Structural and Functional Characterization of Transmembrane Segment IV of the NHE1 Isoform of the Na⁺/H⁺ Exchanger*

Received for publication, August 20, 2004, and in revised form, January 14, 2005
Published, JBC Papers in Press, January 26, 2005, DOI 10.1074/jbc.M409608200

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The Na⁺/H⁺ exchanger isoform 1 is a ubiquitously expressed integral membrane protein that regulates intracellular pH in mammals. We characterized the structural and functional aspects of the critical transmembrane (TM) segment IV. Each residue was mutated to cysteine in cysteine-less NHE1. TM IV was exquisitely sensitive to mutation with 10 of 23 mutations causing greatly reduced expression and/or activity. The Phe₁⁶₁ → Cys mutant was inhibited by treatment with the water-soluble sulfhydryl-reactive compounds [2-(trimethylammonium)ethyl]methanethiosulfonate and [2-sulfonatoethyl]methanethiosulfonate, suggesting it is a pore-lining residue. The structure of purified TM IV peptide was determined using high resolution NMR in a CD₃OD:CDCl₃:H₂O mixture and in Me₆SO. In CD₃OD: CDCl₃:H₂O, TM IV was structured but not as a canonical α-helix. Residues Asp₁⁵₀-Leu₁⁶₂ were a series of β-turns; residues Leu₁⁶₅-Pro₁₆₈ showed an extended structure, and residues Ile₁₆₉-Phe₁₇₆ were helical in character. These three structured regions rotated quite freely with respect to the others. In Me₆SO, the structure was much less defined. Our results demonstrate that TM IV is an unusually structured transmembrane segment that is exquisitely sensitive to mutagenesis and that Phe₁⁶₁ is a pore-lining residue.

* This work was supported in part by funds from the Canadian Institutes of Health Research (to L. F.) and from the Canadian Institutes of Health Research and Protein Engineering Network of Centers of Excellence (to B. D. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1Y4E) have been deposited in the Protein Data Bank, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡ Supported by studentship awards from the Alberta Heritage Foundation for Medical Research and the Heart and Stroke Foundation of Canada.

‡‡ Supported by postdoctoral fellowships from the Natural Sciences and Engineering Research Council of Canada and the Alberta Heritage Foundation for Medical Research.

§ Supported by studentship awards from the Alberta Heritage Foundation for Health Research and the Strategic Training Initiative in Membrane Proteins and Cardiovascular Disease.

** Supported in part by Canadian Institutes of Health Research Strategic Training Initiative in Membrane Proteins and Cardiovascular Disease.

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The mammalian Na⁺/H⁺ exchanger isoform 1 (NHE1) is a ubiquitously expressed integral membrane protein that mediates the removal of one intracellular proton in exchange for one extracellular sodium ion (1). NHE1 thereby protects cells from intracellular acidification (2, 3); and stimulation of its activity promotes cell growth and differentiation (2) and regulates sodium fluxes and cell volume after osmotic shrinkage (2, 3). The Na⁺/H⁺ exchanger also plays an important role in the damage that occurs to the human myocardium during ischemia and reperfusion, and it has been shown that inhibition of the exchanger has beneficial effects on the myocardium under these conditions (4). Amiloride and its derivatives are inhibitors of the NHE1 isoform of the Na⁺/H⁺ exchanger, and a new generation of Na⁺/H⁺ exchanger inhibitors is being developed for clinical treatment of heart disease (5).

Although the activity of NHE1 has been extensively examined in many tissues, only recently is information starting to be elucidated on how this antiporter actually binds and transports Na⁺ ions and protons. NHE1 is composed of two domains as follows: an N-terminal membrane domain of ~500 amino acids and a C-terminal regulatory domain of about 315 amino acids (1, 4) (Fig. 1). The N-terminal membrane domain is responsible for ion movement, and it is reported to have 12 transmembrane (TM) segments and 3 membrane-associated segments (6). Transmembrane segment four (TM IV; residues 155–177) has been implicated in the ion transport and inhibitor binding properties of NHE1 (7–9). The sequence of human TM IV of NHE1 is 158FLQSDLVYFLLPPIDAGYFL177. The underlined residues have been shown to affect Na⁺ affinity or the inhibitor resistance of mammalian NHE1 (7–9). Recently, we have shown that prolines 167 and 168 are critical for NHE1 function, targeting, and expression (10). These data provide a strong case for the importance of many amino acid residues of TM IV in the ion binding, structure, and transport properties of NHE1.

