Site-Directed Mutagenesis of a Predicted Cation Binding Site of Na, K-ATPase†

James W. Van Huysse, Elizabeth A. Jewell, and Jerry B. Lingrel*

Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524

Received September 2, 1992; Revised Manuscript Received November 6, 1992

ABSTRACT: Chemical modification and proteolytic digestion studies have identified a transmembrane glutamic acid residue (E953) of the α subunit of the pig kidney Na, K-ATPase as a possible cation binding site [Goldshleger et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6911–6915]. In addition, an adjacent glutamate (E954) is conserved in all species and isoforms and may also be involved in cation binding. To further explore the role of these residues in ion transport, we have utilized a mutagenesis-expression strategy. This approach avoids the introduction of a large chemical moiety into the protein and allows specific amino acid substitutions to be introduced. Glutamic acid residues 955 and 956 of the rat α-1 subunit (corresponding to glutamates 953 and 954 of the pig kidney Na, K-ATPase) were replaced separately and together using site-directed mutagenesis of the rat α-1 cDNA. The mutant cDNAs were expressed in ouabain-sensitive HeLa cells. This system makes it possible to rapidly identify amino acid substitutions which significantly impair enzyme function, as substitutions which do not affect enzyme activity will yield colonies in the presence of ouabain, while substitutions which severely impair function will prevent or limit growth of the ouabain-sensitive HeLa cells. The amino acid replacements (E955Q, E956Q, E955Q–E956Q, E955D–E956D) all resulted in the growth of ouabain-sensitive cells, demonstrating that the modified Na, K-ATPase in each case was functional. To further study the altered enzymes, ouabain-resistant colonies were isolated and expanded into stable cell lines. Ouabain sensitivity and cation stimulation of Na, K-ATPase activity were examined in crude plasma membrane preparations isolated from transfected cells. In membrane preparations from cells transfected with wild-type and all mutant cDNA constructs, ouabain resistance of Na, K-ATPase activity was increased 1000-fold, compared to activity in preparations from untransfected cells. This confirmed the expression of a ouabain-resistant enzyme in all transfected cell lines. In studies of the cation dependence of Na, K-ATPase, activity contributed by the ouabain-sensitive HeLa Na, K-ATPase was eliminated by performing the assays in 5.0 μM ouabain. Cation stimulation of Na, K-ATPase activity was not affected or was only slightly affected in mutants with single amino acid substitutions [$K_{0.5}(Na^+)$ of 3.45 ± 0.22, 2.76 ± 0.22, and 4.92 ± 0.43 mM and $K_{0.5}(K^+)$ of 0.78 ± 0.07, 0.86 ± 0.06, and 0.78 ± 0.09 mM for wild type, E955Q, and E956Q, respectively). The double substitution E955Q–E956Q decreased the apparent affinity for K^+, but not that for Na^+ [$K_{0.5}(Na^+)$ of 4.16 ± 0.11 mM; $K_{0.5}(K^+)$ of 1.45 ± 0.09 mM]. The E955D–E956D substitution had the reciprocal effect, decreasing the apparent affinity for Na^+, with a small effect on apparent K^+ affinity [$K_{0.5}(Na^+)$ of 6.21 ± 0.52 mM; $K_{0.5}(K^+)$ of 1.16 ± 0.08 mM]. The fact that single amino acid substitutions were largely devoid of effect, along with the ability of the enzyme to function even with double amino acid replacements at this site, argues against a prominent role of E955 and E956 in cation binding.

Na, K-ATPase is a membrane protein essential to the survival of most cells of higher organisms. Since the primary function of Na, K-ATPase is the transport of Na^+ and K^+ across cell membranes (Ullrich, 1979; Langer, 1982; Phillipson, 1985), an understanding of the mechanisms of enzyme function requires the identification of sites that bind Na^+ and K^+. Because the ions to be transported are positively charged, it has been suggested that residues with negatively charged carboxyls in their side chains (i.e., glutamic and aspartic acids) play a critical role in the initial binding and translocation of these cations. Studies directed toward the localization of putative cation binding sites within the Na, K-ATPase molecule have used reagents which appear to react specifically with carboxyl groups under certain conditions (Shani-Sekler et al., 1988). Carbodiimides have been the most extensively used compounds for probing potential cation binding sites, although recent evidence suggests that diazomethane analogs may specifically label these residues (Argello & Kaplan, 1991). Under certain conditions, both types of reagents are relatively specific for carboxyl groups, and studies with these compounds have indicated that there is cation-dependent protection against their inactivation of Na, K-ATPase (Robinson, 1974; Yamaguchi et al., 1983; Shani-Sekler et al., 1988; Argello & Kaplan, 1991). In addition, the chemical labeling of only one or two sites of the α chain causes inactivation, suggesting that there are one to two carboxyl groups involved in the cation binding within each α subunit (Argello & Kaplan, 1991; Goldshleger et al., 1992).

Chemical labeling studies with carbodiimides have shown that the presence of Na^+ or K^+ protects against the inactivation of Na, K-ATPase by these agents (Robinson, 1974; Yamaguchi et al., 1983; Shani-Sekler et al., 1988). One explanation for the ability of Na^+ and K^+ to prevent the inhibition of Na, K-ATPase by carbodiimides is that both cation and chemical label bind to the same glutamic or aspartic acid residues, and therefore, Na^+ and K^+ can antagonize the binding of the chemical modification reagent. On the other hand, the ability of other enzyme substrates (such as ATP and Mg^{2+}) to protect against the inactivation of the enzyme by carbodiimides, along with the tendency of carbodiimides to form cross-links within

† Supported by the Program of Excellence in the Molecular Biology of the Heart and Lung at the University of Cincinnati (USPHS Grant HL41496) and by USPHS Grant HL28573.

* To whom correspondence should be addressed.

0006-2960/93/0432-819$04.00/0

proteins (Gorga, 1985a,b; Pedemonte & Kaplan, 1986a,b), argues against the notion of specificity of these agents for cation binding sites and suggests that the observed inhibition is due to steric hindrance by large chemical groups or to the formation of protein cross-links.

