Critical effects on catalytic function produced by amino acid substitutions at Asp^{804} and Asp^{808} of the α1 isoform of Na,K-ATPase

James W. Van Huysse, Theresa A. Kuntzweiler, Jerry B. Lingrel*

Dept. of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0524, USA

Abstract At two intramembrane carboxyl-containing amino acids of the sheep α1 isoform of Na,K-ATPase (Asp^{804} and Asp^{808}), both charge-conserving (Asp to Glu) and charge-deleting (Asp to Asn, Leu and Ala) replacements were made and the altered enzymes studied. Nucleotide changes encoding the amino acid substitutions were placed in a cDNA encoding a ouabain-resistant enzyme (sheep α1 RD) and the encoded enzymes were expressed in ouabain-sensitive HeLa cells. Transfections with cDNAs carrying all Asp^{804} substitutions, along with those carrying Asp^{808}Ala, Asp^{808}Asn, and Asp^{808}Leu replacements failed to confer ouabain resistance to the cells, indicating critical roles for Asp^{804} and Asp^{808}. Only the expression of the Asp^{808}Glu enzyme produced ouabain-resistant HeLa cells, demonstrating that the altered protein was functional. When the inactive proteins Asp^{804}Ala and Asp^{808}Ala were expressed using an alternative selection system (the protein carrying the amino acid substitution was the ouabain-sensitive wild-type sheep α1 Na,K-ATPase, which was expressed in ouabain-resistant 3T3 cells), intact cells were able to bind extracellular ouabain with high affinity ($K_d = 1$–30 nM), indicating that the inactive proteins were synthesized and folded properly in the plasma membrane. The results demonstrate that carboxyl side chains at positions 804 and 808 are critical for enzyme catalytic function.

Key words: Na,K-ATPase; Site-directed mutagenesis; Cation dependence; Ouabain; Protein processing

1. Introduction

The Na,K-ATPase is a membrane-spanning enzyme that is essential for the survival of higher eukaryotic cells. This protein transports Na$^+$ and K$^+$ across the plasma membrane in a ratio of 3:2, creating ionic gradients which in turn drive physiological processes by cotransport or countertransport of the cations with other substances. For example, the sodium gradient provides the impetus for the translocation of glucose, amino acids and other ions across the plasma membrane via sodium-coupled transport [1–3]. Thus, physiological functions such as the absorption of solutes and water across the gastrointestinal epithelium or the reabsorption of these substances across the epithelial membranes of the kidney depend upon the ionic gradients produced by the Na,K-ATPase. In addition, the enzyme contributes to the establishment of membrane potential [4,5] and is critical for the regulation of cell volume and osmotic pressure. Finally, the Na,K-ATPase is the receptor for cardiac glycosides, mediating the effects of these drugs on cardiac function.

In order to comprehend the mechanism by which Na,K-

ATPase functions, it is essential to determine which residues bind the transported cations. Early studies suggested that the cation ligating amino acids are located within the plasma membrane itself, and some investigators used lipid-soluble chemical agents that insert into the plasma membrane and selectively bind amino acids with negatively charged carboxylate side chains [6–8]. Several of these lipophilic chemical reagents produce an inhibition of the enzyme that is prevented in the presence of the cations, suggesting that the cation binding site is a carboxylate amino acid within the plasma membrane. In a similar study, microsomal membrane preparations were trypsinized, leaving only the resistant membrane-embedded segments, which still occlude Na$^+$ and K$^+$, and the occlusion is inhibited by a lipophilic chemical modifier that binds carboxylate residues [9,10]. Furthermore, the resistant portions of the untreated enzyme can still passively transport potassium. Thus, it appears that the membrane carboxylate amino acids are critical for cation binding and transport.

Both conservative and non-conservative amino acid substitutions have been made at nearly all of the putative membrane carboxylate residues, which are illustrated in Fig. 1. We and others have replaced the amino acid corresponding to Glu^{327} of the sheep α1 subunit with alternative residues. Jewell-Motz and Lingrel [11] made the initial observation that a substitution at this site to an alanine or aspartic acid has a critical effect on enzyme function, as evidenced by failure of a ouabain-resistant enzyme carrying each substitution to confer ouabain resistance to HeLa cells. The same study also showed that the enzyme is functional when a glutamine is substituted, and that the cation dependence properties of the functional mutant were not dramatically altered. In addition, Vilsen [12] reported a normal turnover for a Glu^{327}Gln mutant, and even a radical leucine substitution also produced a functional enzyme [11]. Finally, studies of the non-functional mutants with substitutions at position 327 are consistent with the importance of this site in conformational transitions, but not necessarily in cation binding per se [14].

The carboxyl groups of most other membrane negatively charged residues are not required for normal or nearly normal function. Glu^{953} and Glu^{954} in the putative H9 transmembrane segment have been replaced both conservatively [15] and non-conservatively [16], and there is no significant effect on Na$^+$ or K$^+$ dependence of ATPase activity, even when both residues are substituted simultaneously. Likewise, Jewell-Motz and Lingrel [11] showed that amino acid substitutions eliminating the negative charge and side chain oxygens at Asp^{926} produce a functional enzyme with mild alterations in the cation dependence of Na,K-ATPase activity. Finally, Feng and Lingrel [17] and Vilsen [18] showed that substitutions corresponding to Glu^{779}Ala of the sheep α1 isoform have only mild effects on the cation dependence properties

*Corresponding author. Fax: (1) (513)-558-1190.
E-mail: Lingrejb@uc.edu

and turnover of the enzyme. Therefore, neither the negative charge nor the side chain oxygens at positions 327, 779, 925, 953 and 954 are essential for enzyme function, and no single amino acid of this group is absolutely required for the enzyme to bind and transport cations.