In this communication, we examine both structural and functional aspects of TM IV of the NHE1 isoform of the Na⁺/H⁺ exchanger. We use cysteine-scanning mutagenesis to characterize which amino acids are important in function and are

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1 The abbreviations used are: NHE1–8, Na⁺/H⁺ exchanger isoforms 1–8; cNHE1, cysteine-less NHE1; Me₂SO, dimethyl sulfoxide; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; HA, hemagglutinin; HNHA, 15N-edited ‘H-N’ correlation spectroscopy; HSQC, heteronuclear single quantum coherence spectroscopy; MALDI-MS, matrix assisted laser-desorption ionization mass spectrometry; MTSES ([2-sulfonatoethyl]methanethiosulfonate); MTSET, [2-trimethylammonium]ethylmethanethiosulfonate; NOE, nuclear Overhauser effect; NOYES, nuclear Overhauser enhancement spectroscopy; r.m.s.d., root-mean-square deviation; TM, transmembrane segment; TOCSY, total correlation spectroscopy; HPLC, high pressure liquid chromatography.
To test the effect of MTSET and MTSES on activity of the NHE1 mutants, we used the standard Na+/H+ exchanger assay with ammonium chloride-induced acidification of the cells. In this case cells were acidified twice as described above. After a first control acidification and recovery, either MTSET or MTSES was added to a final concentration of 10 mM for 10 min in Na+-free buffer. The cells were subsequently washed three times in Na+-free buffer prior to the second ammonium chloride-induced acidification and recovery. To calculate residual activity, Equation 1 was used

\[
\text{\% residual activity} = \frac{\text{pH change after (reagent)}}{\text{pH change without (reagent)}} \times 100
\]

(Eq. 1)

Production and Purification of TM IV—We produced TM IV as a fusion protein with the immunoglobulin binding domain of streptococcal protein G (Gc1 domain). DNA encoding the residues of human NHE1 amino acids 155–180 (135FLQSDVFFFLPLLPIILADGYYFLPLR-160) was synthesized synthetically in the core facility of the Department of Biochemistry. Two self-annelling primers were designed to flank the protein sequence with Met residues and to change the codon preference to that favored in Escherichia coli. The primers were 5'-CTCAATGTTTCTCGAGAAGGTTCGGTGGTTTTTCGCTGCGCAGATTATC-

TCTGGATGCGGGCAGATTTTCGAGCCTGGCATGCCGATCCGACCCG-3' and 5'-TCGAGCCCCGCTGCAGCAGCGCGGACAGAATAGCCCGCATGATGGAATACGGCCAGAAGACAGAAAGAAAAAAGATACGGC-

CTCGAGAAATGC-3'. The primers were designed to have overhanging "sticky" Nhel and XhoI ends so as to insert into the GEO-1 vector (14, 15). The annealed primers were ligated into the cut vector and transformed in E. coli BL21 DE3 (pLysS) for protein production. The correct clones were verified by restriction enzyme digests and DNA sequencing. Cultures were induced with isopropyl 1-thio-β-D-galactopyranoside and treated to release the soluble protein as described earlier (14). For routine production of the protein, cultures were started from a single colony and grown in logarithmic stages until 2.0 liters. At A600 of 0.8, cultures were centrifuged, the eluted proteins concentrated using an Amicon concentration and then resuspended in 150 mM NaCl, 50 mM NaHPO4. They were then de-salted by using a Sephadex G-25 column in 10 mM NH4HCO3, pH 8.0, and then were lyophilized to dryness. To cleave the transmembrane segment IV free of Gc1, standard techniques were employed. Briefly, a given purified batch of fusion peptide was lyophilized twice out of 0.05% (w/v) trifluoroacetic acid-trifluoroacetic acid (99%, Aldrich) in water, dissolved to ~2 mg/ml in 50% (v/v) trifluoroacetic acid in water with excess cyanogen bromide (97%, Aldrich), and allowed to react in darkness for ~16 h at room temperature. The reaction mixture was quenched with an equal volume of deionized water and subsequently lyophilized. Reverse-phase HPLC was used to separate the various peptide products of the cleavage, and fractions containing the TM IV segment were pooled and lyophilized at least twice prior to use. The correct fragment identity was determined by MALDI mass spectrometry.

Preliminary experiments produced unlabeled TM IV. Later experiments produced labeled TM IV for more detailed characterization of the peptide structure. For these experiments, E. coli cells were grown in a minimal medium containing 87 mM NaH2PO4, 24 mM KH2PO4, 4 mM MgSO4, 1.8 μM FeSO4, 55.5 mM glucose, pH 7.5. The medium was supplemented with 1 g of (15NH4)2SO4.