Recent evidence suggests that there is a single common binding site for cations and *N,N'*-dicyclohexylcarbodiimide (DCCD) located within a 19-kDa trypsin digestion product of pig kidney Na, K-ATPase (Karlish et al., 1990; Goldshleger et al., 1992). Karlish et al. showed that, after trypsin digestion of purified enzyme in the absence of Ca^{2+} and in the presence of Rb^+, the α subunit is digested, while the β subunit is left intact. The largest tryptic digestion product of the α chain is a 19-kDa membrane-embedded fragment spanning asparagine 831 to the carboxyl terminus. The entire mixture of trypsin-digested products ("19-kDa membranes", which contain the 19-kDa fragment, as well as other fragments) has Rb^+ and Na^+ occlusion capacities similar to those of undigested enzyme (Karlish et al., 1990), and digestion of the β chain with other proteases does not result in the loss of Rb^+ occlusion (Capasso et al., 1992). The 19-kDa fragment itself can be digested in the presence of Ca^{2+}, and the amount of this fragment remaining can be varied by termination of the digestion in the presence of Ca^{2+} at different time points. The amount of 19-kDa fragment (and possibly that of other remaining fragments) correlates almost exactly with Rb^+ occlusion, and the mixture of products occludes both Na^+ and Rb^+ in a ratio of 3:2 (Karlish et al., 1990). Although trypsin digestion removes the ATP binding domain, phospholipid vesicles reconstituted with the trypsinized membrane preparation can passively transport Rb^+. In addition, Goldshleger et al. (1992) recently showed that the 19-kDa fragment contains a single DCCD binding site, that Rb^+ protects against the labeling of this fragment by DCCD (Rb^+-protectable DCCD labeling), and that Rb^+-protectable DCCD labeling of the 19-kDa fragment correlates with the DCCD-induced inactivation of Rb^+ occlusion by the entire trypsin-digested enzyme. Furthermore, there is one molecule of DCCD bound to 19-kDa membranes per Rb^+ binding site and 2 mol of DCCD bound per mole of phosphoenzyme or per mole of α chain. These data suggest that a single DCCD-labeled site within the 19-kDa fragment may be one out of at least two primary determinants of cation (Rb^+) binding on the α chain, with the second site being outside of the 19-kDa fragment, but not yet localized. Goldshleger et al. (1992) have recently sequenced part of the 19-kDa fragment from the pig kidney α subunit and have shown that the DCCD label was bound primarily to E953 and possibly to the adjacent glutamic acid (954).

The glutamate at amino acid 954 of the pig kidney Na, K-ATPase is conserved across all species and isoforms studied to date (Shull et al., 1985; Kawakami et al., 1985; Shull et al., 1986; Ovchinikov et al., 1986), while a glutamate is present in the first position (E953) in all but two species which have been sequenced. In *Artemia salina* and *Drosophila melanogaster*, a phenylalanine occupies the position corresponding to E953 (Baxter-Lowe et al., 1989; Lebovitz et al., 1989). The high degree of conservation of glutamates at these two positions, combined with the above DCCD labeling/trypsin digestion studies, suggests that either E953, E954, or both residues may be critical amino acids in Na, K-ATPase and may be cation binding sites.

The goal of the current study was to examine the effects of individual and simultaneous substitutions at these two residues on the function of Na, K-ATPase. The substitutions were made via site-directed mutagenesis in a cDNA encoding a ouabain-resistant isoform, and the mutant enzymes were assayed for their ability to confer ouabain resistance to ouabain-sensitive HeLa cells. Finally, the sodium- and potassium-dependence properties of the altered enzymes were examined in crude plasma membrane preparations of transfected cells.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture reagents and supplies were obtained from Gibco and Fisher, and ouabain was obtained from Calbiochem. Nytran membranes (Magna NT) for Northern analysis were purchased from Micron Separations, Inc., Westboro, MA. Most of the reagents for the ATPase assay were purchased from Aldrich, and molecular biology reagents were obtained from New England Biolabs, Sigma, Fisher, Promega, and United States Biochemical. All radioisotopes were purchased from New England Nuclear. Other reagents and supplies were of the highest quality available.

Mutagenesis and Cloning. Mutations were introduced into the rat α-1 cDNA sequence at positions corresponding to two amino acids which are potential cation binding residues (E955 and E956 in the rat α-1 isoform). To facilitate mutagenesis, several unique restriction sites were introduced into the rat α-1 cDNA. These sites divided the cDNA into small cassettes which could be shuttled between the M13mp19 vector used for mutagenesis and the remainder of the cDNA. The sites were introduced by substitution of alternate, frequently used codons, and the substitution changed the DNA sequence but not the amino acid sequence. The cassette to be mutated was subcloned from the rat α-1 cDNA into an M13mp19 vector, and the procedure of Kunkel (1985) was used to make mutations. In all of the current studies, the mutated cassette had 5′ *Eco*RI and 3′ *Hin*dIII sites and spanned the last base of the codon for amino acid 936 (arginine) to 31 bases 3′ of the codon for the carboxy terminal amino acid (tyrosine). The former restriction site is unique within the cDNA and is a naturally occurring restriction site, while the latter was created by a GC-for-CA substitution at base numbers 28 and 29 3′ of the carboxy terminal tyrosine. Both single (E955Q, E956Q) and double (E955Q–E956Q) Glu-to-Gln substitutions were made, along with a double Glu-to-Asp substitution (E955D–E956D). Following mutagenesis, strains of M13mp19 containing mutations were identified by single-base tracking, and the mutated cassette was sequenced in its entirety in these strains to ensure that the desired mutation had been incorporated and that no additional mutations were introduced (Sanger et al., 1977). The mutated cassette was next subcloned into a shuttle vector containing the remaining portion of the rat α-1 cDNA. The entire cDNA was then removed via a restriction digest and was ligated into the pRC/CMV expression vector. Following ligation, the construct was sequenced across the mutation site and across the points of ligation to once again confirm the presence of the mutation and to ensure that the ligation sites were intact.

Transfection and Tissue Culture. The expression vector containing a mutated or wild-type rat α-1 cDNA was transfected into HeLa cells using calcium phosphate precipitation (Chen & Okayama, 1987, 1988). As negative transfection controls, cells were transfected without DNA or with a pRC/CMV vector containing a ouabain-sensitive cDNA (rat α-3). Ouabain selection (1 μM concentration) began 16–24 h after removal of precipitated DNA, and selection was maintained throughout isolation of resistant colonies and expansion of cell lines. Approximately 10–14 days after the

beginning of selection, individual colonies were isolated with cloning cylinders and were expanded into cell lines. All cells were grown at 37 °C under 5% CO_2 in Dulbecco's modified Eagle medium (pH 7.4) containing 10% calf serum, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B. Media was changed every 3 days. An untransfected HeLa cell line was maintained in the absence of ouabain, and these cells were also used in ouabain sensitivity studies.

Ouabain was used to rapidly screen for critical mutations as follows. Beginning 16–24 h after the end of the calcium phosphate precipitation, ouabain was added to the media to achieve a final concentration of 1 μM. At this concentration ouabain is lethal to untransfected HeLa cells, since the endogenous HeLa Na, K-ATPase is completely inhibited by 1 μM ouabain, and since these cells must pump Na^+ in order to survive. In order to enable the transfected cells to survive in the presence of ouabain, they can be transfected with a cDNA which encodes a protein able to confer ouabain resistance. However, if the same cDNA is mutated to create an amino acid substitution, and that substitution severely impairs the function of the transfected enzyme, the transfected cells will not survive. Thus, critical mutations could be identified as those which do not permit the survival of the transfected HeLa cells in 1 μM ouabain. If there had been a transfection which did not produce colonies resistant to ouabain, an alternative selection procedure would be necessary. Since the expression vector for all DNA constructs contained the gene encoding neomycin resistance, transfected cells could have been alternatively selected by growth in the neomycin analog G418.

