The Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1) is an integral membrane protein that regulates intracellular pH by removing one intracellular H\(^+\) in exchange for one extracellular Na\(^+\). It has a large N-terminal membrane domain of 12 transmembrane segments and an intracellular C-terminal regulatory domain. We characterized the cysteine accessibility of amino acids of the putative transmembrane segment IX (residues 339–363). Each residue was mutated to cysteine in a functional cysteine-less NHE1 protein. Of 25 amino acids mutated, 5 were inactive or nearly so after mutation to cysteine. Several of these showed aberrant targeting to the plasma membrane and reduced expression of the intact protein, whereas others were expressed and targeted correctly but had defective NHE1 function. Of the active mutants, Glu\(^{346}\) and Ser\(^{351}\) were inhibited >70% by positively charged [2-(trimethylammonium)-ethyl]methanethiosulfonate, suggesting that they are pore lining and make up part of the cation conduction pathway. Both mutants also had decreased affinity for Na\(^+\) and decreased activation by intracellular protons. The structure of a peptide representing amino acids 338–365 was determined by using high resolution NMR in dodecylphosphocholine micelles. The structure contained two helical regions (amino acids Met\(^{340}\)–Ser\(^{344}\) and Ile\(^{353}\)–Ser\(^{359}\)) kinked with a large bend angle around a pivot point at amino acid Ser\(^{351}\). The results suggest that transmembrane IX is critical with pore-lining residues and a kink at the functionally important residue Ser\(^{351}\).

The mammalian Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1)\(^a\) is a ubiquitous integral membrane protein that regulates intracellular pH by mediating removal of one intracellular proton in exchange for one extracellular sodium ion (1). NHE1 has many cellular and physiological functions. It promotes cell growth and differentiation (2), protects cells from intracellular acidification (2, 3), and also regulates sodium fluxes and cell volume after challenge by osmotic shrinkage (4). In addition, Na\(^+\)/H\(^+\) exchanger activity has been demonstrated to be involved in modulating cell motility and invasiveness of neoplastic breast cancer cells (5), and it has been shown to be critical to cell motility in some cell types (6). The Na\(^+\)/H\(^+\) exchanger also plays a critical causal role in heart hypertrophy and in the damage that occurs during ischemia and reperfusion. Inhibition of the exchanger with a new generation of Na\(^+\)/H\(^+\) exchanger inhibitors protects the myocardium during these disease states (7–10).

NHE1 has an N-terminal membrane ion translocation domain of ~500 amino acids and a C-terminal regulatory domain of ~315 amino acids (1, 8). Wakabayashi et al. (11) used a combination of hydrophobicity analysis and cysteine-scan-ning mutagenesis to predict a topology with 3 membrane-associated segments and 12 integral transmembrane (TM) segments (Fig. 1A). A recent homology model, based on the Escherichia coli antiporter NhaA crystal structure (12), also predicts 12 TM segments, but assignments of transmembrane segments are different in comparison with the Wakabayashi topology (13).

The exact details of cation coordination and translocation through the central ion pore of NHE1 have only recently begun to emerge. In this respect, we have identified TM IV (14) and TM VII (15) (with topology assignments according to Ref. 11) as functionally critical. More recently, we solved the structures of these TM segments and identified specific essential residues (16, 17). Prolines 167 and 168 of TM IV were essential to NHE1 function (14) and Phe\(^{161}\) was shown to be a pore-lining residue involved in transport using cysteine-scanning mutagenesis (16). The peptide was composed of an N-terminal \(\beta\)-turn region, followed by an extended (nonhelical) structure.

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\(^{b}\)The coordinate and structure factors (code 2zk3c) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://wwww.rcsb.org/j). The atomic coordinates and structure factors (code 2zk3c) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://wwww.rcsb.org/j).

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\(^{4}\)Recipient of support as a Canada Research Chair in Structural Biology.

\(^{5}\)Recipient of an Alberta Heritage Foundation for Medical Research Scientist award. To whom correspondence should be addressed: Dept of Biochemistry, 347 Medical Science Bldg., University of Alberta, Edmonton, Alberta T6G 2H7 Canada. Tel.: 780-492-1848; Fax: 780-492-0886; E-mail: fliegel@ualberta.ca.