NMR Spectroscopy and Structure Calculation—One- to two-mg samples of unlabeled TM IV peptide were prepared in CD3OD: trifluoroethanol: H2O mixtures; CD3OD:CDCl3:H2O (4:4:1 v/v/v); and Me$_{3}$SO. Note that for CD3OD:CDCl3:H2O, screw-cap NMR tubes (535-TR-7, Wilmad Labglass, Buena, NJ) were used because they are not solvent resistant. Chemical shifts were referenced internally to DSS at 0.5 ppm, with indirect referencing employed for 15N (16). One-dimensional 1H NMR spectra were acquired at 500 MHz on a Varian INOVA spectrometer at various temperatures for each solvent.

Samples for extended analysis were prepared in Me$_{3}$SO and in CD3OD:CDCl3:H2O (4:4:1 v/v/v) at ~2 mM peptide and 1.0 mM DSS. One-dimensional 1H, natural abundance 15N HSQC, TOCSY (60-ms...
RESULTS

Activity and Expression of NHE1 Mutants—Fig. 1 illustrates a general model of the Na\textsuperscript+/H\textsuperscript+ exchanger (Fig. 1A) and a schematic model illustrating TM IV (Fig. 1B). To examine which amino acids of TM IV were critical for the activity of the Na\textsuperscript+/H\textsuperscript+ exchanger and which amino acids were pore-lining, we used the cysteine-less Na\textsuperscript+/H\textsuperscript+ exchanger (cNHE). Each residue in TM IV of cNHE1 was individually mutated to a cysteine residue. Initial experiments examined whether these mutant forms of the Na\textsuperscript+/H\textsuperscript+ exchanger had activity. Most surprisingly, TM IV was exquisitely sensitive to mutation. Fig. 2 shows the effects of mutation of amino acids of TM IV to cysteine on NHE activity. Substitution of any of the amino acids with cysteine in TM IV resulted in significant reductions of the measurable activity of the protein in all mutants (p < 0.05). In particular, residues Phe\textsuperscript{155}, Leu\textsuperscript{156}, Ser\textsuperscript{158}, Asp\textsuperscript{159}, Phe\textsuperscript{162}, Phe\textsuperscript{164}, Pro\textsuperscript{167}, Pro\textsuperscript{168}, Asp\textsuperscript{172}, Tyr\textsuperscript{173}, and Phe\textsuperscript{176} retained less than 20% of the control activity. These mutants were not used for further characterization of activity. We have demonstrated previously that Pro\textsuperscript{178} is not critical for function of NHE1 (10).

Fig. 3 illustrates a Western blot of total cell extracts from API cells stably expressing the single cysteine mutants. Both the mutant and wild-type exchangers displayed the same pattern of immunoreactive bands, with a larger band at ~110 kDa that represents the glycosylated form of the mature Na\textsuperscript+/H\textsuperscript+ exchanger and a smaller band at ~95 kDa that represents an immature form of the exchanger that is not fully glycosylated (10). Both the native wild-type NHE and the cysteine-less NHE1 showed strong immunoreactive bands of 110 kDa size and a weaker 95-kDa, unglycosylated NHE1. The amount of mature 110-kDa NHE is quantified below each lane in Fig. 3, relative to cNHE1. Cells lines containing the mutants F155C, Q157C, L163C, L166C, I170C, A173C, G174C, Y175C, L177C and possibly S158C and F176C have relatively normal levels of activity. Substitution of any of the amino acids with cysteine in TM IV was exquisitely sensitive to mutation. Fig. 2 shows the effects of mutation of amino acids of TM IV to cysteine on NHE activity. Substitution of any of the amino acids with cysteine in TM IV resulted in significant reductions of the measurable activity of the protein in all mutants (p < 0.05). In particular, residues Phe\textsuperscript{155}, Leu\textsuperscript{156}, Ser\textsuperscript{158}, Asp\textsuperscript{159}, Phe\textsuperscript{162}, Phe\textsuperscript{164}, Pro\textsuperscript{167}, Pro\textsuperscript{168}, Asp\textsuperscript{172}, Tyr\textsuperscript{173}, and Phe\textsuperscript{176} retained less than 20% of the control activity. These mutants were not used for further characterization of activity. We have demonstrated previously that Pro\textsuperscript{178} is not critical for function of NHE1 (10).