Northern Analysis. Total RNA was extracted and isolated from one confluent 100-mm tissue culture dish according to the procedure of Chomczynski and Sacchi (1987). Extracted RNA (15 μg) was then denatured in 1.0 M glyoxal, 54% DMSO, and 0.01 M sodium phosphate, pH 6.8 at 50 °C for 60 min (total volume of each sample in buffer was 30 μL). Northern analysis was next performed using a modification of the procedure of Church and Gilbert (1984). The above samples were loaded into a 0.1% agarose/0.01 M sodium phosphate (pH 6.8) gel for separation of RNA fragments, and the RNA was transferred overnight to a nylon membrane. Blots were probed with a ^{32}P-labeled, 275-bp *Bam*HI fragment of the expression vector containing the polyadenylation sequence immediately 3′ to the inserted cDNA. Membranes were then washed twice with 0.5% bovine serum albumin/1 mM EDTA/5% SDS/0.04 M $NaHPO_4$ (pH 6.8), twice with 1 mM EDTA/1% SDS/0.04 M $NaHPO_4$ (pH 6.8), and once with 0.1 × SSC/0.5% SDS [1 × SSC, 0.15 M NaCl/0.015 M sodium citrate (pH 7.0)]. All washes were carried out at 65 °C for 30 min. Filters were blotted dry, and the signal was detected by autoradiography at −70 °C.

Crude Plasma Membrane Preparations of HeLa Cells. Plasma membranes of HeLa cell cultures were isolated according to procedures described previously (Jewell & Lingrel, 1991). Briefly, HeLa cells (five 175-cm^2 flasks) were washed once with ~30 mL of phosphate-buffered saline and treated with 0.05% trypsin. Excess trypsin was immediately removed, and cells were incubated at room temperature until they began to become dislodged with mild shaking (~10 min). They were then resuspended immediately in cold (4 °C) tissue culture medium and centrifuged at 1000*g* for 1 min. The pellet was resuspended in 8 mL of cold 1 mM $NaHCO_3$/2 mM $CaCl_2$/5 mM $MgCl_2$ (buffer A). Cells were allowed to lyse in this medium for 15 min and were homogenized in a glass Dounce homogenizer. Samples were centrifuged at 1000*g* for 1 min to remove debris and nuclei. The supernatant was immediately decanted and saved, taking care not to disturb the pellet. The pellet was resuspended in 2 mL of buffer A and centrifuged, and the supernatant was combined with that from the first centrifugation. Next, 2 mL of 6.0 M NaI/75 mM $MgCl_2$ was added to the combined supernatant under constant stirring (the $MgCl_2$ was added to the NaI solution immediately before NaI treatment of the supernatant), allowing the mixture to stir for 30 min at 4 °C as described previously (Lane et al., 1973). Samples were centrifuged at 48000*g* for 30 min at 4 °C, and the pellet was resuspended in 5 mL of 10 mM Tris/1 mM EDTA (buffer B, pH 7.4), centrifuged again, and resuspended in 1 mL of buffer B. Membrane suspensions were then frozen and stored at −70 °C. Protein concentration in the final preparation was assayed at a later date, using a modified method of Bradford (1976).

ATPase Assays and Determination of Na, K-ATPase Activity. ATPase assays were performed on the above membrane preparations according to a previously described procedure (Brown, 1982; Jewell & Lingrel, 1991). This assay measures the release of $^{32}P_i$ from [γ-^{32}P]ATP and was linear over the entire 30-min incubation period for preparations of all wild-type and mutant cell lines. Less than 20% of the total ATP was hydrolyzed under the assay conditions. In order to determine the mutant or wild-type rat α-1 Na, K-ATPase activity, membrane preparations were assayed in the presence of both 5 μM and 10 mM ouabain. Since endogenous HeLa Na, K-ATPase activity is completely inhibited by 5 μM ouabain (see Figure 3), and the expressed rat α-1 isoform is minimally affected at this concentration (Figure 3), the activity corresponding to the transfected rat α-1 isoform could be analyzed free of the endogenous component. The rat α-1 Na, K-ATPase activity is completely inhibited at 10 mM ouabain, leaving only background activity contributed by ATPases other than Na, K-ATPase. Thus, subtraction of residual ATPase activity at 10 mM ouabain concentration from activity at 5 μM ouabain yields only the transfected Na, K-ATPase activity. Background ATPase activity (not inhibited by 10 mM ouabain) was not more than 40% of the total ATPase activity at 5 μM ouabain. To determine the ouabain sensitivity of the expressed rat α-1 Na, K-ATPase activity, ouabain concentration was varied in the following fixed conditions: 100 mM NaCl, 10 mM KCl, 40 mM choline chloride, 3 mM Tris–ATP, 3 mM $MgCl_2$, 5 mM histidine, and 0.1 M EGTA (pH 7.4).

ATPase activity remaining at 10 mM ouabain was not Na-dependent or K-dependent, and was equal to the ATPase activity at 0 mM NaCl or 0 mM KCl in 5 μM ouabain (data not shown). Therefore, in studies of Na^+ dependence of Na, K-ATPase activity, ATPase activity at 0 mM NaCl in 5 μM ouabain was subtracted from the activity at each NaCl concentration in 5 μM ouabain. This produced a Na^+ stimulation curve of only the wild-type or mutant rat α-1 enzyme. Studies of K^+ stimulation of Na, K-ATPase activity were performed in an identical fashion. The conditions for NaCl dependence were as follows: 10 mM KCl, 3 mM Tris–ATP, 3 mM $MgCl_2$, 5 mM histidine, and 0.1 M EGTA (pH 7.4), and variable NaCl and choline chloride concentrations. The sum of ion concentrations ([NaCl] + [KCl] + [choline chloride]) was kept constant at 150 mM. Conditions for the determination of K^+ dependence were as follows: 30 mM NaCl, 3 mM Tris–ATP, 3 mM $MgCl_2$, 5 mM histidine, and 0.1 M EGTA (pH 7.4), and the KCl and choline chloride concentrations were varied. The sum of ion concentrations

was kept fixed at 150 mM in the same manner as for Na^+ dependence (above).

Data Analysis. Data for the determination of NaCl and KCl dependence were analyzed by both noncooperative and highly cooperative models of cation binding, and calculations were made using both eqs 1 and 2 to calculate apparent

$$v = V_{max}/(1 + K_{ligand}/[S])^n \quad (1)$$

$$v = V_{max}[S]^n/(K' + [S]^n) \quad K_{0.5} = K'^{1/n} \quad (2)$$

affinities for noncooperative and highly cooperative binding, respectively, where n is the number of sites ($n = 3$ for Na^+; $n = 2$ for K^+). This model (Garay & Garrahan, 1973) assumes that there are identical noninteracting sites for Na^+ (three sites) and K^+ (two sites). A highly cooperative model was also used to analyze the data, and under this model apparent affinities were calculated according the eq 2. Data were analyzed by nonlinear regression analysis using the Sigma Plot Scientific Graph System by Jandel Scientific. The results for both models are presented in Tables I and II, whereas the figures for sodium and potassium dependence (Figures 4 and 5) were derived using eq 1 only (noncooperative model).

