Random Mutagenesis Reveals a Novel Site Involved in Inhibitor Interaction within the Fourth Transmembrane Segment of the Na^+/H^+ Exchanger-1[†]

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ABSTRACT: We constructed and expressed human Na^+/H^+ exchanger (NHE-1 isoform) cDNAs randomly mutagenized within the sequence encoding the transmembrane region of the exchanger. Using acute intracellular acidifications in the presence of the NHE-1 inhibitor amiloride (300 μM), we selected a clone expressing a NHE-1 protein exhibiting a 3.3-fold increase in K_i for amiloride (10 μM instead of 3 μM). Sequencing its cDNA revealed one point mutation resulting in a Gly174Ser substitution near the carboxy-terminal end of the putative fourth transmembrane domain of NHE-1. The introduction of this mutation into the wild-type NHE-1 cDNA and its expression reproduced the features of the mutant. Site-directed Gly174Ala and Gly174Asp substitutions resulted, respectively, in no change and in an approximately 4-fold decrease in the amiloride affinity. An additional mutation (Leu163Phe) in transmembrane segment four has previously been shown to result in a decreased sensitivity to amiloride and its derivatives. The Leu163Phe/Gly174Ser double mutant possesses a strongly reduced affinity for various inhibitors (17 μM for amiloride, 2 μM for MPA, and 20 μM for HOE694) and also a decreased affinity (28 mM instead of 14 mM) for sodium. Although distant in the transmembrane segment, Leu163 and Gly174 residues are both not hydrogen-bonded, being one helix turn from proline residues, and are therefore located in highly flexible regions of the protein. This flexibility and the availability of free carbonyls may play an important role in the interaction with the inhibitors and transported cations.

The mammalian Na^+/H^+ exchanger isoforms are integral plasma membrane ion transporters which utilize the energy provided by the inwardly directed sodium gradient to catalyze the electroneutral exchange of one extracellular sodium ion for one intracellular proton [for review, see Aronson (1985)]. Among these transporters, the NHE-1 isoform (NHE stands for Na^+/H^+ exchanger) is widely expressed in eukaryotic cells. It plays a crucial role in intracellular pH and cell volume regulation as well as in the early response to various mitogenic and cell-stimulating agents [for review, see Counillon and Pouysségur (1993)]. The transport sites of this antiporter can accommodate sodium, lithium, and protons, resulting in the ability of the antiporter to function in a reversible manner if the sodium transmembrane gradient is artificially inverted. The NHE-1-mediated Na^+/H^+ exchange is blocked relatively specifically by amiloride and its N5-substituted derivatives which compete with sodium on the external transport site [for review, see Kleyman and Cragoe (1988)]. The other NHE isoforms (NHE-2−4) (Orlowski et al., 1992; Tse et al., 1992,1993a), expressed specifically in epithelia such as kidney and small intestine, play an important role in transepithelial sodium and acid−base equivalent vectorial transport [for review, see Tse et al. (1993b)]. These isoforms are inhibited much less efficiently by the amiloride-related compounds, and by newer inhibitors, belonging to the HOE694 family which have been shown to exhibit a very high discriminative potency for the different NHE isoforms (Counillon et al., 1993a). Since the Na^+/H^+ exchangers appear to be involved in many vital physiological processes, the basic understanding of their function and regulation represents a field of investigation of utmost interest. Moreover, the importance of this field is highlighted by the involvement of the cardiac NHE-1 isoform in acute pathological situations such as postischemic myocardial fibrillation and cell death (Lazdunski et al., 1985; Scholz et al., 1995). Therefore, information obtained from such studies may provide important clues for the future design of specific pharmacological agents for interaction with the various Na^+/H^+ exchanger isoforms.

The cloning of a cDNA encoding the NHE-1 isoform, as well as the production of a polyclonal serum against its C-terminal region, provided information about the primary structure and topological structure of this exchanger (Sardet et al., 1989, 1990). However, progress in the elucidation of the structure−function relationships of this transporter are presently limited by the same problems which are usually encountered for the study of many other eukaryotic transmembrane transporters. The low level of expression of this protein *in vivo* and the lack of efficient overexpression

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systems (Fafournoux et al., 1991) represent a severe limitation for attempts of large-scale purification of this protein, and unless unexpected technical progress occurs, the production of high-quality crystals and the subsequent determination of three-dimensional structure are at present unlikely.

As an alternative method, we have previously described the use of a genetic approach, which allows the identification of critical residues involved in the function of the antiporter (Counillon et al., 1993b). Instead of requiring protein purification, this strategy relies on the high potency of somatic cell genetics to select cells expressing Na^+/H^+ exchangers bearing structural alterations affecting functional sites of the protein. One of the major limitations of this genetic approach is the extremely low probability of spontaneous mutations occurring at the locus of interest, resulting in a low frequency of mutant isolation (less than 1 in a population of 10^7 cells). To increase this frequency, we employed here an alternative mutagenesis strategy which involved the expression of pools of randomly mutagenized cDNA encoding the NHE-1 isoform in the antiporter-deficient PS120 fibroblast cell line (Pouysségur et al., 1984). Selective tests were applied to the resulting population of clones, allowing the isolation of a novel amiloride-resistant mutant emerging from approximately 3000 stably transfected clones expressing functional Na^+/H^+ exchangers. The mutation responsible for this amiloride-resistant phenotype is located close to the C-terminal end of the fourth putative transmembrane segment of NHE-1. On the basis of this information, we have characterized this region in greater detail, and using site-directed mutagenesis, we demonstrate that the fourth transmembrane segment of NHE-1 plays a crucial role in the interaction of the transporter with amiloride and with the transported cation sodium.

MATERIALS AND METHODS

Site-Directed Mutagenesis. A 1.1 kb *Hind*III-*Sac*I fragment of the pEAPK expression vector (i.e. the pEAP expression vector with the NHE-1 cDNA 3′ noncoding region deleted) (Wakabayashi et al., 1992; Counillon et al., 1994) (Figure 1) encoding the first 345 amino acids of the human NHE-1 isoform was subcloned in the pTZ18 vector (Bio-Rad) and used as a template for single-strand site-directed mutagenesis according to Kunkel (1985). The codon changes used to create the various NHE-1 mutants are represented in Table 1. Mutagenized cDNA fragments were confirmed by sequencing and cloned into the pEAPK expression vector by restriction cutting using the *Hind*III and *Sac*I enzymes (Eurogentech) and subsequent ligation (Eurogentec T4 DNA ligase). Prior to transfection in PS120 fibroblasts, the presence of the various mutations in the pEAPK expression vectors was confirmed by DNA sequencing.