We examined the sensitivity to MTSET or MTSES of the mutant Na\textsuperscript+/H\textsuperscript+ exchangers that had greater than 20% residual activity of the cysteine-less NHE1 (Fig. 4). Of the active Na\textsuperscript+/H\textsuperscript+ exchangers, only F161C was affected by treatment with either MTSET or MTSES. This resulted in decreases in Na\textsuperscript+/H\textsuperscript+ exchanger activity of ~60 and 80%, respectively. To
confirm that Phe161 was a critical residue, we created the mutant F161K, which introduced a positive charge into this location in the wild-type NHE1 background. This mutant retained only \( \frac{1}{4} \) of wild-type NHE activity (Fig. 2), had reduced expression (not shown), and reduced surface processing (Table I) relative to wild-type NHE1 supporting the importance of Phe161 in NHE1 function.

Subcellular Localization of the Mutant and Wild-type Na\(^{+}/H^+\) Exchangers—We have earlier found that mutation of amino acids of the Na\(^{+}/H^+\) exchanger sometimes causes the protein to be targeted to an intracellular location (10, 25). We therefore used quantitative measurement of the intracellular localization of NHE1 within AP-1 cells. Cells were treated with sulfo-NHS-SS-biotin and then lysed and solubilized, and labeled proteins were bound to streptavidin-agarose beads. An equal amount of the total cell lysate and unbound lysate was separated by size using SDS-PAGE followed by Western blotting with anti-HA antibody to identify tagged NHE1 protein.

Production and Characterization of TM IV—To examine the structure of TM IV, we produced it as a fusion protein with GB1. On SDS-PAGE, the fusion protein appeared as a band of \( \approx 8 \) kDa in size (not shown). This was slightly less than the predicted size of 11 kDa. We confirmed the identity of the protein by mass spectrometry, suggesting that the protein ran with a somewhat anomalous weight, typical of many membrane proteins. The yield of the GB1-TM IV fusion protein was typically \( \approx 25 \) mg/liter of cells. After CNBr treatment to free TM IV, it was then purified by reverse phase HPLC. We confirmed the identity of TM IV by mass spectroscopy. For the unlabeled TM4, the expected mass (with a C-terminal homo-
serine lactone) is 3138.8 atomic mass units, and the observed mass in retained preparative HPLC fractions ranged over 3138.4–3134.0 atomic mass units (parent peak mass differed slightly from fraction to fraction; ionic adducts also observed). Impurities at 4405–4411 and 9910–9915 atomic mass units were detected in less than 25% of the total pooled fractions each, at intensities ranging from 0.5 to 2% relative to the parent peak plus ionic adducts. For the uniformly $^{15}$N-labeled peptide, the expected mass was 3165.8, and observed mass range was 3165.6–3171.6 atomic mass units. Impurities at mass 3085–3086, 4428–4430, and 4816–4820 atomic mass units were detected in less than 10% of the total pooled fractions each, at intensities ranging from 1 to 4% relative to the parent peak plus ionic adducts. Purity was therefore estimated to be greater than 95% for each of the peptides after pooling of the HPLC fractions.

**Choice of NMR Conditions**—To determine the structure of TM IV, we tested several solvent mixtures. The trifluoroethanol:H$_2$O mixtures and methanol appeared more promising than either the CD$_3$OH:CDCl$_3$:H$_2$O mixture or Me$_2$SO, in that wider dispersions of chemical shifts were observed in the backbone H$^\alpha$ (7–9 ppm) and H$^\beta$ (3.5–5 ppm) regions of one-dimensional $^1$H spectra (data not shown). Unfortunately, the peptide precipitated out of each of these solutions within days, making multidimensional NMR data acquisition impractical. In the range of 5–40 °C, 30 °C gave rise to well resolved, minimally broadened $^1$H spectra (data not shown). Therefore, structural studies were carried out in CD$_3$OH:CDCl$_3$:H$_2$O and Me$_2$SO at 30 °C. These ~2 mM peptide samples with 0.5 mM DSS as the internal chemical shift reference are stable at room temperature, providing reproducible spectra over the course of several months.