RESULTS

The goal of the current study was to examine the effects of amino acid substitutions at E955 and E956 on Na, K-ATPase function. A mutagenesis-selection scheme was devised to determine whether substitutions at these two residues had a critical effect on enzyme function, using the following rationale. The endogenous Na, K-ATPase in HeLa cells is relatively ouabain-sensitive and is inhibited by 1 μM ouabain. HeLa cells require a functional Na^+ pump in order to survive, and untransfected cells will not survive in 1 μM ouabain, since the endogenous HeLa enzyme cannot function at this ouabain concentration. On the other hand, it is possible to transfect HeLa cells with a cDNA encoding a relatively resistant enzyme which is unaffected by this concentration of the cardiac glycoside, enabling the cells to survive. Under these conditions, survival of the cells requires that the ouabain-resistant enzyme be functional. Therefore, if an amino acid substitution is made in the resistant isoform, and the substitution abolishes or severely limits enzyme function, resistance will not be conferred by the altered protein. Using this strategy, mutations encoding the current amino acid replacements were made in a cDNA encoding a normally ouabain-resistant enzyme, and the ability of the modified enzyme to permit HeLa cell survival was then examined. Failure of the altered enzyme to confer ouabain resistance indicates that the amino substitution was introduced at a critical site, while cell growth indicates a relatively mild effect or the lack of an effect on enzyme function.

Using the above strategy, all mutations were made in a rat α-1 cDNA, which encodes an enzyme which is $\sim$1000-fold more resistant to ouabain than enzyme containing either of the remaining rat isoforms or nonrodent isoforms. HeLa cells transfected with cDNAs encoding E955Q, E956Q, and E955Q–E956Q substitutions grew as well in 1 μM ouabain as those transfected with wild-type constructs (Figure 1). Under the conditions of the present study (transfection of a ouabain-resistant isoform into a ouabain-sensitive cell line), establishment of resistance in these cells was a result of the expression of a functional ouabain-resistant enzyme. Control transfections (calcium phosphate precipitation without DNA or with a cDNA encoding the rat α-3 isoform) did not produce

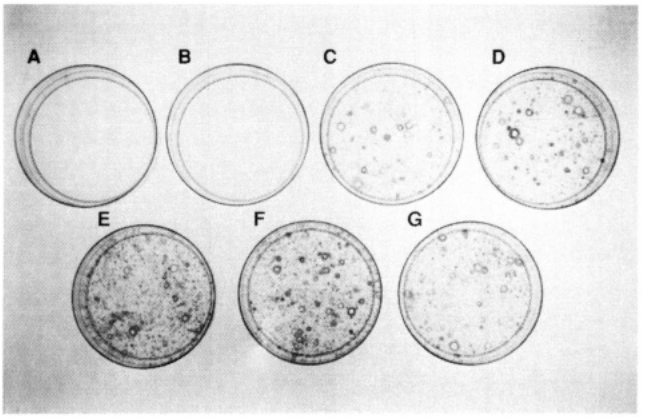

FIGURE 1: Tissue culture plates in which HeLa cells were transfected and selected in 1 μM ouabain for 5 weeks. (A) Mock transfectin using only transfection buffer without added DNA. (B) Transfection with 25 μg of pRC/CMV vector containing a cDNA encoding a relatively ouabain-sensitive isoform (rat α-3). (C) HeLa cells transfected with 25 μg of pRC/CMV vector with a wild-type rat α-1 cDNA insert. (D–G) HeLa cells transfected with 25 μg of vector with a rat α-1 cDNA insert encoding the amino acid replacements E955Q, E956Q, E955Q–E956Q, and E955D–E956D, respectively. Since untransfected HeLa cells are sensitive to 1 μM ouabain, only those cells expressing a functional, ouabain-resistant Na, K-ATPase enzyme should survive in 1 μM ouabain. Amino acid substitutions which critically impair enzyme function should not permit cell growth. In a parallel transfection followed by G418 selection (0.6 mg/mL), transfection of the cDNA construct containing the rat α-3 isoform produced numerous (>75) colonies resistant to G418, demonstrating successful transfection of that cDNA construct (data not shown).

any cells viable in 1 μM ouabain, demonstrating that resistance was not conferred in the absence of a ouabain-resistant isoform (Figure 1A,B). Each transfected cDNA construct also contained the gene conferring resistance to the antibiotic neomycin. When the neomycin analog G418 was used instead of ouabain to select transfected cells, even the vector containing the rat α-3 cDNA conferred resistance (data not shown), demonstrating successful transfection of that cDNA construct.

Since cell growth occurred in 1 μM ouabain after transfections with all cDNAs encoding Glu-to-Gln substitutions, a cDNA construct containing a double Glu-to-Asp substitution (E955D–E956D) was made and transfected. This type of substitution (either Glu to Asp or Asp to Glu) at cation binding sites of the sarcoplasm reticulum (SR) Ca^{2+} ATPase eliminates enzyme activity without exception (Clarke et al., 1990), and loss of enzyme activity with the E955D–E956D substitution (lack of ouabain-resistant colonies) would suggest that at least one of these two amino acids is critical to enzyme function, perhaps via directly binding cations. However, cells transfected with the cDNA encoding the E955D–E956D substitution were ouabain resistant (Figure 1G), demonstrating that the expressed enzyme was functional.

In order to verify that the transfected cDNA was being transcribed into mRNA in each cell line, a Northern analysis was performed. Transcription of the transfected cDNA constructs in HeLa cell lines is shown in Figure 2. Blots were probed with a 275-bp, ^{32}P-labeled DNA fragment which was removed via a *Bam*HI digest from the expression vector pRC/CMV. This fragment contained the polyadenylation sequence immediately 3′ to the inserted cDNA and is unique to the expression vector. Therefore, only cell lines expressing the transfected cDNA as mRNA will hybridize with the probe. The specificity of the probe is shown in lane 1, where mRNA isolated from untransfected HeLa cells showed no hybridization. The expression of mRNA was variable from one cell line to another, even within each group of cell lines transfected

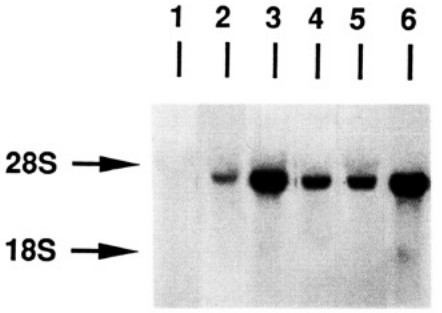

FIGURE 2: Northern analysis of RNA extracted from HeLa cells. Northern blots were probed with a ^{32}P-labeled 275-bp *Bam*HI fragment of the expression vector. This fragment containing primarily the polyadenylation sequence immediately 3′ to the inserted cDNA (bovine growth hormone polyadenylation sequence). Each lane was loaded with a total of 15 μg of RNA. The autoradiogram was exposed for 3 days. Lane 1: RNA extracted from untransfected HeLa cells. Lanes 2–6: each lane contains RNA extracted from HeLa cells transfected with pRC/CMV vector containing a rat α-1 cDNA without mutation (lane 2) or with mutations encoding the amino acid substitutions E955Q, E956Q, E955Q–E956Q, and E955D–E956D (lanes 3–6, respectively). Among different cell lines transfected with the same cDNA construct, the level of transcription did not correlate with the V_{max} for Na, K-ATPase activity (data not shown).

with the same cDNA construct. The intensity of the signal on Northern blots did not correlate with ATPase activity (data not shown). The lack of correlation between transcription and enzyme activity agrees with a previous study using a different expression vector (Jewell & Lingrel, 1991) and suggests that posttranscriptional regulation may play a key role in the control of Na, K-ATPase activity in this system. The exact mechanisms of this control have not been established.