PCR-Mediated Chemical Mutagenesis. The NHE-1 cDNA fragment encoding putative transmembrane segments 2−12 was chosen as a target for PCR-mediated chemical mutagenesis (Figure 1) (Diaz et al., 1991). In order to facilitate further subcloning steps, the *Acc*I restriction site at position 932 in the NHE-1 cDNA coding sequence was eliminated by site-directed mutagenesis, using a silent nucleotide substitution (GTC TAC to GTC TA**T** in the mutant) for the amino acid sequence. The resulting NHE-1 expression vector (pEAPK-Δ*Acc*I) was then used instead of pEAPK. For random mutagenesis, 20 μg of purified pEAPK-Δ*Acc*I plasmid was

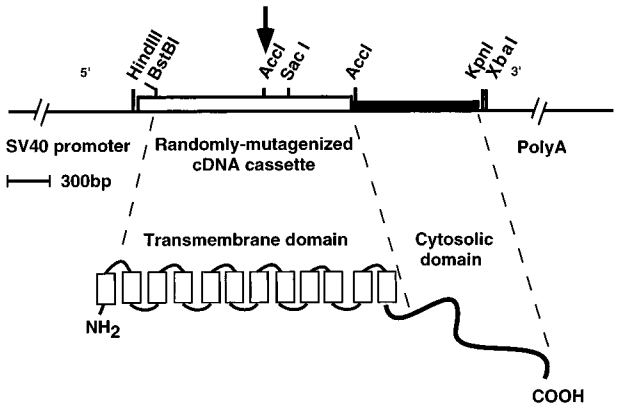

FIGURE 1: NHE-1 cDNA organization and topological model of the protein. (Top) Schematic representation of the NHE-1 cDNA cloned in the pEAPK expression vector. The lines correspond to the expression vector and noncoding regions of the cDNA, and the boxed area corresponds to the coding region of the cDNA. The arrow indicates the *Acc*I restriction site which is removed in the pEAPK-Δ*Acc*I vector used to construct the randomly mutagenized pool of cDNAs. (Bottom) Topological model of the NHE-1 Na^+/H^+ isoform showing the organization of the protein in the transmembrane and cytosolic domains. Rectangles represent the putative transmembrane segments. The dotted lines indicate the correspondence between the cDNA and the protein sequence.

Table 1: Compilation of All the Amino Acid Substitutions Engineered in the NHE-1 Mutants Presented in This Work[a]

mutation	codon change
L163A	CTC → GCC
L163R	CTC → CGC
L163W	CTC → TGG
L163Y	CTC → TAC
G174A	GGC → GCC
G174D	GGC → GAC
G174S	GGC → AGC

[a] The nucleotide changes resulting in the codon changes are underlined.

ethanol-precipitated, resuspended in 100 μL of 250 mM sodium acetate (pH 4.3) containing 1 M sodium nitrite, and incubated at 25 °C for various times. At 10, 20, 50, 80, and 120 min, 1 μg DNA aliquots were mixed with 10 μL of 2.5 M sodium acetate (pH 7) and precipitated in ethanol. After two rinses with 70% ethanol, 10 ng of the DNA aliquots was amplified by 27 cycles of PCR (Stratagene Taq polymerase) using the 5′ primer 5′-GGGCTGCTGCCTGT-TCTCAGG-3′ (503 primer) and the 3′ primer 5′-GAT-GTCTTCGATGCCTGTCAG-3′ (309 primer), producing a 1 kb fragment encoding the whole transmembrane region of NHE-1. PCR-amplified fragments were then digested with the *Bst*BI and *Acc*I restriction enzymes, releasing a DNA fragment encoding putative transmembrane segments 2−12 (Figure 1). This fragment was purified on a low melting agarose gel and ligated into the pEAPK vector. Microdialyzed ligation mixes were then transformed into Xl1-Blue *Escherichia coli* cells by electroporation. The randomly mutagenized NHE-1 constructs present in several bacterial clones were analyzed using various restriction enzymes and by sequencing in order to determine the nitrous acid exposure time resulting in the appropriate mutation frequency.

Preparation of the Pools of the Randomly Mutagenized NHE-1 cDNAs. The PCR-amplified cDNAs obtained after 50 min of nitrous acid incubation (creating approximately 1 mutation per kilobase of sequence) were ligated into the

pEAPK-ΔA*cc*I vector and transfected into Xl1-Blue *E. coli* cells as described above. Three independent ligation and transformation experiments resulted in the production of approximately 1500, 1600, and 2500 independent bacterial colonies. Following each ligation and transformation, the plasmids present in 10 colonies were analyzed by restriction cutting, in order to verify that more than 90% of them were possessing an intact full length insert. Additionally, we checked to be sure that transformation of the religated vector alone did not produce any colonies under the same experimental conditions. In order to ensure that equal representation for each cDNA clone was obtained following this procedure, each pool of colonies was then harvested separately in LB medium and frozen at −20 °C following the addition of glycerol to a final concentration of 50%. For each pool, 100 μL aliquots of the frozen stocks (containing more than 10^6 bacteria as tested by plating of serial dilutions and estimation of the number of colonies) were amplified overnight in 200 mL cultures, and plasmid DNA was prepared. Each pool of DNA (termed 1500, 1600, and 2500) was transfected in respectively 9, 9, and 15 100 mm plates.

Cell Culture and Transfection. Mutant Chinese hamster fibroblasts lacking Na^+/H^+ exchange activity (PS120 cell line) were grown in Dulbecco's modified Eagle's medium supplemented with 50 μg/mL streptomycin, 50 units/mL penicillin, and 7.5% fetal calf serum at 37 °C, in a humidified atmosphere of 5% CO_2 and 95% air. For a typical expression experiment, 5×10^5cells per 100 mm petri dish were transfected using the calcium phosphate precipitation method (Wigler et al., 1979) with 20 μg of wild-type, site-directed, or randomly mutagenized human NHE-1 cDNA with its 5′ and 3′ noncoding regions and inserted in the expression vector (pEAPK or pEAPK-Δ*Acc*I).

