effect of Mg-ATP and Mg-P₃ on ouabain binding to Na,K-ATPases. Amount of [³H]ouabain bound to equal amounts of membrane preparations under different ligand conditions (see “Experimental Procedures”). These values were normalized such that the amount bound in the presence of Mg,P₃ was 100%.

**Fig. 3.** Effects of cations on ouabain binding to Na,K-ATPase with substitutions in Asp⁸⁰⁴. The symbol representation is as follows: sheep α₁ (open symbols); Asp⁸⁰⁴→Ala (●), Asp⁸⁰⁴→Asn (▲), and Asp⁸⁰⁴→Glu (○). The conditions are as described under “Experimental Procedures.” The nonspecific binding in the presence of 30 mM [³H]ouabain and 20 μM unlabeled ouabain was used as a zero. The data were normalized with the binding in the presence of no competing ligand representing 100%.

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**Asp⁸⁰⁴ and Asp⁸⁰⁸ Coordinate Cations in the Na,K-ATPase**

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Asp<sup>804</sup> and Asp<sup>808</sup> Coordinate Cations in the Na,K-ATPase

**DISCUSSION**

Previous reports have indicated that Asp<sup>804</sup> and Asp<sup>808</sup> are “essential” for Na,K-ATPase activity, based on the incapacity of Asp<sup>804</sup> or Asp<sup>808</sup> substituted enzymes to support cell growth (18, 21, 27). Due to the chemical characteristics of the side chains at these positions and the transmembrane location, these residues have been assumed to be part of the cation binding site in the Na,K-ATPase. However, these initial observations do not directly demonstrate that the cation interactions are altered by these amino acid replacements. Structure-function studies aimed at defining residues within an active site by modifying the protein sequence require discrimination between non-specific general effects on protein structure and specific effects on the binding properties of a particular ligand. The present report attempts to show that individual replacement of Asp<sup>804</sup> or Asp<sup>808</sup> specifically alters the enzyme-cation interactions without dramatically affecting enzyme folding, processing, conformational transitions, or capacity to interact with various other ligands.

**Structural Functional Status of Asp<sup>804</sup> and Asp<sup>808</sup> Substituted Enzymes—**To investigate the functional role of these carboxyls, we first determined the basic structural status of the substituted enzymes. Asp<sup>804</sup> or Asp<sup>808</sup> substituted enzymes bind ouabain with high affinity which indicates that the structures of the extracellular loops interacting with the drug, namely H1-H2, H3-H4, and H7-H8 (25, 29, 45) are similar to the wild type protein. Furthermore, ouabain binding to whole cells expressing the modified proteins demonstrated that the enzymes are correctly targeted to the plasma membrane, allowing an indirect effect of these substitutions on protein processing to be disregarded as an explanation for their incapacity to support cell growth. The Asp<sup>804</sup> and Asp<sup>808</sup> substituted enzymes were also able to bind ATP and undergo phosphorylation by Mg-P<sub>i</sub>. These data not only suggest that the cytoplasmic portions of the enzymes are correctly folded, but also that the mutant proteins can undergo major conformational transitions associated with the normal enzyme mechanism. Thus, it appears that the inability of these substitutions to support cell growth is associated with the specific functional roles of these residues in the mechanistic cycle of the Na,K-ATPase.

Cation effects on ouabain binding showed that K<sup>+</sup> is clearly unable to prevent the binding of the drug to the substituted proteins. The partially competitive effect of K<sup>+</sup> on ouabain binding under our experimental conditions is likely due to the displacement of the equilibrium: E<sub>2</sub>P + 2K<sup>+</sup> ⇌ E<sub>2</sub>(2K) + P<sub>i</sub>, to the right. The affinity for ouabain of the E<sub>2</sub>(2K) form is much lower than that of E<sub>2</sub>P (30, 32, 46–48). The simplest interpretation of our findings is that the substituted proteins, where Asp<sup>804</sup> and Asp<sup>808</sup> were replaced, are unable to bind K<sup>+</sup>. A more complex explanation involves an interaction between the modified enzymes and K<sup>+</sup> which is not translated into a lower affinity for the drug, i.e. the enzyme is “locked” in E<sub>2</sub>P or although the enzyme is dephosphorylated it does not display a low affinity binding site on the extracellular surface. This complex rationale seems unlikely considering that Na<sup>+</sup> can drive the substituted enzymes into a E<sub>2</sub>(Na) form. Therefore, the loss of K<sup>+</sup>-antagonism supports the involvement of Asp<sup>804</sup> and Asp<sup>808</sup> in the binding of this cation during transport.