In order to rule out any effects on ouabain binding, ouabain sensitivity studies were performed on membrane preparations. The lack of effect of the mutations on ouabain sensitivity is shown in Figure 3. While untransfected HeLa cell membranes had an I_{50} of $\sim 10^{-7}$ M, the I_{50}'s of the wild-type rat α-1, E955Q, E956Q, E955Q–E956Q, and E955D–E956D enzymes were all ~1000-fold greater than that of untransfected HeLa cells, and none of the mutant I_{50}'s were significantly different from that of wild-type rat α-1. The similar shift in the ouabain inhibition curve for wild-type and mutant enzymes rules out any gross effect of the amino acid substitutions on ouabain binding. The fact that cell lines expressing each of the mutant enzymes exhibited a shift in ouabain sensitivity of 3 orders of magnitude confirms the expression of a ouabain-resistant isoform in each cell line.

Since the amino acids of interest in this study have been suggested to be cation binding residues, the cation dependence of Na, K-ATPase activity was examined in membrane preparations from wild-type and mutant enzymes. The sodium dependence of Na, K-ATPase activity in typical membrane preparations is shown in Figure 4, and the accompanying kinetic data for wild-type and mutant enzymes are summarized in Table I. The K_{Na^+} determined by eq 1 in Experimental Procedures (noncooperative model of Na^+ and K^+ binding) and and $K_{0.5}$ (cooperative model, eq 2) for Na, K-ATPase containing the wild-type rat α-1 isoform agree with previously published values (Jewell & Lingrel, 1991). In the E955Q mutant, the $K_{0.5}$ for NaCl was slightly decreased (3.45 ± 0.22 mM for wild type vs 2.76 ± 0.22 for E955Q) while it was increased in the mutants E956Q (4.92 ± 0.43 mM), E955Q–E956Q (4.16 ± 0.11 mM), and E955D–E956D (6.21 ± 0.52 mM). Thus, the largest effect was in the E955D–E956D mutant, where the $K_{0.5}$ increased to 180% of the wild-type value. The results were qualitatively similar regardless of which model was used to calculate the kinetic constants.

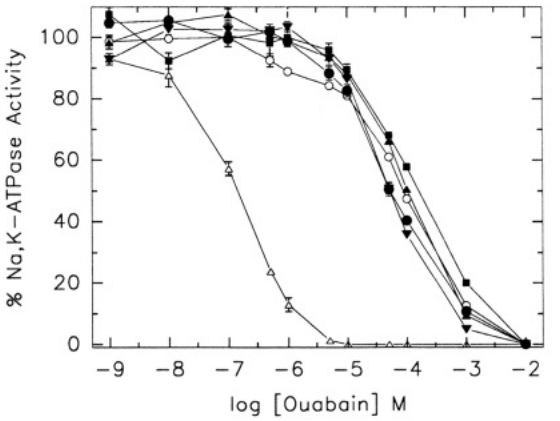

FIGURE 3: Ouabain sensitivity of Na, K-ATPase activity in crude plasma membrane preparations isolated from HeLa cells which were untransfected (△), transfected with a wild-type rat α-1 cDNA (○), or transfected with a mutated rat α-1 cDNA encoding the following amino acid substitutions: (▲) E955Q; (■) E956Q; (●) E955Q–E956Q; (▼) E955D–E956D. ATPase activity at 10 mM ouabain was equal to that in 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 percent Na, K-ATPase activity. Values for each enzyme are the mean ± SEM of an assay performed in triplicate from one membrane preparation of cells derived from a single HeLa colony. I_{50} values for untransfected HeLa cells or cells transfected with the wild-type rat α-1 cDNA agree with previously published data (Price et al., 1990; Jewell & Lingrel, 1991). The specific Na, K-ATPase activities in the absence of ouabain in each of the preparations shown were 8.9, 16.9, 19.4, 9.1, 9.2, and 7.0 μmol of P_i·(mg of protein)$^{-1}$·h^{-1} for untransfected HeLa Na, K-ATPase, wild-type rat α-1 isoform, and mutant rat α-1 isoforms with the amino acid replacements E955Q, E956Q, E955Q–E956Q, and E955D–E956D, respectively.

The stimulation of Na, K-ATPase activity by potassium chloride is shown in Figure 5, and the kinetic data are summarized in Table II. As was the case for Na^+ dependence, the results were similar no matter which model of ligand binding was used to calculate the kinetic constants. The apparent affinities of the wild-type rat α-1 enzyme for potassium agreed with previously published values (Jewell & Lingrel, 1991). The stimulation of Na, K-ATPase activity by KCl was similar among membrane preparations containing wild-type enzyme and those containing single amino acid replacements ($K_{0.5}$ of 0.78 ± 0.07 mM KCl for wild type vs 0.86 ± 0.06, and 0.78 ± 0.09 mM for E955Q and E956Q, respectively). However, unlike the minimal effects of the E955Q–E956Q substitution on apparent Na^+ affinity, the $K_{0.5}$ for KCl of this double-mutant enzyme increased nearly 2-fold (1.45 ± 0.09 mM). The E955D–E956D substitution had a small effect, increasing the $K_{0.5}$ by 49% (1.16 ± 0.08 mM).

DISCUSSION

The present results demonstrate that single amino acid substitutions at E955 and E956, potential cation binding sites, have little or no effect on Na, K-ATPase function. When the properties of the wild-type rat α-1 enzyme were compared with those of enzymes containing the single amino acid substitutions E955Q and E956Q, in each case there was no significant effect of the substitution on cation dependence of Na, K-ATPase activity, nor was there any effect on ouabain sensitivity. This demonstrates that a carboxyl-containing side chain is not required at positions 955 and 956 for normal enzyme function. In addition, not only do single Glu-to-Gln substitutions result in a functional enzyme but double Glu-to-Gln and Glu-to-Asp substitutions (E955Q–E956Q, E955D–