Selection Procedures. Transfected PS120 fibroblasts were submitted to 1 h-long 50 mM NH_4^+ loading (Boron and De Veer, 1976), followed by a rapid rinse and by a 1 h recovery in a medium containing 120 mM NaCl. This procedure allows the transfectants which express a functional Na^+/H^+ exchanger to survive the NH_4^+-induced acute acidification, while the nontransfected cells are killed by this procedure (Pouysségur, 1985). Similar tests were repeated twice a week until clones stably expressing NHE-1 were obtained. For the analysis of the biochemical features of the NHE-1 molecules obtained by site-directed mutagenesis, the clones were trypsinized and mixed in order to obtain cellular populations. For the selection of amiloride-resistant clones expressing a NHE-1 molecule altered by the random mutagenesis process, the clones expressing a functional NHE-1 molecule were submitted to the same intracellular acidification protocol and then incubated in a recovery medium containing 300 μM amiloride. The amiloride-resistant clones surviving this selection procedure when applied twice a week during 2 weeks were collected individually in 12-well plates, Li^+-loaded during 1 h with a 120 mM LiCl-containing medium, and incubated for 1 h in a pH 5.5 sodium-free medium. This selection procedure (Pouysségur et al., 1984), which is based on the fact that the Na^+/H^+ exchanger can function in a reversible manner, was calibrated to result in the killing of about 50% of a PS120 cellular population stably expressing the wild-type NHE-1 isoform and was designed to eliminate the clones in which amiloride resistance was due to the overexpression of the wild-type NHE-1 molecule. Alternately, the NHE-1 cDNA obtained by site-directed mutagenesis which exhibited no functional expression upon acid load tests (Leu163Tyr mutant) was cotransfected together with the pcDNAneo expression vector (Invitrogen) using a ratio of 9:1 (pEAPK:pCDNAneo), and the cells were then selected during 20 days using 400 μg/mL neomycin.

Pharmacological Characterization of the Mutated NHE-1 Isoforms. The various transfectants were seeded on 24-well plates and after 24−48 h, were incubated in the NH_4^+ loading medium for 1 h. Following two rapid rinses with choline chloride buffer, the initial rates of $^{22}Na^+$ uptake were determined by incubating the cells for 3 min or less in the $^{22}Na^+$ uptake media containing 1 μCi/mL carrier-free $^{22}Na^+$ (Amersham), 120 mM choline chloride, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 15 mM Hepes (pH 7.4), 1 mM ouabain, and various concentrations of the NHE-1 inhibitors. Na^+/H^+ exchanger-independant $^{22}Na^+$ uptake was determined by incubating the cells in the uptake medium containing 500 μM MPA inhibitor, hence completely blocking the exchanger activity. Under these conditions, the basal level of $^{22}Na^+$ uptake represents less than 1% of the exchanger's maximal activity. When the effect of various concentrations of NaCl was tested in competition experiments, the concentration of choline chloride was adjusted to maintain osmolarity, and the uptake time was 30 s in order to ensure initial rate conditions. The influx was then stopped by rinsing the cells four times with ice-cold phosphate-buffered saline (150 mM NaCl and 5 mM sodium phosphate at pH 7.4); the cells were then solubilized in 0.1 N NaOH, and the radioactivity was determined by γ counting. When necessary, the protein contents in the wells were determined on 50 μl samples of solubilized cells using the BCA assay (Pierce).

Immunodetection and Deglycosylation of NHE-1. As described (Counillon et al., 1994), membrane samples were prepared from PS120 fibroblasts expressing the mutated NHE-1 Leu163Tyr molecule. Endoglycosidase H (New England Biolabs) digestion of the wild type and the Leu163Tyr mutant was performed overnight. Total membrane proteins (20−200 μg) were solubilized in protein sample buffer, resolved by SDS−PAGE and transfered to nitrocellulose (Hybond C, Amersham). As described, the blot was probed with a 1:500 dilution of a polyclonal anti-NHE-1 rabbit antibody (RP-C28) and developed using the enhanced chemiluminescence system (ECL, Amersham).

Cloning and Sequencing of the Mutated cDNAs. Total RNA was extracted from the amiloride-resistant fibroblasts using a guanidinium isothiocyanate/pH 4.5 phenol solution (Eurogentec) and ethanol precipitated. Aliquots of 4 μg of total RNA were reverse transcribed (New England Biolabs M-MuLV reverse transcriptase) using the oligonucleotide sequence 5′- TGGCAGGATGCGCTCGGAAGG-3′ as a specific primer. One-tenth aliquots of the reverse transcriptase incubation mix were PCR-amplified using Goldstar Taq polymerase (Eurogentech), the 5′-biotinylated 503 5′-primer, and the 309 3′ primer. Single-stranded PCR product was then obtained by binding the biotinylated double-stranded PCR product to streptavidin-coaded Dynabeads (Dynal kit) followed by alkaline denaturation and sequenced using T7 DNA polymerase (Pharmacia) according to Sanger et al. (1977).

RESULTS

Random Mutagenesis

The protocol used to create a set of randomly mutated NHE-1 cDNAs involved the treatment of plasmid DNA with a chemical mutagen, nitrous acid, followed by PCR amplification (Diaz et al., 1991) of the cDNA region encoding the NHE-1 transmembrane domain (Figure 1). In order to calibrate this mutagenesis process, we performed sequencing reactions on PCR fragments amplified after different incubation times in nitrous acid, and subcloned in the pEAPK-Δ*Acc*I expression vector. We decided to use the DNA sample which had been treated for 50 min with nitrous acid, resulting in the creation of mutations at a frequency of about 1/kb to build a collection of randomly mutagenized NHE-1 cDNAs. Nitrous acid treatment results in the deamination of dA to dHX, dG to dX, and dC to dU. Therefore, following DNA replication by Taq polymerase, A·T pairs are expected to be substituted by G·C pairs, and conversely, G·C pairs by A·T pairs. Although Taq polymerase can by itself introduce additional base substitutions, they are expected to occur at a somewhat lower frequency than 1 mutation/kb, and this protocol therefore provides a relatively well-controlled spectrum of possible substitutions. Hence, we concluded that a set of at least 5000 cDNA clones would be sufficient to ensure an efficient representation of the nitrous acid/PCR-generated mutations.