The dramatic alterations in the protein-cation interaction resulting from the replacement of either Asp<sup>804</sup> or Asp<sup>808</sup> clearly shows their involvement in cation binding to the Na,K-ATPase; however, a less pronounced effect of these substitutions on the stability of the E<sub>2</sub>P conformation was also observed by their reduced apparent affinities for P<sub>i</sub>. In particular, two substitutions of Asp<sup>804</sup> increased the AC<sub>50</sub> value for P<sub>i</sub>, to greater than 10-fold compared to the wild type. Thus, one indirect consequence of disrupting a K<sup>+</sup> interaction site appears to be the destabilization of the E<sub>2</sub> form of the Na,K-ATPase. These results illustrate the communication between the cation binding site of the H5-H6 hairpin (Asp<sup>804</sup> and Asp<sup>808</sup>) and the phosphorylation site (Asp<sup>809</sup>) in the cytoplasmic domain connected to H5 (Fig. 5).

**Structural Functional Significance of Asp<sup>804</sup> and Asp<sup>808</sup>—**Previous studies have shown that other transmembrane glutamic and aspartic residues do not seem to contribute to the cation binding cage in the Na,K-ATPase (17–22, 24). Asp<sup>804</sup> and Asp<sup>808</sup> are unique carboxyl residues in the transmembrane region of the Na,K-ATPase that may be part of the cation binding sites of the enzyme.

Models describing the cation binding sites of the Na,K-ATPase often implicate two negatively charged residues within the structure of the binding pocket. These proposed models were based on earlier studies which indicate that the transport.
of potassium cations is voltage independent; whereas, the translocation of sodium ions is voltage dependent (13). Therefore, if K⁺ movement is electroneutral, two negative charges are required to balance the charges of the two K⁺ ligands (12, 13). Thus, it is tempting to hypothesize that Asp⁸⁰⁴ and Asp⁸⁰⁸ are these two negative charges. Although this simple explanation is inviting, recent reports have shown that both K⁺ and Na⁺ transport are voltage dependent, challenging the number of negatively charged residues involved in the binding sites, i.e., three versus two (49, 50). In addition, the dielectric constant of the membrane spanning environment is unknown which limits the prediction of whether Asp⁸⁰⁴ or Asp⁸⁰⁸ exist in a protonated or charged form. In fact, high pKₐ values have been determined for transmembrane carboxyls not involved in salt bridges or proton transport (in conjunction with a proton-acceptor group) (51). The current understanding of the enzyme structure does not allow us to hypothesize on pKₐ values for these carboxyls or their possible neutralizing role during cation transport. However, the mutagenesis and characterization studies shown here, definitively show that the side chains of Asp⁸⁰⁴ and Asp⁸⁰⁸ are important in either chemical form (protonated or charged) for the transport of K⁺.

In accordance with our data, the essential role of Asp⁸⁰⁴ and Asp⁸⁰⁸ in cation coordination or transport is supported by the conservation of these amino acids in all species and isoforms of the Na,K-ATPase (4). In addition, Asp⁸⁰⁸ is also conserved in other P-type ATPases while Asp⁸⁰⁴ is replaced with Asn in the SR Ca-ATPase, Glu in the H,K-ATPase, and is absent in the plasma membrane Ca-ATPase (51, 52). Moreover, it must be noted that all of the substituted proteins, with the exception of the conservative substitution, Asp⁸⁰⁸ → Glu, resulted in a similar loss of K⁺-antagonism of ouabain binding. In the case of Glu⁷⁷⁹ and Glu⁷⁷⁹, two residues which are important for cation dependent steps but not in the direct coordination of K⁺, different substitutions resulted in varied effects on ouabain binding or Na,K-ATPase activity (20, 24). Thus, both the conservative nature of these residues and the consistent effects resulting from the replacement of these residues suggest their essential role in coordinating K⁺ during transport.