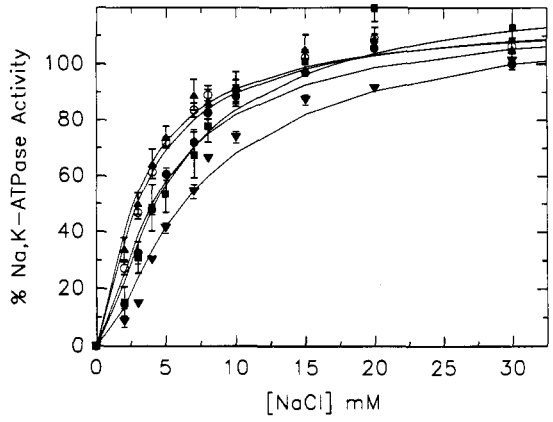

FIGURE 4: Sodium stimulation of Na, K-ATPase activity in crude plasma membrane preparations isolated from HeLa cells which were transfected with wild-type rat α-1 cDNA (○) or transfected with a mutated rat α-1 cDNA encoding the following amino acid substitutions: (▲) E955Q; (■) E956Q; (●) E955Q–E956Q; (▼) E955D–E956D. The Na, K-ATPase activity shown is only that of the enzyme expressed by the transfected cDNAs (defined in Experimental Procedures). The expressed Na, K-ATPase activity at each NaCl concentration was divided by that at 50 mM NaCl, and this quotient was multiplied by 100 to calculate percent Na, K-ATPase activity. Values for each enzyme are the mean ● SEM of an assay performed in triplicate of a membrane preparation of cells derived from a single HeLa colony. The data were analyzed by nonlinear regression according to a noncooperative model of Na^+ binding (see Data Analysis in Experimental Procedures). For cell lines in this figure, the V_{max}'s of the expressed Na, K-ATPase activity determined by this analysis were 27.1, 11.3, 5.3, 16.0, and 9.1 μmol of P_i·(mg of protein)$^{-1}$·h^{-1} for Na, K-ATPase containing the wild-type rat α-1 isoform and mutant rat α-1 isoforms with the amino acid replacements E955Q, E956Q, E955Q–E956Q, and E955D–E956D, respectively. A summary of kinetic data for all cell lines is shown in Table I. Kinetic constants for enzyme containing the wild-type rat α-1 isoform agree with previously published data (Jewell & Lingrel, 1991).

Table I: Summary of Kinetic Constants Derived from Cooperative and Noncooperative Models of Na^+ Binding[a]

	apparent affinities of expressed enzyme	
transfected cDNA	$K_{0.5}$ (mM)[b]	K_{Na^+} (mM)[c]
wild-type rat α-1	3.45 ± 0.22 (4) [0.28]	1.11 ± 0.13 (4) [0.06]
E955Q	2.76 ± 0.22 (4) [0.30]	0.76 ± 0.08 (4) [0.08]
E956Q	4.92 ± 0.43 (4) [0.69]	1.72 ± 0.19 (4) [0.21]
E955Q–E956Q	4.16 ± 0.11 (3) [0.45]	1.32 ± 0.04 (3) [0.18]
E955D–E956D	6.21 ± 0.52 (3) [0.32]	2.36 ± 0.28 (3) [0.23]

[a] The values shown are the $K_{0.5}$ (cooperative model) for NaCl or K_{Na^+} (noncooperative model) generated by assuming two different models of ligand binding. The data are the mean ± SEM of kinetic constants derived from separate trials, and the number of trials is in parentheses. The number of trials includes at least two different cell lines for each transfected enzyme (each cell line was produced by a single transfected colony). Within each trial the Na, K-ATPase activity was measured in triplicate at each NaCl concentration shown in Figure 4, nonlinear curve fitting was performed on the set of triplicate values, and the standard deviation of this fit was calculated. The value in brackets is the average standard deviation of the fit for all trials involved. [b] Cooperative model (highly cooperative interaction between Na^+ sites). Data were fit by the equation $v = V_{max}\,[Na^+]^3/(K'_{0.5} + [Na^+]^3)$, where $K_{0.5} = (K'_{0.5})^{1/3}$. [c] Noncooperative model (no interaction between Na^+ sites). Data were fit by the equation $v = V_{max}/(1 + K_{Na^+}/[Na^+])^3$.

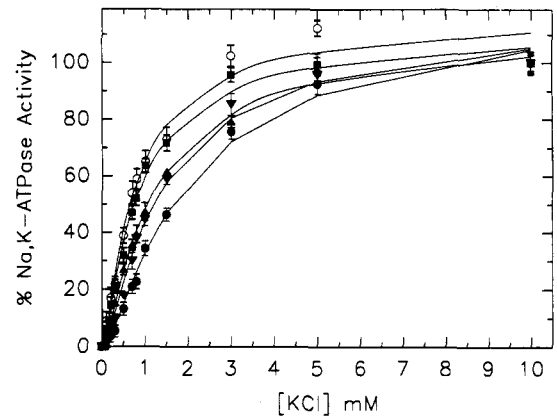

FIGURE 5: Potassium stimulation of Na, K-ATPase activity in crude plasma membrane preparations prepared from HeLa cells which were transfected with wild-type rat α-1 cDNA (○) or transfected with a mutated rat α-1 cDNA encoding the following amino acid substitutions: (▲) E955Q; (■) E956Q; (●) E955Q–E956Q; (▼) E955D–E956D. The Na, K-ATPase activity shown is only that of the enzyme expressed by the transfected cDNAs (defined in Experimental Procedures). The expressed Na, K-ATPase activity at each KCl concentration was divided by that at 10 mM KCl, and this quotient was multiplied by 100 to calculate percent Na, K-ATPase activity. Values for each cDNA construct are the mean ± SEM of an assay performed in triplicate of a membrane preparation of cells derived from a single HeLa colony. The data were analyzed by nonlinear regression according to a noncooperative model of K^+ binding (see Data Analysis in Experimental Procedures). For cell lines in this figure, the V_{max}'s for the expressed Na, K-ATPase activity determined by this analysis were 17.0, 16.0, 5.0, 17.2, and 12.7 μmol of P_i·(mg of protein)$^{-1}$·h^{-1} for Na, K-ATPase containing the wild-type rat α-1 isoform and the rat α-1 isoform with the amino acid replacements E955Q, E956Q, E955Q–E956Q, and E955D–E956D, respectively. A summary of kinetic data for all cell lines is shown in Table II. Kinetic constants for enzyme containing the wild-type rat α-1 isoform agree with previously published data (Jewell & Lingrel, 1991).

Table II: Summary of Kinetic Constants Derived from Cooperative and Noncooperative Models of K^+ Binding[a]

	apparent affinities of expressed enzyme	
transfected cDNA	$K_{0.5}$ (mM)[b]	K_{K^+} (mM)[c]
wild-type rat α-1	0.78 ± 0.07 (3) [0.06]	0.42 ± 0.06 (3) [0.03]
E955Q	0.86 ± 0.06 (3) [0.07]	0.46 ± 0.04 (3) [0.04]
E956Q	0.78 ± 0.09 (3) [0.11]	0.40 ± 0.08 (3) [0.05]
E955Q-E956Q	1.45 ± 0.09 (3) [0.06]	0.94 ± 0.08 (3) [0.08]
E955D-E956D	1.16 ± 0.08 (3) [0.11]	0.65 ± 0.01 (3) [0.09]

[a] The values shown are the $K_{0.5}$ for KCl (cooperative model) or K_{K^+} (noncooperative model) generated by assuming two different models of ligand binding. The data are the mean ± SEM of kinetic constants derived from separate trials, and the number of trials is in parentheses. The number of trials includes at least two different cell lines for each transfected enzyme (each cell line was produced by a single, distinct transfected colony). Within each trial, the Na, K-ATPase activity was measured in triplicate at each KCl concentration shown in Figure 5, nonlinear curve fitting was performed on the set of triplicate values, and the standard deviation of this fit was calculated. The value in brackets is the average standard deviation of the fit for all trials involved. [b] Cooperative model. Data were fit by the equation $v = V_{max}\,[K^+]^2/(K'_{0.5} + [K^+]^2)$, where $K_{0.5} = (K'_{0.5})^{1/2}$. [c] Noncooperative model. Data were fit by the equation $v = V_{max}/(1 + K_{K^+}/[K^+])^2$.