At the present time there are two putative membrane-embedded carboxylate amino acids (Asp[804] and Asp[808]) where the effects of substitutions on enzyme function have not been examined in detail. The goal of the studies presented here is to evaluate the importance of these two residues. In the current study, enzymes with substitutions at Asp[804] and Asp[808] were initially expressed in a system where relatively high levels of protein have been obtained [19], i.e. the ouabain-resistant sheep α1 RD isoform expressed in HeLa cells. This makes it possible to determine (1) whether the mutant enzymes were active and if so, (2) whether the functional properties of the active mutant proteins were altered. For enzymes with radical substitutions that were found to be inactive (Asp[804]Ala and Asp[808]Ala), a different selection system was used (i.e. expression of a ouabain-sensitive wild-type sheep α1 Na,K-ATPase in ouabain-resistant 3T3 cells), in order to determine whether the inactive proteins were produced and folded properly into the plasma membrane, based on the ability of intact cells expressing the altered proteins to bind ouabain with high affinity.

2. Materials and methods

2.1. Mutagenesis and subcloning

Mutations encoding the amino acid substitutions Asp[804]Ala, Asp[804]Glu, Asp[804]Leu, Asp[804]Asn, Asp[808]Ala, Asp[808]Glu, Asp[808]Leu and Asp[808]Asn were introduced [20] into a 763-base *HindIII/BglII* cassette (spanning the sequence encoding Lys[691] to Ile[946]) which had been excised from the sheep α1 RD cDNA and subcloned into an M13mp18 vector. After base changes encoding the amino acid substitutions at Asp[804] and Asp[808] were made, the mutant cassette was sequenced in order to verify the changes and to ensure that no additional changes occurred due to polymerase errors. The cassette was subsequently subcloned into a pKC4 eukaryotic expression vector containing the remainder of the sheep α1 RD cDNA and was also subcloned into a pKC4 vector containing the analogous sequence of the wild-type sheep a1 cDNA, i.e. one encoding the native protein without the Asp[111]Arg and Asn[122]Asp substitutions. The former cDNA encodes a ouabain-resistant Na,K-ATPase, while the latter encodes a ouabain-sensitive protein. Since both ends of the *HindIII/BglII* cassette occur within the cDNA, the points of ligation were sequenced after the subcloning step, in order to ensure that there were no bases gained or lost.

2.2. Expression of amino acid-substituted enzymes to determine activity

Untransfected HeLa cells were grown in Dulbecco's modified eagle medium with 10% calf serum and antibiotics (100 U/ml penicillin G, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B), and cells were incubated at 37°C in 10% CO_2. Transfections with mutant RD sheep α1 constructs were carried out using a previously described method [21,22]. 2 days after removal of the precipitated DNA, ouabain was added to the media to achieve a final concentration of 1 μM and all media contained this concentration thereafter. After colonies were clearly established, but before they began to show evidence of reseeding, they were isolated and expanded into stable cell lines. At least four independent clones were picked for each transfection that produced ouabain-resistant cells.

2.3. Sequence analysis of the DNA in ouabain-resistant HeLa cells

In order to confirm that the cDNA carried by the ouabain-resistant HeLa cells contained the appropriate nucleotide substitution, DNA was isolated from the resistant cells and a segment of the integrated cDNA encoding the putative H6 transmembrane domain was amplified by PCR and sequenced. Cells were grown to confluence on a 24-

well plate and the DNA was extracted [23]. The above segment of DNA was amplified in two separate rounds of thermal cycling, using an initial set of primers that spanned the sequence encoding amino acids 624–1004, followed by a second round, using primers internal to the first set (amino acids 667–961). The sense primer for the first round was 5' CGT GGG CAT CAT CTC AGA AGG G 3', while the antisense primer was 5' CGT CGC CTG ATG ATG AGC TTC C 3'. In the second reaction, the primers were ACT CCC GAG CAA CTG GAT G and GAA AGG AAA GCA GCA AGG in the sense and antisense strands, respectively. The first reaction was carried out using standard PCR buffer (Boehringer-Mannheim), 1 pM primers and 20% of the DNA extracted from one well that was confluent with cells. The denaturation, annealing and extension conditions were 94°C (30 s), 60°C (1 min) and 72°C (1 min) respectively, repeated for seven cycles. The second round was carried out using 10 μl of the first reaction and the same conditions except that the annealing temperature was 55°C and the reaction was repeated for 35 cycles. The samples containing amplified DNA were then filtered with a Centricon-100 microfiltration apparatus and analyzed with an automated sequencer.