\(^{6}\)The abbreviations used are: NHE1, Na\(^+\)/H\(^+\) exchanger isoform 1; cNHE1, cysteine-less NHE1; TM, transmembrane (segment); HA, hemagglutinin; MTSET ([2-(trimethylammonium)ethyl]methanethiosulfonate); MTSES ([2-sulfonatoethyl]methanethiosulfonate); NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; DPC, dodecylphosphocholine; HSQC, heteronuclear single quantum coherence spectroscopy; TOCSY, total correlation spectroscopy; r.m.s.d., root mean square deviation; DSS, (d\(_6\))-2,2-dimethyl-2-silapentane-5-sulfonic acid.
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Oligonucleotides used for site-directed mutagenesis of TM IX

| Mutation | Oligonucleotide sequence | Restriction site |
|----------|--------------------------|-----------------|
| Y339C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | PvuII |
| M340C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | AlwNI |
| A341C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | AlFIII |
| Y342C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | Spfl |
| L343C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| A344C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| A345C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| E346C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| S347C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| F348C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| H349C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| L350C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| S351C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| S352C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| I353C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| M354C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| A355C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| L356C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| S357C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| A358C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| S359C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| G360C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| I361C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| Y362C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| A345C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| A346C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |
| G360C   | 5'-CTCCCTCTACGCTGAGCTCTACTTGG-3' | SphI |

Mutated nucleotides are in lowercase letters. Mutated amino acid residues are indicated in boldface type, and new restriction sites are underlined. Restriction sites deleted are indicated by parentheses.

Induced around Pro\textsuperscript{467}–Pro\textsuperscript{468}, and a C-terminal helical region (16). In contrast, TM VII was an interrupted helix with several residues critical for function (17). Of importance, functionally essential residues were localized at and near the flexible break point between helices.

Another important segment of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger is TM IX. TM IX is composed of residues 339–363 (Fig. 1B). Several studies have suggested that it is critical to NHE1 function. One study used chimeric NHEs to examine TM segments that confer sensitivity to pharmacological antagonists (18). Using hybrid NHEs of the sensitive NHE1 and resistant NHE3, TM IX was shown to be important in mediating sensitivity to the antagonists. Another study showed that alteration of His\textsuperscript{349} of TM IX reduced the sensitivity to amiloride compounds (19). In contrast, TM VII was an interrupted helix with several residues critical for function (17). Of importance, functionally essential residues were localized at and near the flexible break point between helices.

Another important segment of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger is TM IX. TM IX is composed of residues 339–363 (Fig. 1B). Several studies have suggested that it is critical to NHE1 function. One study used chimeric NHEs to examine TM segments that confer sensitivity to pharmacological antagonists (18). Using hybrid NHEs of the sensitive NHE1 and resistant NHE3, TM IX was shown to be important in mediating sensitivity to the antagonists. Another study showed that alteration of His\textsuperscript{349} of TM IX reduced the sensitivity to amiloride compounds (19). Two other studies have shown by site-specific mutagenesis that the TM IX and Glu\textsuperscript{346} of TM IX are important in NHE function and drug sensitivity (20, 21).

In this study, we examine both structural and functional aspects of TM IX of the NHE1 isoform of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger. We use cysteine-scanning mutagenesis of TM IX in the full-length NHE1 protein to characterize which amino acids are important in function and are likely pore lining. Cysteine-scanning mutagenesis has been used to determine pore-lining residues in numerous membrane proteins (22–27), including our own study of TM IV of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger (16). Two sulfhydryl-reactive reagents that are often used in these studies are MTSET and MTSES, which are membrane-impermeant (28–30) and react with potentially pore-lining residues surrounded by water. Parallel structural characterization by NMR and CD spectroscopy is used to examine the structure of the TM segment in isolated peptide form. Considerable literature has suggested that the study of isolated TM segments reflects the structure and function of the intact membrane protein (31–35) especially when stabilized in membrane-mimetic dodecylphosphocholine (DPC) micelles (36, 37). Our study demonstrates that TM IX has two helical segments and a sharp kink at a Ser\textsuperscript{351} pivot point. Both Ser\textsuperscript{351} and Glu\textsuperscript{346} appear to be critical pore-lining residues based on cysteine-scanning mutagenesis and accessibility analysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Lipofectamine\textsuperscript{TM} 2000 reagent was from Invitrogen, and PWO DNA polymerase was obtained from Roche Applied Science. MTSET and MTSES were from Toronto Research Chemicals, Toronto, Ontario, Canada. Deuterated DPC was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada).