Isolation of Amiloride-Resistant Clones

The different pools of mutated cDNAs were transfected in PS120 fibroblasts as described in Materials and Methods, and amiloride-resistant stable transfectants were obtained by a two-step selection procedure involving (i) the selection of cells expressing a functional NHE-1 molecule followed by (ii) among these clones the selection of clones resistant to intracellular acidifications in the presence of a relatively high concentration (300 μM) of the NHE-1 competitive inhibitor, amiloride. Among the about 3000 stably transfected PS120 clones expressing the NHE-1 molecule obtained after the first step of this selection, 105 were amiloride-resistant, surviving the second step of selection. As we considered this number to be too high to be only due to the emergence of clones expressing a mutated antiporter affected in its amiloride binding properties, we hypothesized that this phenomenon was due to NHE-1 overexpression in a high proportion of the stably transfected cells. We eliminated these overexpressers using a lithium-loading selection technique, and the 15 clones surviving this procedure were pharmacologically characterized by determining the inhibition of their initial rates of $^{22}Na^+$ uptake by various concentrations of amiloride. Three clones termed 1600-7, 2500-15, and 2500-A exhibited a lower sensitivity to the inhibitor when compared to those of the controls and therefore were very likely to contain mutation(s) affecting amiloride binding. The other clones showed a pharmacological profile identical to that of the wild type, associated with about 3−5 fold higher $^{22}Na^+$ initial rates than the control NHE-1-transfected population and the pharmacologically altered clones. These clones exhibited a moderate level of antiporter overexpression that had not been eliminated by the counterselection procedure.

Pharmacological Characterization of the Amiloride-Resistant Clones Obtained by Random Mutagenesis

The pharmacological profiles of the three amiloride-resistant clones were determined in more detail using various concentrations of amiloride, N5 (methyl propyl) amiloride (MPA), and HOE694. These three clones were found to be absolutely identical with respect to sensitivity to transport inhibition by amiloride, MPA, and HOE694, strongly indicating that these three clones express the same mutated antiporter. Although these mutant clones had been selected from different petri dishes transfected with distinct DNA pools (the 1600 and 2500 pools), ruling out the possibility that they originated from the same initial cellular clone, we concluded that they were likely to be encoded by the same mutated cDNA, which was generated in multiple copies following the PCR amplification of the nitrous acid-treated DNA, and therefore, only one of these clones was further analyzed by sequencing. As shown in Figures 2 and 3, the apparent K_i of these mutants for amiloride is significantly increased when compared to that of the wild-type NHE-1 [10 versus 3 μM for the wild type; the groups are statistically different (Student's unpaired *t*-test) with $p = 0.003$ (two-tail)], similar for MPA (0.12 versus 0.09 μM), and increased by 2.7-fold for HOE694 (0.5 versus 0.18 μM). The mutants were selected on the basis of amiloride resistance, which may account for their differential sensitivity to this drug.

Identification of the Mutation Responsible for the Amiloride-Resistant Phenotype

The cDNA encoding transmembrane segments 2−12 of the 1600-7 clone amiloride-resistant NHE-1 was generated and amplified using RT-PCR. The PCR product was directly sequenced as described in Materials and Methods, and its nucleotide sequence was compared to the wild-type human NHE-1 cDNA sequence. A guanine to adenine substitution was identified at position 522 in the mutated cDNA, resulting in the change of glycine at position 174 to a serine residue in the putative fourth transmembrane segment (TM IV) of the antiporter.

Site-Directed Mutagenesis of the NHE-1 TM IV

Substitutions of Gly174. In order to verify that the glycine to serine substitution at position 174 of NHE-1 was responsible for the change in amiloride affinity observed for the 1600-7 mutant, we introduced this mutation in the wild-type NHE-1 cDNA, and as expected, the transfection of this cDNA in the PS120 cells resulted in the functional expression of a NHE-1 molecule possessing the pharmacological features of the original 1600-7 mutant (Figure 2).

The glycine to serine substitution observed in TM IV is expected to result in relatively modest structural changes, the most obvious consequences being a slight decrease in the backbone flexibility and the introduction of a larger, polar side chain. In order to test whether these effects contributed to the decrease of amiloride binding capabilities, we created two other mutants possessing an alanine (larger side chain reducing the backbone flexibility) and an aspartic acid (larger and charged side chain) instead of a glycine at position 174 (Table 1). While the Gly174Ala mutant had a pharmacological profile similar to that of the wild-type NHE-1, the

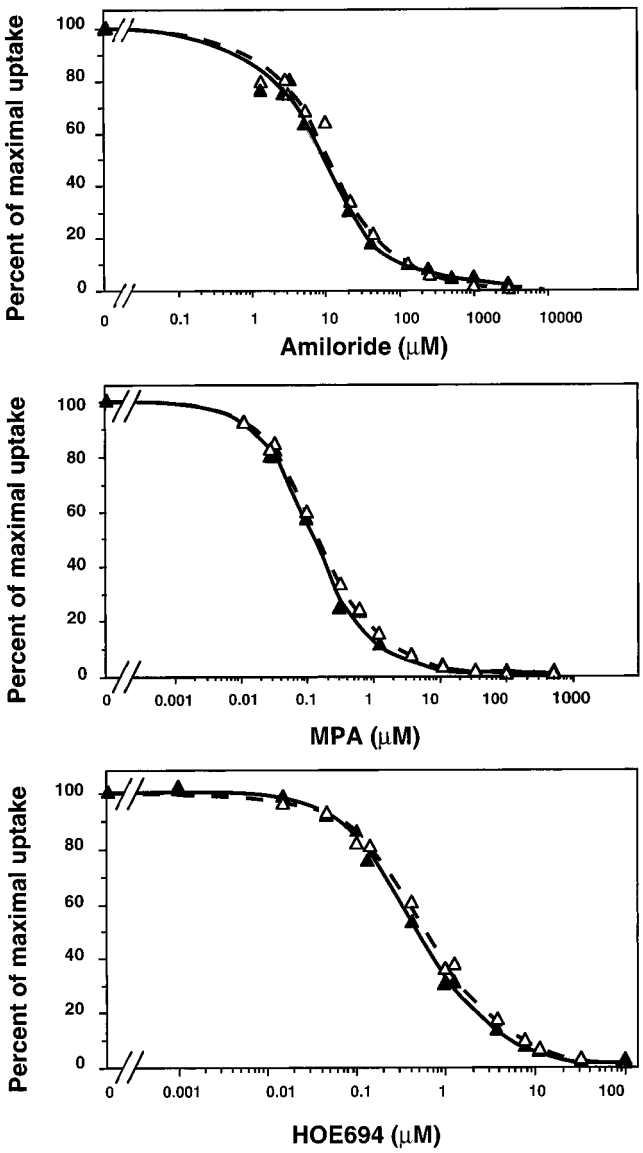

FIGURE 2: Dose-response curves for the inhibition of $^{22}Na^+$ uptake by amiloride, MPA, and HOE694 showing the identity between the 1600-7 and the Gly174Ser mutants. Inhibition of initial rates of $^{22}Na^+$ uptake by various concentrations of the above-mentioned compounds was determined as described in Materials and Methods, and the rates are compared for the 1600-7 mutant obtained by random mutagenesis (△, dotted lines) and the Gly174Ser (▲) (NHE-1 isoform(s) containing the point mutation detected in the 1600-7 mutant sequence. It can be observed that the dose−response curves of these two mutants are identical within the experimental error range (10−20%).