2.4. Expression of inactive enzymes to examine [^{3}H]ouabain binding

A recently developed system for the selection of cells expressing non-functional enzymes in 3T3 cells [14] was used to determine whether the substituted enzymes were processed correctly. This protocol involves the expression of a Na,K-ATPase which binds ouabain (sheep α1 wild-type) in 3T3 cells containing an endogenous enzyme (rodent α1) which exhibits a 1000-fold lower affinity for cardiac glycosides. The 3T3 cells were grown under identical conditions used for HeLa cells, except that the 3T3 cells were incubated in 5% CO_2. Mutations encoding the desired amino acid substitutions were placed in a cDNA encoding the wild-type sheep α1 enzyme and the cDNA was transfected. Since the wild-type sheep α1 enzyme is ouabain-sensitive and thus is unable to confer ouabain resistance, the ouabain selection scheme could not be used to select stable transfectants. Instead, 1 μg of a vector carrying a neomycin resistance gene (pMCl/ Neo) was co-transfected along with 25 μg of the sheep α1 cDNA construct encoding the mutant enzyme. On the day that the precipitation of DNA was terminated, cells on a p-100 plate were washed 3 times, trypsinized, and 1% were then transferred to a p-150 plate containing 25 ml of media with 0.6 mg/ml of the neomycin analog G418. Continuous selection with G-418 was applied from this point. After approx. 10 days of growth, neomycin-resistant (G418-resistant) colonies were isolated and placed on duplicate 24-well plates. Colonies of cells which coexpressed the sheep α1 Na,K-ATPase were identified by screening one plate with an anti-sheep α1 antibody which does not cross-react with the endogenous 3T3 Na,K-ATPase (whole-cell Western analysis). Cells lines which were positive for coexpression of the sheep α1 enzyme were then expanded from the second 24-well plate, and the ability of whole cells to bind [^{3}H]ouabain was examined as previously described [14].

2.5. Crude plasma membrane preparations of HeLa cells

Microsomal membranes were prepared as described previously [24], except the final pellet was resuspended in 10 ml of 1 mM EDTA (buffer B, pH adjusted to 7.5 with Tris) using a glass Dounce homogenizer, and centrifuged at 48 000×g for 30 min at 4°C. The supernatant was discarded, the pellet was resuspended in 1.0 ml of buffer B, and the resuspension was stored at −80°C until the time of assay. Protein concentration was measured using a previously described method [25].

2.6. Quantification of exogenous protein by immunoblot assay

The amounts of sheep α1 RD and Asp[808]Glu enzymes expressed in microsomal membrane preparations were determined using an antibody that reacts specifically with the sheep α1 enzyme and does not cross react with the human (HeLa) Na,K-ATPase [26]. Membrane preparations were spotted onto nitrocellulose filters and probed as previously described [19], and the concentration of the exogenous protein was calculated by densitometric comparison of the standard and sample signals following autoradiography.

2.7. ATPase assays

In order to measure ATPase activity, the malachite green assay of Lanzetta et al. [27] was used to measure the inorganic phosphate released after incubation of 1 μg (total protein) of each membrane
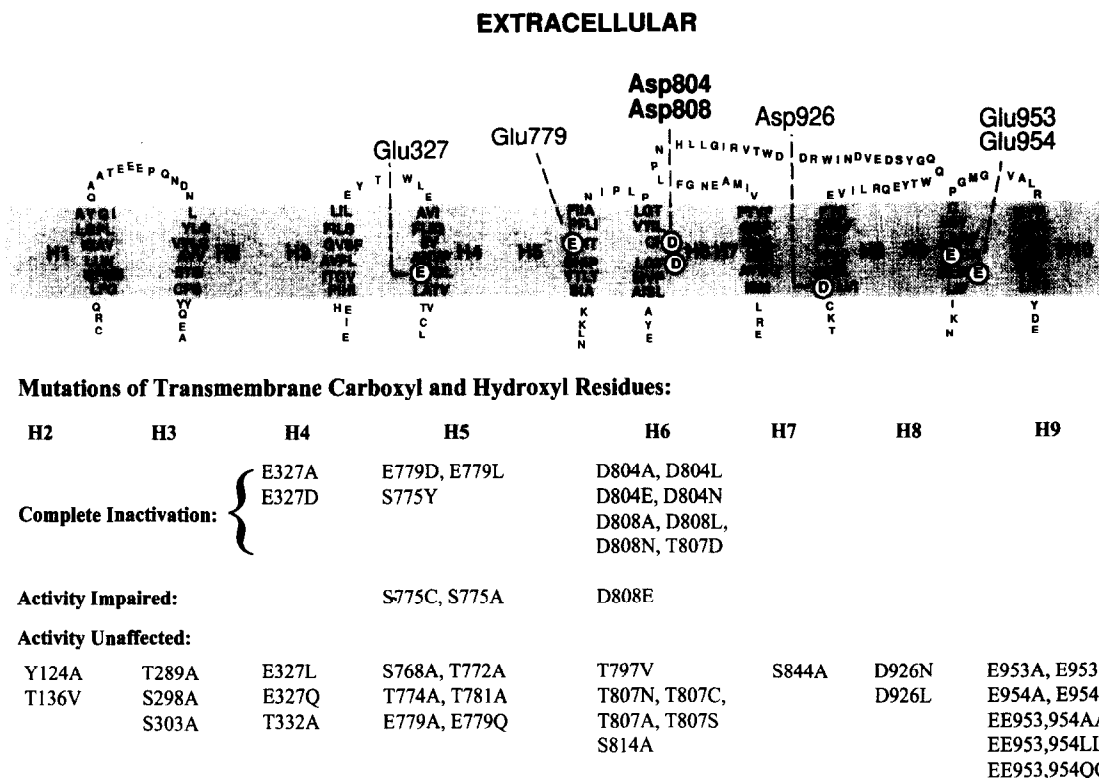
EXTRACELLULAR

Asp804
Asp808   Asp926   Glu953
                                   Glu954
Glu327   Glu779

Mutations of Transmembrane Carboxyl and Hydroxyl Residues:

H2	H3	H4	H5	H6	H7	H8	H9
Complete Inactivation:		E327A E327D	E779D, E779L S775Y	D804A, D804L D804E, D804N D808A, D808L, D808N, T807D			
Activity Impaired:			S775C, S775A	D808E			
Activity Unaffected:							
Y124A	T289A	E327L	S768A, T772A	T797V	S844A	D926N	E953A, E953Q
T136V	S298A	E327Q	T774A, T781A	T807N, T807C,		D926L	E954A, E954Q
	S303A	T332A	E779A, E779Q	T807A, T807S			EE953,954AA
				S814A			EE953,954LL
							EE953,954QQ

Fig. 1. Amino acids of the Na,K-ATPase located within the plasma membrane and containing a carboxylate side chain, based on a 10 transmembrane model derived primarily from hydropathy data. The amino acids in the H6 transmembrane domain were examined in the present study (shown in bold). A summary of the functional consequences of amino acid substitutions within each transmembrane region is included. Criteria for each functional category were as follows. Complete inactivation: failure of a cDNA encoding a ouabain resistant enzyme and also encoding the indicated amino acid substitution to confer ouabain resistance to ouabain-sensitive cells, following transfection. Activity impaired: greater than a 10-fold change in the apparent affinity for at least one cation or greater than a 50% reduction in turnover rate. Activity unaffected: none of the above. Data for Glu[327] were derived from [11–13,18]; for Glu[779]: [11,13,17,18]; for Asp[804] and Asp[808]: [11,13,18] and the present study; Thr[807], unpublished observations and [18]; Asp[926]: [11]; Glu[953] and Glu[954]: [15,16]; all others: [13].

preparation for 30 min at 37°C. Ouabain inhibition curves were initially performed in order to determine the I_{50} for the endogenous HeLa, RD sheep α1 and Asp[808]Glu mutant enzyme in the current system. Conditions for these determinations were (final concentration): 40 mM histidine (pH 7.5), 2 mM MgCl$_2$, 0.1 mM EGTA, 130 mM NaCl, 20 mM KCl, 2 mM ATP and 1 μg (total protein) of microsomal membrane preparation per tube. The ATPase activity was determined at various concentrations of ouabain ranging from zero to 1×10^{-2} M, and the residual ATPase activity at 10^{-2} M ouabain was defined as background. The reactions were carried out in a total reaction volume of 250 μl, following preincubation in ouabain for 30 min at 4°C.

After it was established that the endogenous HeLa Na,K-ATPase activity could be completely inhibited at a ouabain concentration of 1×10^{-5} M without affecting the activity of the RD sheep α1 or mutant Asp[808]Glu proteins (Fig. 3), all subsequent studies were carried out at 1×10^{-5} M ouabain. This eliminated the activity of the endogenous HeLa enzyme, while at the same time preserving the activity of the enzyme encoded by the transfected cDNAs. In Na$^+$ stimulation studies, the concentration of NaCl was varied from zero to 100 mM, while the K$^+$ concentration was kept constant at 20 mM. The conditions for NaCl dependence were the same as for ouabain inhibition curves, except that the NaCl concentration was varied and choline chloride was added to keep the ionic strength (Na$^+$, choline and K$^+$) fixed at 150 mM. For K$^+$ stimulation studies, the KCl concentration was varied from 0 to 20 mM, while the NaCl concentration and ionic strength were fixed at 130 and 150 mM, respectively. Data for the sodium and potassium dependence of ATPase activity were analyzed by non-linear regression analysis, using both cooperative and non-cooperative models of binding for curve fitting [16].

3. Results

For all of the amino acid substitutions in this study a rapid screen was used to determine whether the mutant enzyme was functional. This screen was based on the expression of a ouabain-resistant enzyme in HeLa cells, which have a ouabain-sensitive endogenous Na,K-ATPase. Mutations encoding the desired amino acid substitutions were made in a cDNA carrying the ouabain-resistant sheep α1 RD isoform. Since HeLa cells require a functional Na,K-ATPase for cellular homeostasis, and since their endogenous Na,K-ATPase is inhibited by ouabain, they will not survive and form colonies unless the exogenous enzyme is active when the cells are selected in 1 μM ouabain. Thus, the presence of colonies demonstrates that the amino acid substituted enzyme is functional, while the absence of colonies indicates that the exogenous enzyme is inactive or is critically impaired.

Using the above selection system, transfections with all cDNA constructs encoding amino acid substitutions at Asp[804] failed to produce ouabain-resistant colonies, demonstrating that only the wild type aspartic acid will produce enough enzyme activity to confer ouabain resistance and thus position 804 in the sheep α1 RD enzyme is a critical site for enzyme function. Likewise, transfections with cDNAs carrying the substitutions Asp[808]Ala, Asp[808]Asn and
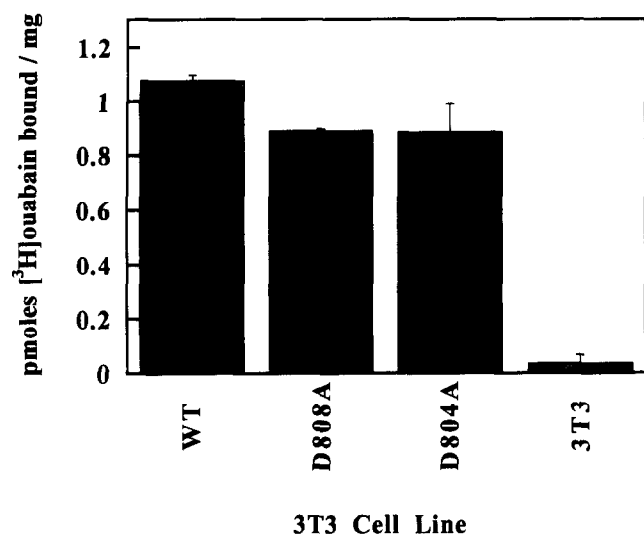
3T3 Cell Line