E956D) produce a functional enzyme as well. In view of recent studies suggesting that E955 may be a primary determinant of cation binding (Goldshleger et al., 1992) and other studies demonstrating the absolute conservation of the glutamate at position 956 (Shull et al., 1985, 1986; Kawakami et al., 1985; Kawakami et al., 1986; Ovchinikov et al., 1986; Takeyasu et al., 1988; Baxter-Lowe et al., 1989; Lebovitz et al., 1989), it is especially significant that both sodium and potassium stimulation of Na, K-ATPase activity in all mutant

enzyme preparations with single amino acid substitutions was similar to that of the wild-type enzyme. Glutamates at these two positions are therefore not essential for the cation-dependent properties of Na, K-ATPase.

The current study suggesting that E955 and E956 are not critical residues seems to contrast with the findings of Goldshleger et al. (1992) suggesting that E955 is a cation binding amino acid. This study demonstrated that chemical labeling at E955 (E953 of the pig kidney Na, K-ATPase) is prevented by Rb^+ and that the labeling correlates with inactivation of Rb^+ occlusion by 19-kDa membranes (the entire trypsin-digested enzyme which contains, among others, the 19-kDa fragment). However, our findings and those of chemical labeling studies could be reconciled by any one of several explanations. First, as Goldshleger et al. have discussed, the Rb^+-protectable labeling by *N,N'*-dicyclohexylcarbodiimide could be due to a change in enzyme conformation induced by Rb^+, which may cause a reduction in the affinity of the enzyme for DCCD. Thus, the Rb^+ protection of DCCD labeling does not necessarily mean that Rb^+ and DCCD bind to the same sites. Also, the effects of DCCD on Rb^+ occlusion in previous studies could be due to steric hindrance created by placing a bulky chemical label at E955. The binding of a large chemical modifying group to E955 could hinder the transition of the enzyme from one conformation to another or could prevent cations from binding to residues near to E955. In either scenario, the chemical labeling of E955 would correlate with, but not directly cause, inactivation of Rb^+ occlusion. Mutagenesis studies do not involve the addition of any chemical moieties to the enzyme, and hence the current studies are free of the steric effects of large ligands bound to the residue of interest. Our studies demonstrating either no effect (K^+ dependence) or only a mild effect (Na^+ dependence) on cation binding with single amino acid substitutions are consistent with E955 and E956 being in proximity to another residue which binds cations, but these data do not support the suggestion that E955 and E956 are cation binding residues.

It is also important to mention that in the DCCD labeling/trypsin digestion studies there appear to be two separate DCCD binding sites present in 19-kDa membranes. As already discussed, the first site has been identified as E953 in the pig kidney Na, K-ATPase (E955 in the present study). While the exact location of a second site has not been determined, it is thought to be located outside of the 19-kDa fragment, since there is only one DCCD site per 19-kDa fragment (Goldshleger et al., 1992). Goldshleger et al. discussed the DCCD labeling of two additional trypsin digestion products, apparently distinct from the 19-kDa fragment. Both products are derived from the amino terminal half of the enzyme, with one containing transmembrane segments M1 and M2, while the second contains the M3 and M4 segments. They speculated that the second DCCD binding site is located within the M3/M4-containing fragment and that the labeling associated with the M1/M2-containing fragment is due to cross-linking with the M3/M4-containing product. This was based on the assumption that the original DCCD labeling occurs at carboxyl groups and on the fact that there are no carboxylate side chains in the M1 and M2 transmembrane segments. Regardless of the location of the second site, the DCCD modification of the unknown site could have a direct impact on Rb^+ occlusion if the site were a cation binding residue. Alternatively, cross-linking between a fragment containing the unknown DCCD-labeled residue and another trypsin-digestion fragment could indirectly prevent cation binding if a cation site were located on either cross-linked fragment. Whether the effect is direct or indirect, it is possible that it is the DCCD labeling of the unknown site which alters Rb^+ occlusion, and the labeling of E955 may be inconsequential. A lack of an effect of DCCD binding at E955, in combination with a significant effect of DCCD labeling at a second residue, would not only explain the correlation between DCCD labeling of E955 and inactivation of Rb^+ occlusion but would also explain the slight effect (Na^+ dependence) or absence of effect (K^+ dependence) of the E955Q and E956Q substitutions on apparent cation affinities in the current study. In all of the above studies of Rb^+ occlusion, it is the occlusion by the 19-kDa membranes which was measured and not the occlusion by the 19-kDa fragment itself. Therefore, a second DCCD labeling site may have been present in all occlusion studies of the trypsin-digested enzyme, and the effects of DCCD binding to that site have not been excluded.

A final explanation for the apparent contrast in the findings of the present study and those of DCCD labeling studies is that either or both of the residues E955 and E956 are in fact cation binding amino acids, but the Glu-to-Gln substitutions in the current study are too conservative to significantly alter cation binding. Although our study demonstrates that a carboxyl-containing side chain at positions 955 and 956 is not necessary for normal or nearly normal enzyme function, it is still possible that both of these residues bind cations, but that each is only required to contribute one side-chain oxygen to the ligand binding site, instead of two carboxyl oxygens. Our substitution of each glutamate with a glutamine preserves the carbonyl oxygen, making it possible that a side chain containing a single oxygen, instead of one containing a carboxyl group, is the minimum requirement for normal cation binding properties of Na, K-ATPase. Indeed, replacement of a glutamate with a glutamine at one of the four putative carboxyl-containing cation binding residues of the SR Ca^{2+}-ATPase (E908) had no effect on enzyme activity in microsomal vesicles prepared from transfected cells (vesicle Ca^{2+} uptake 111% of control), while a change of glutamate to alanine or to aspartic acid abolished activity [0% of control (Clarke et al., 1990)]. Thus, Glu-to-Gln substitutions at known cation binding residues do not always abolish or dramatically affect enzyme activity. Nonetheless, additional data from the current studies of the E955D–E956D mutant of Na, K-ATPase, in combination with data from previous studies of the SR Ca^{2+}-ATPase, argue against a primary role for E955 and E956 of the Na, K-ATPase in cation binding. In the SR Ca^{2+}-ATPase, there is evidence for the direct involvement of four negatively charged residues in cation binding (Clarke et al., 1990), although recent studies point out the possibility that one of the four (E309) may actually mediate conformational transitions of the enzyme (Andersen & Vilsen, 1992). At each of these amino acids, a single Asp-to-Glu or Glu-to-Asp substitution abolishes enzyme activity and eliminates Ca^{2+} transport in microsomal vesicles prepared from transfected cells (Clarke et al., 1990). In contrast, when Na, K-ATPase contains an E955D–E956D substitution, the enzyme is still functional, as evidenced by the measurable Na, K-ATPase activity of the E955D–E956D mutant, and by the survival in 1 μM ouabain of HeLa cells expressing this altered protein. Thus, a double Glu-to-Asp substitution at positions 955 and 956 still permits the enzyme to function. On the other hand, even a single Glu-to-Asp or Asp-to-Glu replacement is not tolerated at cation binding residues in the SR Ca^{2+}-ATPase without complete loss of enzyme activity.