**Site-directed Mutagenesis**—Mutations were made to an expression plasmid containing a tagged human NHE1 isoform of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger. The plasmid (pYN4+) contained the cDNA for the entire coding region of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger with a hemagglutinin (HA) tag that we have previously shown does not affect the function of the protein (14). The mutations made (Table 1) changed the indicated amino acids to cysteine, using the background of the functional cysteineless NHE1 protein that we have described earlier (16). Site-directed mutagenesis was done by amplification with PWO DNA polymerase (Roche Applied Science) followed by using the Stratagene (La Jolla, CA) QuikChange\textsuperscript{TM} site-directed mutagenesis kit. The mutagenesis created or deleted a restriction enzyme site for use in screening transformants. The fidelity of DNA amplification was confirmed by DNA sequencing.

**Cell Culture and Transfections**—Stable cell lines of all mutants were made as described earlier (14) via transfection with Lipofectamine\textsuperscript{TM} 2000 reagent (Invitrogen). AP-1 cells were used that lack an endogenous Na\textsuperscript{+}/H\textsuperscript{+} exchanger (16). Selection was done using 800 \mu g/ml geneticin (G418), and stable cell lines for experiments were regularly re-established from frozen stocks at passage numbers between 5 and 9.
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SDS-PAGE and Immunoblotting—Western blot analysis was used to confirm NHE1 expression (14). Cell lysates were made from stable cell lines, and equal amounts of up to 100 μg of each sample were resolved on a 10% SDS-polyacrylamide gel. Nitrocellulose transfers were immunostained using a primary antibody of anti-HA monoclonal antibody (Roche Applied Science). The secondary antibody was peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, Ontario, Canada). The Amersham Biosciences enhanced chemiluminescence blotting and detection system were used to visualize immunoreactive proteins. NIH Image 1.63 software (National Institutes of Health, Bethesda) was used for densitometric analysis of x-ray films.

Cell Surface Expression—We measured cell surface expression to ensure that all mutant proteins were properly targeted to the cell surface (14). Sulfo-NHS-SS-biotin (Pierce)-labeled cell surface proteins and immobilized streptavidin resin was used to remove plasma membrane-labeled Na⁺/H⁺ exchanger. Equal amounts of total and unbound proteins were analyzed by Western blotting against the HA tag, and densitometry was as described earlier (14). The amount of NHE1 on the cell surface was calculated by comparing both the 110- and 95-kDa immunoreactive species in Western blots of the total and unbound fractions. It was not possible to efficiently elute proteins bound to immobilized streptavidin resin reproducibly.

Na⁺/H⁺ Exchange Activity—Na⁺/H⁺ exchange activity was measured as described previously (16). Ammonium chloride was used to induce acute acid load, and the initial rate of Na⁺-induced recovery of cytosolic pH (pHᵢ) was measured using 2',7'-bis(carboxyethyl)-5-(6) carboxylfurorescin-AM (Molecular Probes Inc., Eugene, OR) and a PTI Deltascan spectrofluorometer. Recovery from acidosis was in the presence of 135 mM NaCl. There was no difference in the buffering capacities of the various stable cell lines. Where indicated, activity of the Na⁺/H⁺ exchanger mutants was corrected for targeting of the protein to the cell surface and for the level of protein expression. Results are the means ± S.E., and statistical significance was determined using the Mann-Whitney U test.

To test the effect of MTSET and MTSES on NHE1 activity of mutants, we used the standard Na⁺/H⁺ exchanger assay with slight modification. In this case cells were acidified two times with ammonium chloride 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. Cells were subsequently washed three times in Na⁺-free buffer prior to the second ammonium chloride-induced acidification and recovery. To calculate residual activity during recovery from acidosis Equation 1 was used,

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

(Eq. 1)

In some experiments we checked the Na⁺ and proton affinity of mutant NHE1 proteins. The determination of kinetic parameters of the Na⁺/H⁺ exchanger was essentially as described earlier (38). Na⁺ concentrations were varied while maintaining osmolality with N-methyl-d-glucamine. For these experiments cells were acidified to the same level with 50 mM NH₄Cl. To determine the proton affinity, intracellular pH was varied by acidifying to various degrees using ammonium chloride concentrations from 5 to 50 mM as described earlier (38). Buffering capacity of the cell lines was used to determine proton flux essentially as described earlier (38). Cation affinity was calculated using SigmaPlot and fitting lines to a three-parameter sigmoidal fit.