Gly174Asp mutant exhibited a decrease in its ability to bind the various inhibitors, even to a slightly greater extent than the Gly174Ser mutant (Table 2). These results suggest that the reduced affinity for amiloride caused by substitutions at position 174 is more likely due to the introduction of a polar group than simply to the introduction of a larger side chain at this position.

Substitutions of Leu163. We have previously reported that a change of a leucine residue to a phenylalanine at position 163 in the TM IV of the NHE-1 isoform (human sequence) had been found in another amiloride-resistant Na^+/H^+ exchanger (Counillon et al., 1993b). The simplest hypotheses which are likely to explain the effect of the Leu163Phe mutation are the following.

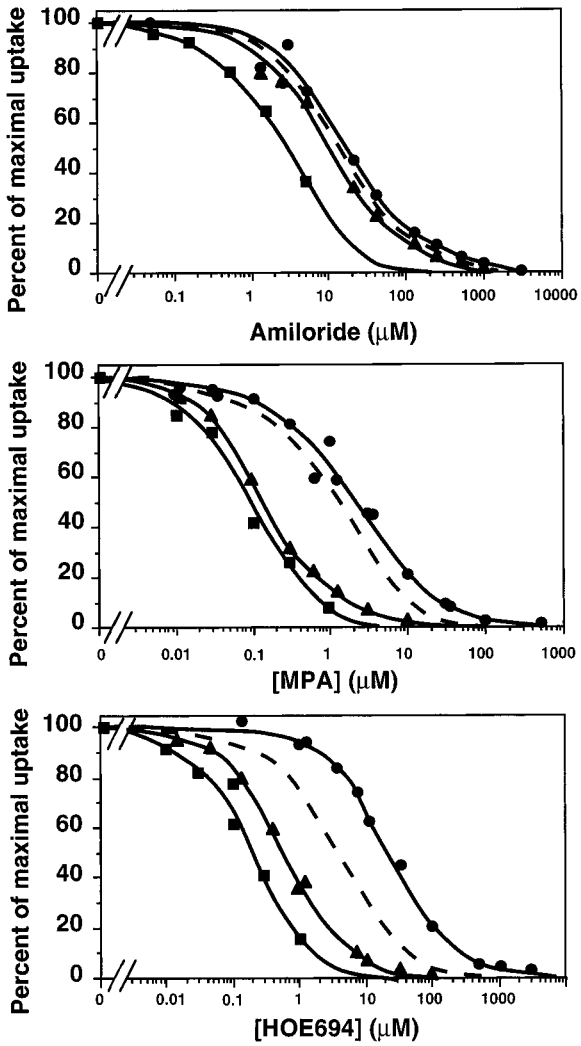

FIGURE 3: Dose−response curves for the inhibition of $22Na^+$ uptake by amiloride, MPA, and HOE694. Summary of the dose−response curves for amiloride, MPA, and HOE694 of the wild-type NHE-1 (■), the Gly174Ser mutant (identical to the 1600-7, 2500-15, and 2500-A mutants, ▲), the Leu163Phe mutant [dotted lines; see Counillon et al. (1993b) for the complete curves], and the Leu163Phe/Gly174Ser double mutant (●). Inhibition of initial rates of $^{22}Na^+$ uptake by various concentrations of the above-mentioned compounds was determined as described in Materials and Methods. For each experiment, the measurements were performed in duplicate, and the results shown here have been compiled from at least three independent experiments. Under these conditions, the variations for each experimental point were in the range of 10−20%. For more clarity, error bars are omitted.

(i) The decrease in affinity is due to steric hindrance, the phenylalanine side chain being significantly more bulky than the leucine side chain. (ii) The decrease in affinity is due to the weakly polar aromatic ring of phenylalanine. (iii) Amiloride does not directly interact with this region of the antiporter, and the decrease in affinity is due to a more global change in the protein conformation resulting from the change of a leucine to a phenylalanine at this particular position.

In order to test these hypotheses, we engineered a set of NHE-1 mutants in which the Leu163 has been substituted by various residues (Table 1). Alanine and tryptophan were introduced to test the steric hindrance hypothesis, since they have smaller and larger volumes, respectively, than the leucine and the phenylalanine found in the wild type and in the amiloride-resistant mutant. The electrostatic effect on amiloride binding was tested by introduction of an arginine

Table 2: Half-Inhibition Constants for Amiloride, N5 (Methyl Propyl) Amiloride (MPA), and HOE694 of all the NHE-1 Mutants Constructed and Characterized in This Work[a]

	half-inhibition constants (μM)		
mutant	amiloride	MPA	HOE694
wild type	3 ± 0.22	0.09 ± 0.016	0.18 ± 0.015
L163A	$8 \pm$ *1.6*	$0.3 \pm$ *0.035*	1.1 ± 0.1
L163R	50 ± 5	$2 \pm$ *0.25*	1.5 ± 0.12
L163W	10 ± 0.7	0.35 ± 0.05	1 ± 0.3
L163Y	ND	ND	ND
G174A	$4 \pm$ *0.6*	$0.1 \pm$ *0.01*	$0.15 \pm$ *0.02*
G174D	$13 \pm$ *1.5*	0.21 ± 0.05	$1 \pm$ *0.27*
G174S	10 ± 0.58	0.12 ± 0.01	0.5 ± 0.09
L163F/G174S	17 ± 2	2 ± 0.27	20 ± 1
L163F	12	1.5	4