While single Glu-to-Gln substitutions at E955 and E956 had only slight effects on the cation stimulation of Na,

K-ATPase, the double substitutions (E955Q–E956Q and E955D–E956D) had more profound effects. When the E955Q–E956Q double mutant was examined for cation dependence of Na, K-ATPase activity, the $K_{0.5}$ for KCl was increased almost 2-fold, while that for NaCl increased by only 19%. A nearly reciprocal effect was seen in the E955D–E956D mutant, with an 80% increase in the $K_{0.5}$ for Na^+ and a 49% increase in that for K^+. Thus, significant effects on cation binding were seen only after simultaneous substitution at E955 and E956, and the mutant enzyme was still functional in each double mutant. Even the largest effect on Na^+ (E955D–E956D) or K^+ (E955Q–E956Q) stimulation is small in comparison to that seen in the one instance where cation binding affinity could be estimated after substitution at a potential cation binding residue (Clarke et al., 1990). After a Glu-to-Asp substitution at E309 of the SR Ca^{2+}-ATPase, enzyme activity was reduced to zero (0% calcium transport in microsomal vesicles), but the binding affinity for Ca^{2+} could be calculated because Ca^{2+}-dependent phosphorylation by ATP could still be measured (Clarke et al., 1990). However, the E309D mutant required 10 times as much $CaCl_2$ to stimulate phosphorylation as that required for the wild-type protein. Therefore, the effects of our E955Q–E956Q and E955D–E956D substitutions on apparent cation affinity in the Na, K-ATPase were only one-fifth as great as that seen for the E309D substitution in the SR Ca^{2+}-ATPase. If the substitutions in both studies occurred at one or more cation binding residues, the disproportionately large effect of the E309D modification in comparison to that seen with our E955Q–E956Q and E955D–E956D alterations may be due to differences in the relative contributions of each residue to its respective cation binding site or to differences in enzyme mechanisms. Alternatively, this disparity in the magnitude of the effect on apparent cation affinities may be due to an indirect effect of the E955Q–E956Q and E955D–E956D substitutions on cation binding in the Na, K-ATPase. Perhaps E955 and E956 are located close to a cation binding site, and substitution at 2 transmembrane residues containing carboxylate side chains (there are only 7 such residues in the model of the α chain containing 10 transmembrane segments) in the vicinity of the binding site indirectly perturbed cation binding. Again, the fact that the single amino acid substitutions E955Q and E956Q had little or no effect on cation binding, along with the fact that E955D–E956D and E955Q–E956Q enzymes are functional, argues against a prominent role of these two residues in cation binding. Regardless of whether the changes in apparent cation affinities in the E955Q–E956Q and E955D–E956D mutants are due to direct intervention at a cation binding site or to some indirect effect, it is interesting that each double mutant had a relatively specific effect on the apparent affinity for potassium or sodium. Future studies of this region of the Na, K-ATPase α subunit may provide insight into the ion specificity of the enzyme.

ACKNOWLEDGMENT

The authors are grateful to Dr. Lois Lane for providing the shuttle and pRC/CMV vectors, for reviewing the manuscript, and for discussions involving the strategy of the present project.

SUPPLEMENTARY MATERIAL AVAILABLE

Protocol used for oligonucleotide-directed mutagenesis (4 pages). Ordering information is given on any current masthead page.

REFERENCES

Andersen, J. P., & Vilsen, B. (1992) *J. Biol. Chem. 267*, 19383–19387.

Argello, J. M., & Kaplan, J. H. (1991) *J. Biol. Chem. 266*, 14627–14635.

Baxter-Lowe, L. A., Guo, J. Z., Bergstrom, E. E., & Hokin, L. E. (1989) *FEBS Lett. 257*, 181–187.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brown, A. M. (1982) in *Red Cell Membranes—A Methodological Approach* (Ellory, J. C., & Young, D., Eds.) pp 223–228, Academic Press, New York.

Capasso, J. M., Hoving, S., Tal, D. M., Goldshleger, R., & Karlish, S. J. D. (1992) *J. Biol. Chem. 267*, 1150–1158.

Chen, C., & Okayama, H. (1987) *Mol. Cell Biol. 7*, 2745.

Chen, C., & Okayama, H. (1988) *Biotechniques 6*, 632.

Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156–159.

Church, G. M., & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1991.

Clarke, D. M., Loo, T. W., & MacLennan, D. H. (1990) *J. Biol. Chem. 265*, 6262–6267.

Garay, R. P., & Garrahan, P. J. (1973) *J. Physiol. 231*, 297–325.

Goldshleger, R., Tal, D. M., Moorman, J., Stein, W. D., & Karlish, S. J. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6911–6915.

Gorga, F. R. (1985a) in *The Sodium Pump*, (Glynn, I. M., & Ellory, O., Eds.) pp 315–320, The Company of Biologists, Ltd., Cambridge, U.K.

Gorga, F. R. (1985b) *Biochemistry 24*, 6783–6788.

Jewell, E. A., & Lingrel, J. B. (1991) *J. Biol. Chem. 266*, 16925–16930.

Karlish, S. J. D., Goldshleger, R., & Stein, W. D. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4566–4570.

Kawakami, K., Noguchi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Inayama, S., Hayashida, H., Miyata, T., & Numa, S. (1985) *Nature (London) 316*, 733–736.

Kawakami, K., Ohta, T., Nojima, H., & Nagano, K. (1986) *J. Biochem. 100*, 389–397.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488–492.

Lane, L. K., Copenhaver, J. H., Lindenmayer, G. E., & Schwartz, A. (1973) *J. Biol. Chem. 248*, 7197–7200.

Langer, G. A. (1982) *Annu. Rev. Physiol. 44*, 435.

Lebovitz, R. M., Takeyasu, K., & Fambrough, D. M. (1989) *EMBO J. 8*, 193–202.

Ovchinnikov, Y. A., Modyanov, N. N., Broude, N. E., Petrukhin, K. E., Grishin, A. V., Arzamazova, N. M., Aldanova, N. A., Monastryrskaya, G. S., & Sverdlov, E. D. (1986) *FEBS Lett. 201*, 237–245.

Pedemonte, C. H., & Kaplan, J. H. (1986a) *J. Biol. Chem. 261*, 3632–3639.

Pedemonte, C. H., & Kaplan, J. H. (1986b) *J. Biol. Chem. 261*, 16660–16665.

Philipson, K. D. (1985) *Annu. Rev. Physiol. 47*, 561.

Price, E. M., Rice, D. A., & Lingrel, J. B. (1990) *J. Biol. Chem. 265*, 6638–6641.

Robinson, J. D. (1974) *FEBS Lett. 38*, 325–328.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Shani-Sekler, M., Goldshleger, R., Tal, D. M., & Karlish, S. J. D. (1988) *J. Biol. Chem. 263*, 19331–19341.

Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature 316*, 691–695.

Shull, G. E., Greeb, J., & Lingrel, J. B. (1986) *Biochemistry 25*, 8125–8132.

Takeyasu, K., Tamkun, M. M., Renaud, K. J., & Fambrough, D. M. (1988) *J. Biol. Chem. 263*, 4347–4354.

Ullrich, K. J. A. (1979) *Annu. Rev. Physiol. 41*, 181–195.

Yamaguchi, M., Sakamoto, J., & Tonomura, Y. (1983) *Curr. Top. Membr. Transp. 10*, 203–217.