**Resonance Assignment**—Standard sequential assignment methods were applied (26), using TOCSY and double-quantum filtered correlation spectroscopy experiments along with H$^N$ values observed by natural abundance $^{15}$N-HSQC experiments for spin-system assignment and NOESY connectivity data to walk through the sequence. This provided assignment of all backbone H$^N$ and H$^\beta$, except for the N-terminal H$^N$, all side chain H$^\beta$ resonances, and most other side chain proton resonances. The C-terminal residue was identified as a homoserine lactone rather than free homoserine, because its H$^\beta$ and side chain H$^\beta$ chemical shifts correspond to $\alpha$-amino-$\gamma$-butyrolactone rather than to i-homoserine (27). This was upheld by the molecular weight indicated by MALDI-MS of the purified peptide when considering the fact that homoserine lactone residues are not subsequently undergoing conversion to the free homoserine moiety, because this reaction is reported to be very slow at the peptide C terminus even with catalytic basic conditions (28). Due to spectral overlap even at 800 MHz, caused primarily by repetition in the primary sequence, a few side chain protons could not be resolved. In CD$_3$OH:CDCl$_3$:H$_2$O, aromatic ring protons could not be identified, and H$^\alpha$ shifts for residues Leu$^{165}$, Pro$^{167}$, Ile$^{169}$, Pro$^{178}$, and Leu$^{179}$, and H$^\beta$ of Pro$^{167}$ were unresolved. In Me$_2$SO, all aliphatic side chain resonances were resolved, and all ring H$^\alpha$ resonances; in the
H$_2$O, as evinced by the greatly reduced number of medium range NOE contacts (Fig. 6), we analyzed in detail only the CD$_3$OH:CDCl$_3$:H$_2$O structural features present. The characteristics of the final ensemble of retained structures are given in Table II.

**DISCUSSION**

**Functional Analysis of TM IV—Transmembrane segment four (TM IV; residues 155–177) of the NHE1 isoform of the Na$^+$/H$^+$ exchanger has been implicated in the ion transport and inhibitor binding properties of the protein (7–9). We have recently shown (10) that the double proline pair Pro$^{167}$ and Pro$^{168}$ is critical for the function of NHE1. They were suggested to be critical in the maintenance of an appropriate structure of TM IV and necessary for normal NHE1 transport function. One method to determine the functional role of individual amino acids of a transmembrane segment of a membrane protein is cysteine-scanning mutagenesis in combination with reaction with sulphydryl reagents. Cysteine-scanning mutagenesis takes advantage of the fact that the sulphydryl moiety is the most reactive functional group in a protein (29). It has been used to determine pore-lining residues in numerous membrane proteins (30–32), including the lactose permease of *E. coli* (33), the mouse acetylcholine receptor (34), the human glucose transporter Glut1, and the human anion exchanger isoform 1 (AE1) (35). Cysteine-scanning mutagenesis uses the highly reactive sulphydryl moiety to determine the accessibility of side chains of amino acids. Two sulphydryl-reactive reagents (36) that are often used in these studies are MTSET and MTSES, which are membrane-impermeant (37, 38), react with pore-lining residues surrounded by water, and cannot reach residues within the hydrophobic bilayer.

Initial experiments substituted each of the amino acids in TM IV of cysteine-less NHE1 with cysteine residues. We found that TM IV was exquisitely sensitive to mutation. Twelve of the cysteine mutants had less than 20% of the activity of the control cNHE1. Western blotting revealed that in some cases the reduction in activity was due to a lower level of expression of the protein (Fig. 3); however, in most cases correction for the amount of plasma membrane protein (Table I and Fig. 2) did not explain the loss of activity. Several of the mutants were expressed mainly as unglycosylated protein, and many but not all of these had greatly reduced activity. We have found previously that mutation of some amino acids can greatly affect the glycosylation levels of the proteins. We examined if these mutations affected the surface targeting of the protein. Our findings that many of the mutations of TM IV affected targeting, expression, or activity are similar to the results found with the human anion exchanger (35) and with TM XI of lactose permease (33). In contrast, for the tetracycline-resistant transporter of *E. coli* (39) and in P-glycoprotein (40), it was possible
to mutate all the amino acids of a TM segment and always retain appreciable activity. This susceptibility to mutation appears to vary not only between proteins but also within different transmembrane segments of the same protein. TM XI of lactose permease had several sensitive amino acids (33), whereas TM XII only had one (41). This property could be reflective of both the importance of the residues in the particular segment, and of the importance of the segment itself to the structure and function of the protein. In our case, it was clear that there was a relatively strict requirement for many of the amino acids of TM IV, and this could be indicative of precise structural and functional roles.

Treatment with both positively charged MTSET and negatively charged MTSES revealed that Phe161 is accessible to and reacts with these sulfhydryl-reactive reagents (Fig. 4). The most likely explanation for this reactivity is an interaction at a site that lines the ion translocation pore. The reagents then at least partially block the pore and inhibit ion transport (35).

Thus, Phe161 in TM IV is the first residue to be unambiguously identified as lining the ion transport pore of NHE1. Mutation of Phe161 to Lys resulted in loss of most of NHE1 activity (Fig. 2), confirming that this is a critical amino acid.