Fig. 2. Binding of [^{3}H]ouabain to intact 3T3 cells that were untransfected (3T3) or transfected with a cDNA encoding the wild-type sheep α1 Na,K-ATPase (WT) or the sheep α-subunit carrying the amino acid substitutions Asp^{804}Ala (D804A) and Asp^{808}Ala (D808A). Conditions for binding were the same as those described in [14] except that the concentration of [^{3}H]ouabain was 70.6 nM. Data shown are the mean ± S.E.M. of two assays performed in triplicate on at least one 3T3 clone for each cell line.

Asp^{808}Leu failed to produce ouabain resistant colonies, demonstrating a critical effect of the encoded substitution in each case. None of these transfections yielded ouabain resistant colonies, even at ouabain concentrations as low as 0.2 μM and potassium ion concentrations as high as 20 mM, while all of the positive-control transfections (sheep α1 RD cDNA) resulted in at least 10 ouabain resistant colonies per p-100 plate. On the other hand, expression of the enzyme with the Asp^{808}Glu substitution produced colonies resistant to 1 μM ouabain, establishing that this protein is functional (transfections with the Asp^{808}Glu cDNA required several weeks to produce ouabain resistant colonies). Negative-control transfections did not yield any ouabain-resistant colonies, demonstrating the absence of spontaneous resistance in this system.

To determine whether the inactivation of the enzyme with substitutions at Asp^{804} and Asp^{808} was due to altered protein synthesis or processing, a separate system was used to express the recombinant α subunits (expression of the ouabain-sensitive wild-type sheep α1 Na,K-ATPase in 3T3 cells). With this system, [^{3}H]ouabain can bind to the exogenous enzyme without interference from the endogenous 3T3 Na,K-ATPase [14], and high-affinity binding to the extracellular surface of intact cells would suggest that the protein is synthesized and folded properly in the plasma membrane. When ouabain binding was measured in preparations of whole cells (Fig. 2), the specific binding of [^{3}H]ouabain to cells expressing α subunits with radical substitutions (Asp^{804}Ala and Asp^{808}Ala) was at least 5-fold greater than non-specific binding. Furthermore, the binding was of high affinity (K_d from 1 to 30 nM for wild-type and both substituted enzymes), demonstrating that ouabain was bound as tightly in the Asp^{804}Ala and Asp^{808}Ala mutants as the wild-type enzyme. Therefore, the substitutions of Asp^{804} and Asp^{808} did not alter the synthesis or targeting of the α subunit, and the inactivation of the enzyme was due to an effect on the catalytic cycle of the protein.

Since the expression of the RD sheep α1 enzyme containing the Asp^{808}Glu substitution conferred ouabain resistance to HeLa cells, stable cell lines were established under ouabain selection and the cells harvested for protein and DNA analysis. The DNA from each line was extracted and the region encoding the H6 transmembrane segment was amplified by PCR and sequenced, in order to confirm the that the transfected cDNA encoded the Asp^{808}Glu substitution. Five lines from the Asp^{808}Glu transfections were examined, and all exhibited a GAG codon (glutamic acid) for the sequence encoding the amino acid at position 808, while all lines from the sheep α1 RD transfections had a GAC sequence at this site (aspartic acid).

In order to examine the functional role of Asp^{808}, we characterized the Na,K-ATPase with the Asp^{808}Glu substitution. In order to rule out gross changes in ouabain sensitivity produced by the amino acid replacement, the inhibition of the mutant enzyme by ouabain was examined (Fig. 3). The inhibition curve for the mutant was biphasic, indicating two com-
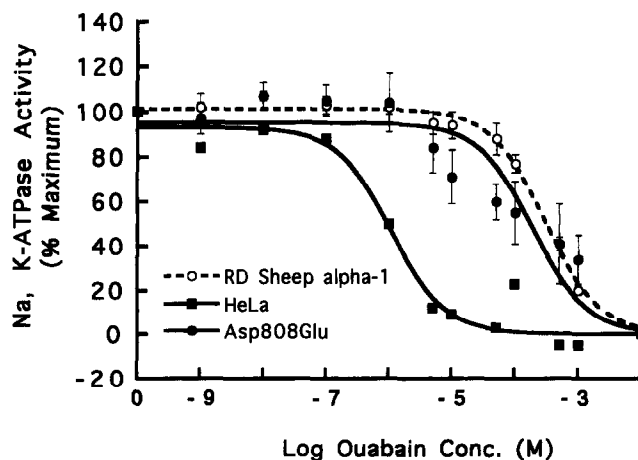

Log Ouabain Conc. (M)