[a] The constants have been obtained from dose−response curves of inhibition of initial rates of $^{22}Na^+$ uptake, as described in Materials and Methods and in the legend of Figure 3. For an n of ≥ 3, standard errors are given in normal characters. For an $n = 2$, data are means plus or minus the range (in italic). ND means nondetermined. L163F mutant data are taken from: Counillon et al. (1993b).

possessing a positively charged guanidinium group, like the NHE inhibitors, and of a residue possessing a side chain that is aromatic and has a polar group, tyrosine. The Leu163Ala, Leu163Arg, and Leu163Trp mutants were fully functional tranporters, while the cells expressing the Leu163Tyr mutant did not exhibit any detectable Na^+/H^+ exchange activity. The Leu163Tyr mutant is expressed and detected in Western blots, but when compared to the control wild-type NHE-1, its electrophoretical mobility corresponds to an immature form of NHE-1 (Counillon et al., 1994) (data not shown), is endoglycosidase H sensitive, and therefore is retained in the cis Golgi or endoplasmic reticulum. The Leu163Ala, Leu163Arg, and Leu163Trp mutants were processed to the cell surface and exhibited modified pharmacological profiles, as listed in Table 2. All these mutants present a decrease in their apparent affinities for the tested inhibitors; thus, a leucine at position 163 is required to maintain high-affinity inhibitor binding.

L163F/G174S Double Mutant. In order to examine the combined effect of amino acid substitutions affecting the two positions which had been independently shown to be involved in the interaction of the antiporters with their competitive inhibitors, we constructed the Leu163Phe/Gly174Ser double mutant and expressed its cDNA in PS120 cells. This double mutant exhibited a level of activity similar to the wild-type antiporter [wild type, 690 ± 52 nmol (mg of protein)$^{-1}$ min^{-1}; Gly174Ser, 668 ± 17 nmol (mg of protein)$^{-1}$ min^{-1}; and Leu163Phe/Gly174Ser, 807 ± 60 nmol (mg of protein)$^{-1}$ min^{-1} ; Figure 4], and showed interesting pharmacological properties associated with a modified affinity for transported cations. As shown in Figure 3, the decrease in the apparent affinities of this double mutant for the HOE694 inhibitor is more pronounced than the decreases observed for both the Leu163Phe and Gly174Ser single mutants, showing additivity. When compared to those of the Leu163Phe single mutant, the modifications observed with respect to amiloride and MPA are in the range of the experimental errors. This point will be commented upon further in the Discussion. Moreover, as illustrated in Figure 4, the affinity for the transported cation sodium is diminished by a factor of 2 in this mutant when compared to that of the wild type (K_m of 27.9 ± 3.6 mM instead of 13.9 ± 2.4 mM) or the single Gly174Ser mutant (K_m of 14.3 ± 0.8 mM).

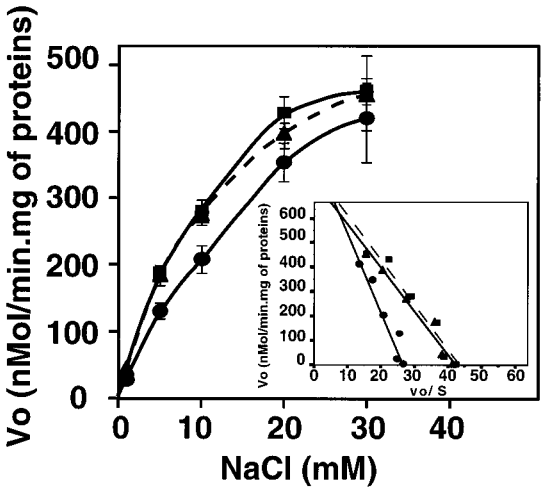

FIGURE 4: Comparison of the V_{max} and K_m for sodium of the wild-type NHE-1 and the Leu163Phe/Gly174Ser double mutant. Experimental data were obtained by measuring initial rates of $^{22}Na^+$ uptake (30 s uptake time) in PS 120 cells transfected with wild-type (■) Gly174Ser (▲, dotted line), or double mutant (●) cDNAs, for different concentrations of extracellular sodium. For each experiment, all the measurements have been performed in duplicate, and the results presented are compiled from at least two independent experiments, each point being the mean of the different experiments. The curves correspond to the interpolation of the mean values. K_m and V_{max} values have been estimated using a nonlinear iterative program that gave a best fit of the experimentally measured activities to the Michaelis−Menten equation (Cigale program version F2.2) and are listed in the Results. (Inset) Eadie−Hofstee plot of the same data.

This result is the first example of characterized mutations affecting the affinities of this transport system for substrate ions.

DISCUSSION

Previous investigations have led to the conclusion that the membrane domain of the Na^+/H^+ exchangers is by itself necessary and sufficient to catalyze ion translocation across the plasma membrane (Wakabayashi et al., 1992). In order to identify functionally important regions of the NHE-1 isoform, we constructed a collection of NHE-1 cDNAs, possessing random mutations in the membrane domain encoding the cDNA sequence. The first putative transmembrane segment, which has the features of a signal peptide (Counillon et al., 1994), and the first extra cellular loop, which is not highly conserved among the different NHE isoforms (Tse et al., 1993b), were not included in the mutagenized DNA cassette (Figure 1). In order to make the molecular analysis of the mutated cDNAs as simple as possible, we intended to limit the initial mutation frequency to approximately 1 mutation/kb, and a highly efficient proton-killing screening technique was employed to select fibroblast clones expressing mutated antiporters. In a previous work, we had used the N5 (methyl propyl)amiloride (MPA) inhibitor as a selective agent to isolate hamster fibroblasts expressing a mutated NHE-1 molecule (Franchi et al., 1986). The previously identified Leu163Phe mutant (human sequence, equivalent to the Leu167Phe substitution initially discovered from the hamster sequence; Counillon et al., 1993b) possessed a significantly more decreased affinity for this amiloride derivative over amiloride itself, suggesting that the Leu163Phe substitution affects the interaction of the antiporter with the N5 substituents of amiloride. In the

present study, we utilized a random mutagenesis technique in association with a selection procedure using amiloride as a NHE-1 inhibitor instead of a N5-substituted derivative such as MPA, in order to isolate at a higher frequency NHE-1 clones mutated on residues more susceptible to interaction with other regions of the amiloride molecule. From approximately 3000 clones expressing NHE-1, this strategy yielded a new amiloride-resistant mutant, possessing the Gly174Ser mutation, which interestingly exhibited an affinity decreased by a factor of 3.3 for amiloride, and a much smaller modification of its pharmacological profile for N5 (methyl propyl)amiloride (MPA). An important source of resistant cells in this series of selection experiments was caused by the apparently high frequency of antiporter overexpression among the transfected clones, and a procedure based on the reversibility of the Na^+/H^+ exchanger had to be used to counterselect these clones. It is interesting to notice that none of the otherwise-identified mutations leading to amiloride resistance (Tables 1 and 2) emerged from the expression of this pool of mutated cDNAs, although some of them could theoretically have been generated and selected by this random mutagenesis process. It can therefore be concluded that this technique did not produce a completely representative pool of randomly mutated cDNAs. For future studies, it may be more suitable to combine different random mutagenesis procedures, hence providing a broader spectrum of possible base replacements.