**Structural Analysis of TM IV**—We examined the structure of TM IV in CD$_3$OD:CDCl$_3$:H$_2$O and in Me$_2$SO at 30 °C. As with many peptides studied by NMR (20, 42) the TM IV segment does not, as a whole, assume a single conformation. Rather, sections within the peptide converge structurally. Consideration of these sections relative to each other then provides insight into the TM IV segment overall. In a given state of the intact NHE1 protein, a single conformation would presumably be preferred by the TM IV segment. However, the structures that we present here represent the most energetically favorable forms of this TM segment in a low dielectric environment mimicking both a lipid bilayer and a protein interior. Therefore, it is reasonable to presume that they would also be favorable within the setting of the NHE1 protein. Indeed, a number of studies have demonstrated strong correspondence between structures of peptides or protein segments obtained in membrane mimetic solvents to structures obtained by solution state NMR in micelles or to entire membrane proteins determined by x-ray crystallography (11–13). Most interestingly, we find that CD$_3$OH:CDCl$_3$:H$_2$O provides a well defined peptide structure, whereas the peptide in Me$_2$SO appears much less structured. This implies that there is a great deal of value in determining membrane peptide structures in multiple solvent conditions, because a single solvent may not induce sufficient sampling of the structured state.

Three sections of four to nine residues of the TM IV segment converge structurally and were defined as core regions by NMRCORE (23) and confirmed by analysis of r.m.s.d. values (Table III). Namely, the stretches of residues Asp$^{159}$–Leu$^{163}$, Leu$^{165}$–Pro$^{168}$a and Ile$^{169}$–Phe$^{176}$b each converge. These are illustrated in Fig. 7 (a–c), and their relationship is demonstrated schematically in Fig. 7d. Each section superposes extremely well, with all convergent residues identified in Fig. 7d for the 600-member ensemble having a C$_\alpha$ r.m.s.d. relative to the average structure in the range of 0.25–0.85 Å. The heavy backbone and side chain atom r.m.s.d. are <1.5 Å over Asp$^{159}$–Leu$^{163}$ and Ile$^{169}$–Gly$^{174}$ as well as for Leu$^{165}$ and Pro$^{167}$ (Table III), which implies good structural convergence at the side chain level for these segments. The boundary points of converged stretches are also very clear upon examination of the r.m.s.d. values in Table III as follows: Ser$^{158}$ and Glu$^{157}$ rapidly diverge from Asp$^{159}$–Leu$^{163}$; Phe$^{164}$ has a much higher r.m.s.d. than the surrounding residues when the peptide structures are superposed at either 159–163 or 165–168; Pro$^{166}$ clearly fits well with 165–168 but not with 169–176, whereas the converse is true of Ile$^{169}$; finally, Leu$^{177}$, Pro$^{178}$, and Leu$^{179}$ demonstrate large jumps in r.m.s.d. values relative to the superposition 170–178. One of the ensemble members is shown in Fig. 7e as a representative structure. Given that the segment must span a membrane with the width on the order of 25–35 Å, a relatively extended ensemble member such as that pictured in Fig. 7e is more representative of the expected biological configuration than one that curls back upon itself. This particular structure must be considered as only demonstrative of the relation between the convergent segments of the TM IV segment, rather than as representative of any global structuring.

The first feature of note is that the TM IV segment certainly does not resemble a canonical transmembrane a-helix. This is despite the fact that there are helix-capping sequences inherent within the TM IV over the segment studied. The N-terminal sequence FLQSDV (155–160) fits well with a type Ib cap, whereas the sequence LDAGYF (171–176) fits a type Va C-terminal cap, with capping nomenclature as defined by Aurora and Rose (43). These cap sequences would imply that a helix does not resemble a canonical transmembrane a-helix. This is true of Ile$^{169}$; finally, Leu$^{175}$, Pro$^{178}$, and Leu$^{179}$ demonstrate strong structural and functional roles.

## Table III

| Residue | r.m.s.d., C$_\alpha$ | r.m.s.d., all heavy atoms |
|---------|---------------------|--------------------------|
| Gln$^{157}$ | 1.83 | 3.43 |
| Ser$^{158}$ | 1.23 | 1.86 |
| Asp$^{159}$ | 0.43 | 1.24 |
| Val$^{160}$ | 0.25 | 0.59 |
| Phe$^{161}$ | 0.30 | 1.42 |
| Phe$^{162}$ | 0.45 | 1.50 |
| Leu$^{163}$ | 0.53 | 1.16 |
| Phe$^{164}$ | 1.54 | 2.65/4.00 |
| Leu$^{165}$ | 0.80 | 1.40 |
| Leu$^{166}$ | 0.57 | 1.94 |
| Pro$^{167}$ | 0.38 | 0.85 |
| Pro$^{168}$ | 0.85/1.43 | 1.55/1.81 |
| Ile$^{169}$ | 3.16/0.76 | 4.36/3.44 |
| Ile$^{170}$ | 0.70 | 1.24 |
| Leu$^{171}$ | 0.59 | 1.41 |
| Asp$^{172}$ | 0.48 | 1.32 |
| Ala$^{173}$ | 0.52 | 0.64 |
| Gly$^{174}$ | 0.55 | 0.56 |
| Tyr$^{175}$ | 0.36 | 2.00 |
| Phe$^{176}$ | 0.70 | 1.77 |
| Leu$^{177}$ | 1.24 | 2.61 |
| Pro$^{178}$ | 1.36 | 1.64 |
| Leu$^{179}$ | 2.66 | 3.53 |