Fig. 3. Ouabain sensitivity of Na,K-ATPase activity in microsomal membrane preparations isolated from HeLa cells which were untransfected (HeLa) or transfected with a sheep α1 RD cDNA which was either unmutated (RD Sheep alpha-1) or mutated to encode the Asp^{808}Glu amino acid substitution (Asp^{808}Glu). ATPase activity at 10^{-2} M ouabain was equal to that under Na$^+$-free conditions, and thus was defined as zero Na,K-ATPase activity. The Na,K-ATPase activity at each ouabain concentration was divided by that in the absence of ouabain, and this quotient was multiplied by 100 to calculate % Na,K-ATPase activity. Values for each enzyme are the mean ± S.E.M. of an assay performed in triplicate on membrane preparations from 3 HeLa clones (RD sheep α1 and Asp^{808}Glu) or from a single clone (HeLa).
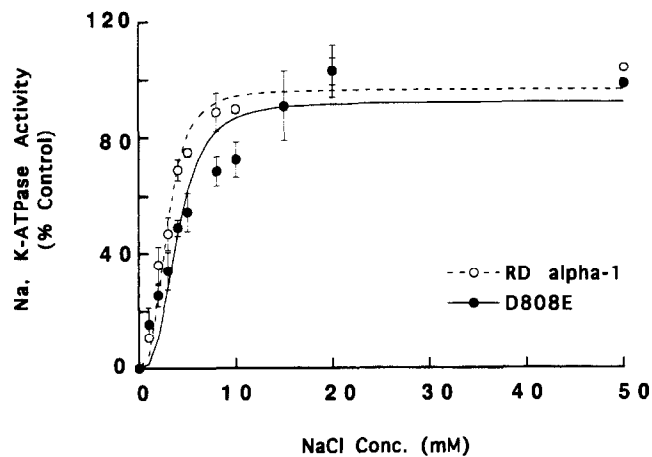
Fig. 4. Sodium dependence of exogenous Na,K-ATPase activity in crude plasma membrane preparations isolated from HeLa cells that were transfected with a sheep $\alpha 1$ RD cDNA which was either unmutated (RD alpha-1) or mutated to encode the $Asp^{808}Glu$ amino acid substitution (D808E). Values for each mutant are the mean ± S.E.M. from assays of membrane preparations from three separate HeLa cell lines, each derived from a single HeLa colony. Within each membrane preparation, the data were analyzed by non-linear regression according to a cooperative model of Na^+ binding [15] and the V_{max} was calculated. The value at each NaCl concentration was divided by the V_{max} for that preparation, and this ratio was multiplied by 100 to give the % control.

ponents of ouabain binding (HeLa and sheep $\alpha 1$ RD). However, both the $Asp^{808}Glu$ and sheep $\alpha 1$ RD proteins had similar overall I_{50}s for ouabain (approx. 10^{-4} M), more than 1000-fold greater than that for the endogenous HeLa protein. Thus, any potential effect of the amino acid substitution on ouabain sensitivity would be relatively small.

The sodium and potassium ion dependence properties of the mutant and RD sheep $\alpha 1$ Na,K-ATPases were also examined (Figs. 4 and 5). The $K_{0.5}$ of the $Asp^{808}Glu$ mutant for sodium (5.07 ± 0.91 mM NaCl) was slightly greater than that of the RD sheep $\alpha 1$ protein (2.85 ± 0.21 mM), while the $K_{0.5}$s for potassium were nearly identical ($Asp^{808}Glu$: 0.59 ± 0.10 mM NaCl vs. 0.65 ± 0.07). The turnover rate (2037 ± 287 min^{-1}) and specific activity (0.97 ± 0.32 µmol/min per mg exogenous protein) of the mutant were reduced, compared to their respective control levels (13 159 ± 1962 min^{-1} and 4.56 ± 0.96 µmol/min per mg). The mechanisms of these reductions cannot be deduced from the current data.

4. Discussion

The present study has identified two critical intramembrane charges at positions 804 and 808 of the Na,K-ATPase, since all substitutions made at position 804 and all nonconservative replacements at position 808 inactivated the protein. Only the conservative change of $Asp^{808}Glu$ resulted in an active enzyme, but even this mutant had a markedly reduced turnover rate and specific activity. Previously, the amino acids Glu, Asn and Leu were substituted at position 804, as were Leu and Asn at position 808 [11,18]. Although each of the earlier replacements produced an inactive Na,K-ATPase, the loss of enzyme function could have been due to alterations in the catalytic cycle, protein synthesis or enzyme processing. In order to determine whether the mutant α-subunits were synthe-

sized and folded correctly into the plasma membrane, two radical substitutions, $Asp^{804}Ala$ and $Asp^{808}Ala$, were made in a wild-type sheep $\alpha 1$ Na,K-ATPase (ouabain-sensitive) and the altered proteins were expressed in 3T3 cells (ouabain-resistant). Since intact 3T3 cells expressing the $Asp^{804}Ala$ and $Asp^{808}Ala$ mutants bound ouabain with high (nM) affinity, the altered enzymes must have been processed correctly into the plasma membrane. Thus, the inactivation of the enzyme by amino acid substitutions at positions 804 and 808 is not due to disruptions in protein synthesis or processing, but must instead be due to direct effects on catalytic function.

Asp^{804} and Asp^{808} are unique among the membrane-embedded, negatively charged amino acids of the Na,K-ATPase (Fig. 1) in that a negatively charged side chain is essential at both sites. At each of the remaining five such residues (Glu^{327}, Glu^{779}, Asp^{926}, Glu^{953} and Glu^{954} in the sheep $\alpha 1$ Na,K-ATPase), at least two substitutions with amino acids lacking a negative charge have produced a functional enzyme, including at least one substitution at each position with a residue lacking side chain oxygens altogether (Fig. 1 [11–13,15–18]). Therefore, neither the charges of these five intramembrane amino acids nor their side chain oxygens are necessary for enzyme function. Since previous studies with chemical labeling agents have suggested that at least one and possibly two membrane carboxyls are required for cation coordination [6–10,28] and since Asp^{804} and Asp^{808} are the only two essential membrane-embedded carboxylate amino acids, both residues are potential cation binding sites.