The fact that Leu163Phe and Gly174Ser, both independently obtained mutations, are located in the same putative transmembrane segment highlights the functional importance of this region of the antiporter. The two sites are quite distant in TM IV (Figure 5), suggesting that the entire transmembrane segment may be involved in inhibitor interaction and transport. The synergistic effect of the double mutation supports this idea. Obviously, this does not exclude the possibility that other residues involved in amiloride interaction are present in other regions of the protein. It may also be stated that this result may be due to a particular structure of the NHE-1 DNA encoding this region, making it more sensitive to mutagenic agents. This seems very unlikely, however, since the mutagenesis conditions which generated the amiloride-resistant variants were very different in these two cases. The Leu163Phe mutant has been mapped from a mutation which occurred in a hamster fibroblast cell line selected for its amiloride-resistant phenotype, the mutation occurring in the hamster genomic DNA present in the cell nucleus. By contrast, the Gly174Ser mutant was generated using an *in vitro* random mutagenesis procedure combining the nitrous acid treatment of purified human cDNA cloned in an expression vector and its amplification using polymerase chain reaction.

The highly amiloride-resistant NHE-3 isoforms (rat and rabbit) (Orlowski et al., 1992; Tse et al., 1992) also have a phenylalanine at a position equivalent to leucine 163 in the human NHE-1 sequence, suggesting that equivalent substitutions in isoforms other than NHE-1 contribute to the observed low affinity for the inhibitors. It is important to notice that NHE-3 possesses a much higher K_i for the NHE inhibitors than the NHE-1 Leu163Phe mutant, indicating that additional regions of the molecule involved in inhibitor binding have diverged during the molecular evolution of the NHE isoforms. Indeed, Orlowski and Kandasamy (1996) have recently shown that TM IV is also involved in inhibitor

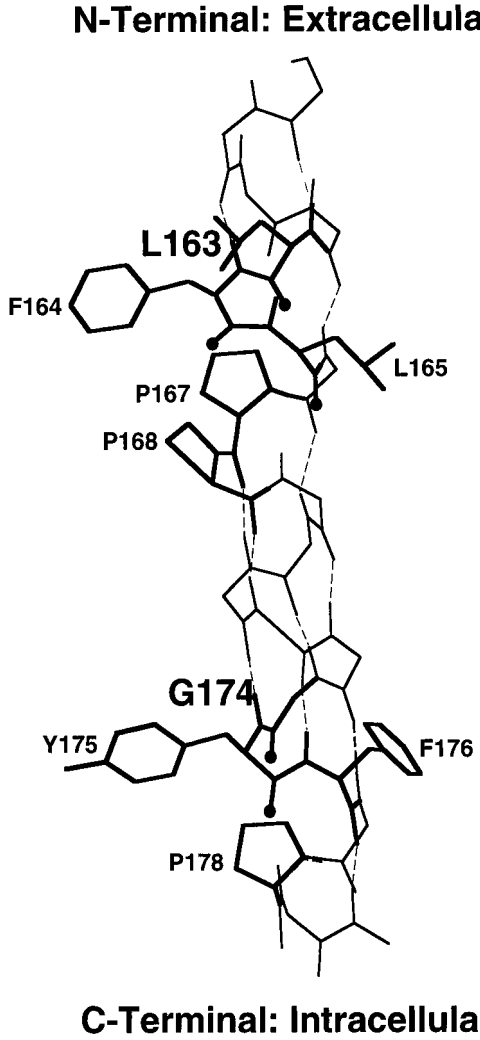

FIGURE 5: α-helical model of TM IV extending from Glu159 at the N-terminal extracellular (top) to Arg180 at the C-terminal intracellular side (bottom) of the membrane. The model was constructed with standard α-helical Φ and ψ angles of −57° and −47°, respectively, and then energy minimized using CHARMm22. Backbone hydrogen bonds between amide protons at position NH*i* and the carbonyls at position O*i*−4 are indicated by the dotted lines. The filled circles indicate carbonyl groups (Leu163, Phe164, Leu165, Gly174, and Tyr17) that are missing hydrogen bond donors since proline has no amide proton. The kink (26° on average) created in the helix by proline also breaks the NH*i*+1···O*i*−3 hydrogen bond. For clarity, not all side chains are illustrated.

interaction. Yun et al. (1993) have shown in a manner consistent with our findings that the introduction by site-directed mutagenesis of the equivalent of the Leu163Phe substitution in the rabbit NHE-2 cDNA resulted in the production of a mutated NHE-2 possessing a decreased affinity for amiloride when compared to that of the wild-type NHE-2 isoform. The case of the Gly174Ser mutation identified in this study is slightly different, since no equivalent of this amino acid substitution is observed in the sequences of the presently cloned amiloride-resistant NHE isoforms. However, the entire stretch of sequence surrounding this position in TM IV including Gly174 is highly conserved, and we can therefore predict that similar substitutions in other NHE isoforms are likely to produce comparable changes in pharmacological profiles and transport features, as it is the case for substitutions of Leu163.

In order to investigate the possible mechanisms by which the Leu163Phe substitution caused a decrease in amiloride

affinity, several amino acid changes have been engineered at position 163. Although not all the possible substitutions were introduced at this position, a range of different residues were used (alanine, phenylalanine, tryptophan, tyrosine, and arginine, as listed in Table 1) in order to cover a broad spectrum of amino acid side chains. The Leu163Phe and Leu163Trp as well as the Leu163Ala mutants exhibit decreased affinities for amiloride and its derivatives (Table 2), ruling out the hypothesis that amiloride resistance is simply the consequence of the introduction of groups larger than leucine at position 163. Additionally, it can be observed from the data presented in Table 2 that all the functionally-expressed mutants at position 163 possess an increased K_i for the NHE-1 inhibitors. It can therefore be concluded from this set of results that a leucine side chain at this position of TM IV is important for the specificity of interaction of the Na^+/H^+ exchangers with their competitive inhibitors.