The stoichiometry of transport requires the simultaneous binding and occlusion of three Na⁺ or two K⁺. Thus, three or two cation binding sites consisting of several coordinating residues are required within the structure of the Na,K-ATPase. One question arises, are Asp⁸⁰⁴ and Asp⁸⁰⁸ part of the same site or do they participate in two separate binding sites? In the case of the SR Ca-ATPase it has been proposed that while Asn⁷⁸⁶ (Asp⁸⁰⁴) is part of the “cytoplasmically facing sites,” Asp⁸⁰⁰ (Asp⁸⁰⁸) is part of the two Ca²⁺ occlusion sites (53). Our analysis of Asp⁸⁰⁴ and Asp⁸⁰⁸ does not allow the discrimination between different cation binding sites. However, we have observed a significant reduction in the Hill coefficient for Na⁺ interactions with the substituted proteins. This suggests that either the cooperativity between the Na⁺ sites is changed or that only two Na⁺ interact with the substituted enzymes. Taken together with our results on K⁺ interactions with these proteins, the second case implies a model in which: (a) each carboxyl is part of a separate binding site and the replacement of one knocks out its corresponding cation site; (b) binding of a single K⁺ does not dephosphorylate the enzyme or prevent ouabain binding; and (c) binding of the two Na⁺ dephosphorylates the enzyme and prevents ouabain binding. Evidence from previous studies that supports this model includes: (a) Mg-Pi can phosphorylate the E₂ form of the enzyme when only one K⁺ site is occupied (54); and (b) two Na⁺ ions can drive the dephosphorylation of E₂P in the Na-ATPase mode of the enzyme (i.e. in the absence of K⁺) (55–57).

Although the substitutions of Asp⁸⁰⁴ and Asp⁸⁰⁸ demonstrate similar effects on K⁺-antagonism of ouabain binding to the extracellular surface, these substitutions vary in two specific ways with respect to their effects on the ATP/Pi binding domain. First, the aspartic acid at position 808 can be replaced with a glutamic acid and retain Na,K-ATPase activity (27). Second, all replacements of Asp⁸⁰⁴, including the conservative change of Asp⁸⁰⁴ → Glu, destabilize the E₂ conformation as displayed by their reduced apparent affinities for Pᵢ. Thus, the functional roles of the carboxyl side chains at positions 804 and 808 overlap with respect to cation binding but differ in their effects on conformational transitions. A carboxyl moiety at position 808 is crucial for cation binding and ATPase turnover. However, both the carboxyl moiety and the side chain length at position 804 are required for cation binding and the conformational changes induced by K⁺. Thus, whether these residues are part of a single cation site or separate sites it appears that K⁺ interactions with Asp⁸⁰⁴ are crucial for stabilization of the E₂ conformation. In this respect, several investigators have noted a biphasic K⁺ interaction with the Na,K-ATPase in which a high affinity site (which plays a conformational role) can be distinguished from a lower affinity site (58–60). For example, a single high affinity K⁺ site can alter the fluorescence of an fluorescein isothiocyanate-labeled form of the Na,K-ATPase (59) and protect the ATP-binding domain from modification by 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (60). Na⁺ interactions reverse these effects. Our data suggest that this high affinity cation site would involve Asp⁸⁰⁴ linking this.
transport site to the P, and ouabain interaction sites.

Structural Connection between the Ouabain, the Cation, and the Phosphorylation Sites on the Na,K-ATPase—Both Asp$^{804}$ and Asp$^{808}$ are located in the sixth transmembrane fragment of the Na,K-ATPase $\alpha$ subunit (Fig. 5). The hairpin formed by the fifth and sixth transmembrane domains seems to play a fundamental role in cation binding (Ref. 61 and work presented), ouabain binding (25, 45), and the voltage dependence of the enzyme (24). Furthermore, evidence exists in support of the idea that this domain moves during the enzyme catalytic cycle as it is triggered by phosphorylation of the protein (9, 10, 62). The role of Asp$^{804}$ and Asp$^{808}$ in coordinating cations during transport supports the structural model for the Na,K-ATPase depicted in Fig. 5 where: (a) the H5-H6 hairpin forms part of the cation occlusion cage; (b) the energy release upon ATP hydrolysis is translated to the cation binding site(s) through the fifth transmembrane domain; and (c) the movement of this hairpin through enzyme phosphorylation/dephosphorylation or cation binding influences ouabain binding through contact points in the H5-H6 extracellular loop.

In summary, the functional characterization of enzymes with modifications in Asp$^{804}$ and Asp$^{808}$ showed parameters similar to the wild type Na,K-ATPase with the exception of their interaction with cations. In particular, the interaction with K$^+$ is removed upon substituting either Asp$^{804}$ or Asp$^{808}$. Thus, we conclude that these residues are part of the cation coordination cage of the Na,K-ATPase linking the ouabain, phosphate, and cation binding domains of this protein.

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