*Superposed on C$_\alpha$ of 159–161 and 163 using LSqKAB (50, 51).*

*Not defined as core using program NMRCORE.*

*Superposed on C$_\alpha$ of 165–168.*

*Superposed on C$_\alpha$ of 170–173, 175–176, and 178.*
\[\beta\text{-turns. Asp}^{159} \text{ is the } i + 2 \text{ residue in one type IV turn, and the first residue in a second, Val}^{160}, \text{ is the } i + 3 \text{ and } i + 1 \text{ residue for these turns, as well as the first residue in a third turn; following along the sequence, Phe}^{164} \text{ and Phe}^{162} \text{ both participate in the turns initiated at Asp}^{159} \text{ and Val}^{160} \text{ and Leu}^{163} \text{ participates as the } i + 3 \text{ residue for Val}^{160}. \text{ Therefore, this series of turns, observed in 81–94\% of the ensemble, serves to converge this five-residue stretch. Most interestingly, Leu}^{165} - \text{Pro}^{168} \text{ are nowhere near as strongly influenced by } \beta\text{-turns. Rather, these residues appear to be quite extended (Fig. 7b), with Pro}^{168} \text{ initiating type I or IV } \beta\text{-turns in } \sim 85\% \text{ of the ensemble. Finally, residues Ile}^{169} - \text{Phe}^{176} \text{ converge strongly, providing the only appreciable } \alpha\text{-helical character over the entire transmembrane segment. The helical stretch extends over } 4 - 6 \text{ residues for } \sim 77\% \text{ of the ensemble members, starting between Ile}^{169} \text{ and Leu}^{171} \text{ and ending at Gly}^{174}, \text{ with each one of these members of the ensemble showing helical character over the four-residue segment of Leu}^{170} - \text{Gly}^{174}. \text{ The C terminus of this convergent stretch is defined by Gly}^{174} \text{ and Tyr}^{175}, \text{ each of which act as the first residue for a sequential pair of type IV } \beta\text{-turns in 92\% or more of the ensemble members.}

\text{Although similar experimental resolution in NMR studies has been achieved using micelles in aqueous solution (46), such an environment would surround the TM IV peptide with hydrophobic detergent tails. In its natural setting, this segment of the NHE1 protein would contact lipid tail groups, surrounding

\text{Fig. 7. Convergent structural motifs pinpointed by NMRCORE (23) in the lowest energy 600 members of the final 1000 structure ensemble satisfying NOE and J-coupling restraints determined for the TM4 peptide in CD}_{2}\text{OH}:\text{CDCl}_{3}:\text{H}_{2}\text{O (4:1 v/v/v). a–c show the lowest energy 60 structures, with backbone atoms colored black and side chains gray. a, defined extended structure over 165–168; and c, helical stretch at 169–176. d, schematic demonstrating convergent stretches (gray) in relation to pivot residues Phe}^{164} \text{ and Pro}^{168}/\text{Ile}^{169} \text{(black) and flexible N and C termini (dashed lines). Note that only proline side chains are drawn. e, stick diagram with Cory, Pauling, Koltun coloring of a single member of the ensemble (i.e. the flexible linkers and termini in d have assumed a discrete configuration) demonstrating the overall extended nature of the peptide.}
polypeptide segments, and a pore region with a relatively high dielectric constant. In the case where a transmembrane segment has a very clearly defined structure, such as a canonical membrane-spanning α-helix, the micellar environment serves as an excellent conformational stabilizer. In the micellar state, we would anticipate that the allowed rotations about Phe164 and Pro165/Phe166 would be greatly reduced. However, this may actually be an artificial constraint upon the peptide structure relative to the solvent setting, which allows increased conformational sampling. We feel that the present structure in a mixture of solvents representing a variety of dielectrics provides a reliable representation of the best structured regions of the TM IV segment. This should be useful in interpretation of future experiments using more complete sections of NHE1, in particular for solid state NMR experiments that are aided by knowledge of the most likely polypeptide backbone configurations (47).