The random mutagenesis approach allowed us to show in the present study that, as with the Leu163Phe mutation, the Gly174Ser substitution results in the amiloride-resistant phenotype. As shown in the Results, the effects of the substitutions introduced at this position suggest that the decrease in affinity observed in the Gly174Ser mutant is likely to be due to the replacement of the glycine by a more polar residue. However, these results do not demonstrate that Gly174 physically interacts with the amiloride molecule. Hence, we cannot rule out the possibility that the observed change in apparent affinity for the inhibitor upon the Gly174Ser substitution is indirect, causing a conformational change affecting the amiloride binding site situated in another region of the protein.

The double mutant Leu163Phe/Gly174Ser exhibits a strong decrease in affinity for the HOE694 compound (Table 2) which is presently the most discriminative NHE inhibitor. When compared to the data obtained for the single mutants, this result clearly shows an additive effect for the combination of these two mutations (Figure 3, lower panel). By contrast, the results are much more difficult to interpret when amiloride or MPA is used as the inhibitors, since the inhibition curves do not show significant differences between the single and the double mutants. The amiloride inhibition curves for the Leu163Phe and the Gly174 Ser mutants are identical within the experimental error range (Figure 3, top panel). The same fact is observed between the wild-type NHE-1 and the Gly174Ser mutant for the MPA inhibition (Figure 3, medium panel). On the basis of these observations, we can therefore predict that any additive effect of the two mutations on the interaction of amiloride and MPA will not be detectable, being also within the experimental error range. This is indeed what happens for the double mutant. Taken together, these results clearly show that no synergistical effect on inhibitor interaction can be produced by the presence of these two mutations in the same NHE-1 molecule, and a clear additive effect for the HOE694 inhibition.

Although biochemical data indicate that amiloride and its derivatives act in competition with the transported cations, neither the Leu163Phe nor Gly174Ser mutant exhibited experimentally detectable changes in its cation binding capability. By contrast, the double mutant exhibits a decreased affinity for its substrate sodium (Figure 4), revealing that TM IV also plays a role in the interaction of the antiporter with its transported cation. Minor modifications in the ion binding capabilities, in the same order of magnitude as the experimental variations, may not have been detected in the case of the Leu163Phe and Gly174Ser single mutants (Figure 4; Counillon et al., 1993b). The data obtained with the double mutant can therefore be explained by the fact that the combination of the two substitutions in the same molecule results in an experimentally detectable reduced affinity for cations. It may also be argued that the combination of both mutations results in a structural change synergistically affecting ion binding, which is not produced by any of the single mutations. It is also important to notice that any PS120 cell expressing a NHE-1-mutated molecule strongly impaired in its ion binding capabilities would not have survived the acid loading selection method, making the discovery of mutants impaired in ion binding very unlikely using the selection procedure described in this report.

One particularly striking feature of TM IV is the unusual presence of 3 absolutely conserved proline residues in its structure [prolines 167, 168, and 178 (human NHE-1 sequence), Figure 5]. Due to the limited information available about membrane protein structures, the functional contributions of proline residues in transmembrane segments are not clearly understood and have been the subject of interesting speculation [for review, see Williams and Deber (1991)]. Prolines introduce a kink in the α-helical segment (Von Heijne, 1991) which may provide space for substrate binding. On the basis of the finding that amino acid substitutions at positions 163 and 174 in NHE-1 TM IV result in modifications of the inhibitor and ion binding properties of the transporter, we propose an alternative role for the proline residues present in this transmembrane segment. The lack of amide hydrogens in proline residues leaves the backbone carbonyl at position O$i-4$ without hydrogen donors. In addition, the kink introduced by the proline also breaks the N$i+1$ to O$i-3$ hydrogen bond. Thus, the carbonyls of both Leu163 and Gly174 are left without hydrogen-donating partners, the residues situated four residues downstream in the TM IV sequence being Pro168 and Pro178, respectively (Figure 5). Likewise, the carbonyls of Phe164, Leu165, and Tyr175 are not hydrogen bonded. A predictable effect of this lack of hydrogen bonding is an increased polypeptide chain flexibility in this region. Hence, a conformational role for prolines may be to impose a distance effect, approximately one helix turn above the proline residue itself. For simplicity, we modeled TM IV in an α-helical conformation (Deber & Li, 1995) in Figure 5. Visible in this representation, both Leu163 and Gly174 are situated at the N-terminal part of structurally similar regions, consisting of one aromatic residue (Phe164 or Tyr175), one aromatic or hydrophobic residue (Leu165 or Phe176), one leucine residue (166 and 177), and a proline residue (Pro167 and -178). These two regions show a high degree of sequence homology among the NHE isoforms. It is therefore tempting to speculate that the amiloride-resistant phenotype can be correlated with alterations of highly conserved regions of TM IV possessing a similar structure and a high degree of flexibility. This flexibility could play a direct role in the conformational changes of the antiporter during its functional cycle. Additionally, it may be hypothesized that the free backbone carbonyls can themselves directly provide hydrogen bonding for inhibitor and cation binding or for conformationally important interactions with other domains of the protein. This hypothesis may not only

be restricted to the NHE family. Indeed, Ser775 in the α−subunit of the sheep Na^+/K^+-ATPase (Arguello & Lingrel, 1995) is likely to be part of the K^+ binding site and is located one turn amino-terminal to a proline residue. The impact of the double proline in folding NHE-1 into a functional state is underlined by preliminary results indicating that double mutants (Ala-Ala or Gly-Gly) of the 173−174 proline doublet are not functionally expressed. The investigation of the individual role of the different proline residues of TM IV will be conducted in future studies.

In this article, we have used random mutagenesis and acid loading selection techniques to gain information about NHE-1 functional sites. The results obtained here reveal a new functional site of the Na^+/H^+ exchanger and demonstrate the importance of the putative TM IV of NHE-1 for amiloride and sodium binding. This approach can be extended by combining complementary random mutagenesis procedures and by using different screening strategies, in order to select mutated NHE-1 molecules exhibiting diversely altered biochemical features such as a modified ion selectivity.

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