The effects of the present site-directed amino acid substitutions at Asp^{804} and Asp^{808} in the sodium pump agree with the effects of alterations at the corresponding residues in the sarcoplasmic reticulum (SR) Ca-ATPase. The two proteins contain several homologous sequences, and in the SR Ca-ATPase, the amino acids corresponding to Asp^{804} and Asp^{808} of the sodium pump are known to be essential sites for enzyme function, since even conservative substitutions abolish the ability of the enzyme to transport Ca^{2+} [29–34]. In addition, these two amino acids are Ca^{2+} binding sites. Thus, due
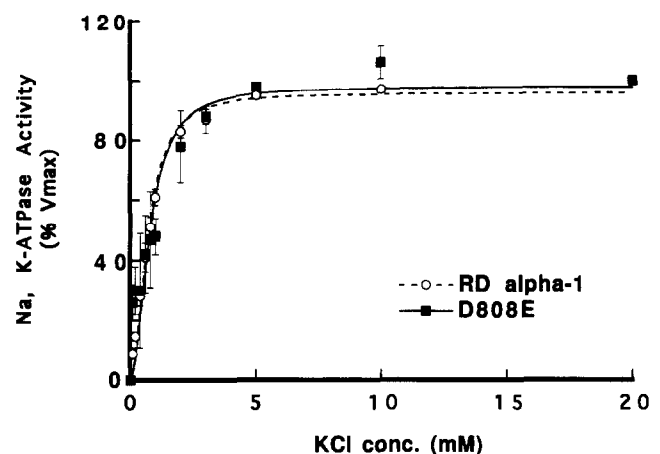

Fig. 5. Potassium dependence of exogenous Na,K-ATPase activity in crude plasma membrane preparations isolated from HeLa cells that were transfected with a sheep $\alpha 1$ RD cDNA which was either unmutated (RD alpha-1) or mutated to encode the $Asp^{808}Glu$ amino acid substitution (D808E). Values for each mutant are the mean ± S.E.M. from assays of membrane preparations from three separate HeLa cell lines, each derived from a single HeLa colony. Data were analyzed as described in Fig. 4.

to the conservation of an oxygenated side chain in these AT-Pases, it is possible that Asp[804] and Asp[808] of the Na,K-AT-Pase are coordinated to Na$^+$ and/or K$^+$ during transport. Recently, Adebayo et al. [35] have shown that substitutions at Asn[879] and Asp[883] of the plasma membrane Ca-ATPase, again corresponding to Asp[804] and Asp[808] of the Na,K-AT-Pase, completely abolish enzyme activity. It was proposed that Asn[879] and Asp[883] are cation binding amino acids in this protein.

In contrast, the effects of substitutions at the remaining membrane carboxyls in the Na,K-ATPase do not mimic the effects of similar substitutions in the SR Ca-ATPase. For example, there are two membrane carboxylate amino acids (Glu[309], corresponding to Glu[327] in the Na,K-ATPase and Glu[771], corresponding to Glu[779]) that are required for the function of the SR Ca-ATPase but are not required in the sodium pump [11–13,17,18,29–34], however, it is possible that the binding and/or transport of the divalent Ca^{2+} ion requires additional membrane negative charges that are not needed for the passage of the monovalent Na$^+$ and K$^+$ cations across the plasma membrane. Alternatively, noncarboxylate oxygen-containing side chains in the Na,K-ATPase may substitute for the Glu[309] and Glu[781] carboxyls of the SR Ca-ATPase in coordinating the monovalent cations. Recently, substitutions at Ser[775] of the Na,K-ATPase, located one helical turn from Glu[779], have been shown to have profound effects on the K$^+$ interaction with the enzyme [19]. Ser[775] is a possible K$^+$ binding site in the sodium pump.

Although 7 of 8 amino acid replacements at Asp[804] and Asp[808] produced an inactive enzyme as assayed by the ability of the substituted protein to confer ouabain resistance to ouabain-sensitive HeLa cells, one substituted Na,K-ATPase (Asp[808]Glu) conferred ouabain resistance. This mutant was significantly impaired, with a specific activity and turnover rate reduced to 21% and 15% of control levels, respectively. The cation dependence of Na,K-ATPase activity was essentially normal, and thus the high affinity binding sites for the transported cations appear to be intact. However, the conservative nature of the amino acid substitution precludes the elimination of Asp[808] from consideration as a potential high affinity cation binding site.

These studies indicate the importance of the H6 transmembrane helix in the catalytic activity of the Na,K-ATPase. This segment, as well as the H5 domain, are emerging as regions that are critically important in the catalytic cycle of the enzyme. For example, the folding of the H5-H6 'hairpin loop' of the enzyme (amino acids Gln[736] to Arg[830]) into the plasma membrane is altered following the binding of K$^+$ or Rb$^+$ [36,37], and it is possible that a shift of the H5-H6 region is critical for cation transport. In addition, Ser[775] (H5 transmembrane segment) may be a K$^+$ binding site, as substitutions at this position have large effects on K$^+$ affinity constants [19]. It is also known that the carboxyl-selective chemical reagent 4-(diazomethyl)-7-(diethylamino)coumarin (DEAC) binds to Glu[779] in the H5 transmembrane region and inactivates the enzyme via disruption of Na$^+$ and K$^+$ binding [28], and indeed amino acid substitutions at this site decrease the apparent affinities for both cations [17,18]. Furthermore, the H5-H6 loop contains Phe[786], Leu[793] and Thr[797], which have been shown to be important determinants of ouabain sensitivity [13,38,39]. Finally, the H5-H6 loop may also be important in energy transduction, since phosphoryla-

tion from ATP appears to alter the insertion of this loop into the plasma membrane, as demonstrated by the increased labeling of Glu[779] by DEAC when the enzyme is phosphorylated [28]. Combined with previous studies, our finding of the critical requirements for membrane carboxyls at positions 804 and 808 suggests a role for these residues in the catalytic functions mediated by the H5-H6 domain.