Correlating Structural and Functional Data—in light of the structural data, plausible explanations for the loss in activity observed for some of the most severe cysteine-scanning mutants can be advanced. Mutations to P167C and P168C practi- cally abrogate all exchanger activity, and mutations of these prolines to other residues did not allow for a return to a normally functioning Na+/H⁺ exchanger (10). Mutation from an amino to a much freer amino acid moiety would readily modify the structural motif observed at this pair of prolines (Fig. 7b). Given the loss of activity, it is likely that the extended structural nature of this motif is crucial in NHE1 folding into the functional form. The Y175C mutation also causes almost complete loss in activity. Most interestingly, Tyr175 is observed to participate as the second residue in an extremely well converged double (i;i + 1) type IV β-turn, just N-terminal to the aromatic residue Phe176. Note that F176C also causes significant loss of activity. Analysis of the torsion angles defined between the Cα–Cβ, Cβ–Cγ, and Cγ–Cδ vectors of Tyr175 and Phe176 for the ensemble of 600 structures shows that the aromatic side chain moieties of Tyr175 and Phe176 have a strong tendency to fall within −50° of each other relative to the polypeptide backbone over this region of the TM IV segment. Because these are large, hydrophobic side chains, it seems likely that they would be at the interface with neighboring transmembrane segments or protruding into the lipid bilayer rather than directly into the channel environment. Substitution of these aromatic groups with the significantly less bulky, polar cysteine side chain would disrupt these interactions, thereby disrupting the NHE1 structure as a whole, leading to the observed loss in activity. However, the mutated F161C side chain does appear to be pore-exposed, so this is not a certainty.

The mutation F161C allows for reactivity with the sulfhydryl-reactive reagent MTSET that inhibits Na+/H⁺ exchanger activity. This implies that residue 161 is facing the NHE1 translocation pore. With this in mind, examination of the structure of the TM IV segment provides a very interesting revelation. Phe161 side chain is actually fully exposed when put in the context of residues Phe155–Ile166 in every member of the ensemble (data not shown). Because rotation of the structurally convergent sections is observed about Phe164 (Fig. 7d), any further analysis of the two C-terminal domains relative to Phe161 would be purely speculative, and it was therefore not possible to determine at this stage which other C-terminal amino acids face the translocation pore. However, the exposed nature of the Phe161 side chain at the extracellular end of the segment is demonstrated in all 600 retained ensemble members satisfying the experimental constraints, meaning that the isolated TM IV segment in membrane mimetic solvent assumes a structure consistent with the reactivity observed for the exchanger as a whole. Over the convergent stretch of Asp159–Leu163 (Fig. 7a), the Asp159 side chain is uniformly observed on the same face of the peptide. Therefore, it seems likely that the anionic Asp159 side chain would also protrude into the pore of the exchanger, allowing it to act in cation coordination during translocation. Mutation of this amino acid eliminated activity and reduced expression of the protein, consistent with a critical role for this amino acid. We have previously postulated that polar residues within transmembrane segments of the Na+/H⁺ exchanger are important in cation coordination and transport (48, 49), and the structure of TM IV supports such a role for this polar residue.

Our study has given the first detailed structural and functional information on TM IV of the NHE1 isoform of the Na+/H⁺ exchanger. TM IV appears to be very sensitive to alterations in its amino acid sequence. We show that although it is a well structured transmembrane segment, its structure is uniquely different from a typical α-helix. Possibly this reflects its pivotal role in cation binding and transport. Phe161 and possibly Asp159 are amino acid residues that protrude into the pore of the channel.

Acknowledgments—Protein identification and purification were performed at the Institute for Biomolecular Design, University of Alberta, Edmonton, Alberta, Canada. We thank the Canadian National High Field NMR Centre for their assistance in the collection of the 800-MHz data. Operation of Canadian National High Field NMR Centre is funded by the Canadian Institutes of Health Research, Natural Sciences and Engineering Research Council of Canada, and the University of Alberta. We also thank Jeff Devries and Gerry McQuaid for maintaining the 500- and 600-MHz spectrometers and Dr. T. Burrow of the Department of Chemistry, University of Toronto, for useful discussions.

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Transmembrane Segment IV of the Na⁺/H⁺ Exchanger
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Structural and Functional Characterization of Transmembrane Segment IV of the NHE1 Isoform of the Na⁺/H⁺ Exchanger
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J. Biol. Chem. 2005, 280:17863-17872.
doi: 10.1074/jbc.M409608200 originally published online January 26, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M409608200

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