References

[1] Crane, R.K. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 99–159.
[2] Geck, P. and Heinz, E. (1981) in: Membrane Transport (Bonting, S.L. and De Pont, J.J.M.M. eds.) pp. 285–310, Elsevier, Amsterdam.
[3] Hoshi, T. and Himukai, M. (1982) in: Transport and Bioenergetics in Membranes (Sato, R. and Kafawa, Y. eds.) pp. 111–135, Plenum, New York.
[4] Hodgkin, A.L. and Katz, B. (1949) J. Physiol. 68, 37–77.
[5] Hodgkin, A.L. and Horowitz, P. (1959) J. Physiol. 148, 127–160.
[6] Shani-Sekler, M.S., Goldshleger, R., Tal., D.M. and Karlish, S.J.D. (1986) J. Biol. Chem. 263, 19331–19341.
[7] Capasso, J.M., Hoving, S., Tal, D.M., Goldshleger, R. and Karlish, S.J.D. (1992) J. Biol. Chem. 267, 1150–1158.
[8] Arguello, J.M. and Kaplan, J.H. (1991) J. Biol. Chem. 266, 14627–14635.
[9] Karlish, S.J.D., Goldshleger, R. and Stein, W.D. (1990) Proc. Natl. Acad. Sci. USA 87, 4566–4570.
[10] Goldshleger, R., Tal, D.M., Moorman, J., Stein, W.D. and Karlish, S.J.D. (1992) Proc. Natl. Acad. Sci. USA 89, 6911–6915.
[11] Jewell-Motz, E.A. and Lingrel, J.B. (1993) Biochemistry 32, 13523–13530.
[12] Vilsen, B. (1993) Biochemistry 32, 13340–13349.
[13] Feng, J. and Lingrel, J.B. (1994) Biochemistry 33, 4218–4224.
[14] Kuntzweiler, T.A., Wallick, E.T., Johnson, C.L. and Lingrel, J.B. (1995) J. Biol. Chem. 270, 2993–3000.
[15] Van Huysse, J.W., Jewell, E.A. and Lingrel, J.B (1993) Biochemistry 32, 819–826.
[16] Van Huysse, J.W. and Lingrel, J.B. (1993) Cell. Mol. Biol. Res. 39, 597–507.
[17] Feng, J. and Lingrel, J.B. (1995) Cell. Mol. Biol. Res. 41, 29–37.
[18] Vilsen, B. (1995) Biochemistry 34, 1455–1463.
[19] Arguello, J.M. and Lingrel, J.B. (1995) J. Biol. Chem. 270, 22764–22771.
[20] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[21] Chen, C. and Okayama, H. (1987) Mol. Cell Biol. 7, 2745–2752.
[22] Chen, C.A. and Okayama, H. (1988) Biotechniques 6, 632–638.
[23] Laird et al. (1991) Nucleic Acids Res. 19, 4293–4294.
[24] Lane, L.K., Copenhaver, J.H., Lindenmayer, G.E. and Schwartz, A. (1973) J. Biol. Chem. 248, 7197–7200.
[25] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[26] Malik, B., Jamieson, G.A., Jr. and Ball, W., Jr. (1993) Protein Sci. 2, 2108–2111.
[27] Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A. (1979) Anal. Biochem. 100, 95–97.
[28] Arguello, J.M. and Kaplan, J.H. (1994) J. Biol. Chem. 269, 6892–6899.
[29] Clarke, D.M., Loo, T.W., Inesi, G. and MacLennan, D.H. (1989) Nature 339, 476–478.
[30] Clarke, D.M., Loo, T.W. and MacLennan, D.H. (1990) J. Biol. Chem. 265, 6262–6267.

[31] Vilsen, B. and Andersen, J.P. (1992) J. Biol. Chem. 267, 3539–3551.

[32] Vilsen, B. and Andersen, J.P. (1992) J. Biol. Chem. 267, 25739–25743.

[33] Andersen, J.P. and Vilsen, B. (1992) J. Biol. Chem. 267, 19383–19387.

[34] Andersen, J.P. and Vilsen, B. (1994) J. Biol. Chem. 269, 15931–15936.

[35] Adebayo, A.O., Enyedi, A., Verma, A.K., Filoteo, A.G. and Penniston, J.T. (1995) J. Biol. Chem. 270, 27812–27816.

[36] Lutsenko, S., Anerko, R. and Kaplan, J.H. (1995) Proc. Natl. Acad. Sci. 92, 7936–7940.

[37] Arystarkhova, E., Gibbons, D.L. and Sweadner, K.J. (1995) J. Biol. Chem. 270, 8785–8796.

[38] Burns, E.L. and Price, E.M. J. Biol. Chem. (1993) 268, 25632–25635.

[39] Palasis, M., Kuntzweiler, T.A. and Lingrel, J.B. (1996) J. Biol. Chem., in press.