Peptide Synthesis and Purification—TM IX peptide (sequence, KSYMAYLSAELFHLSGIMALIASGVVMRPKK; acetyl-capped N terminus, amide-capped C terminus) was purchased from GL Biochem (Shanghai, China) at >95% purity. Purity was assessed by high pressure liquid chromatography, and identity was confirmed using sequential assignment of NMR data and by matrix-assisted laser desorption ionization mass spectroscopy.

NMR Spectroscopy and Structure Calculations—The NMR sample was prepared by dissolving 0.9 ± 0.1 mM synthetic peptide in a 95% H₂O, 5% D₂O solution containing ~75 mM deuterated DPC and 0.25 mM (d₅)-2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) for chemical shift referencing. Solution pH was adjusted to 5.05 without consideration of deuterium isotope effects, and all experiments were conducted at 30 °C. One-dimensional ¹H, natural abundance gradient-enhanced ¹H-¹³C HSQC, two-dimensional ¹H-¹³C TOCSY (60-ms mix; decoupling in the presence of scalar interactions spin lock), and NOESY (225-ms mix) experiments were acquired on the Canadian National High Field NMR Centre Varian INOVA 800-MHz spectrometer. All experiments were used as configured within the Varian BioPack software package. Spectra were processed using NMRPipe (39) and analyzed using Sparky 3 (65). All spectral assignments were carried out by manual peak picking in Sparky, and resonance assignments have been deposited in the BioMagResBank (code 15747).

Structure calculations were performed in the python scripting interface of XPLOR-NIH version 2.18 (40) using NOE restraints from the assigned spectrum. Peak volumes were independently calculated using Gaussian and Lorentzian fit Sparky algorithms, with no allowed peak center motion. Those peaks that were not assigned a volume or were assigned a negative volume by the algorithm (~27.5%) were either manually assigned a summed signal intensity as defined by a user-specified region or fit by Sparky algorithm after contour adjustment. Peak volumes were empirically calibrated to distance ranges of 0–5.0 Å, 1.8–5.0 Å, and 1.8–6.0 Å; the Lorentzian fit volumes were more conservative and used for subsequent analysis. Restraints were divided into bin ranges corresponding to strong (1.8–2.8 Å), medium (1.8–4.0 Å), weak (1.8–5.0 Å), and very weak (1.8–6.0 Å) contacts. Ambiguous assignments and NOE potential scaling were handled as described previously (17). Following 21 rounds of structural refinement by simulated annealing, an additional 6 rounds were performed with dihedral angle potential scaling factors of 5, 50, 100, 100, 50, and 25. Simulated annealing parameters were similar to those described previously (17), but with 15,000 cooling steps.

A single extended polypeptide was generated and subjected to simulated annealing for each round. To handle the families of 100 structures generated per round an in-house tcl/tk script...
Characterization of TM IX of the Na\(^+\)/H\(^+\) Exchanger

![Diagram of TM IX](image)

**RESULTS**

Cysteine-scanning Mutagenesis—Fig. 1A illustrates a general model of the Na\(^+\)/H\(^+\) exchanger (Fig. 1A) after (11) and a schematic model of TM IX is shown in Fig. 1B. We used the cysteineless Na\(^+\)/H\(^+\) exchanger (cNHE) to examine which amino acids of TM IX are critical to activity of the Na\(^+\)/H\(^+\) exchanger and which amino acids are pore lining. We have shown previously that HA-tagged cNHE functions normally (16). Twenty five amino acids of TM IX (339YMAYLSAELFHLSGIMALIASGVVM363) were mutated to cysteine residues in the background of the cysteineless NHE1 protein. These amino acids represent more than a typical number of amino acids that cross a lipid bilayer.

Experiments examined whether these mutant forms of the Na\(^+\)/H\(^+\) exchanger expressed and targeted properly and had activity. Fig. 2A shows a representative Western blot of the expression of the cysteineless NHE1, the wild type and the cysteine-scanning mutants expressed in AP-1 cells. The level of expression relative to the cysteineless Na\(^+\)/H\(^+\) exchanger is shown below each mutant. Most mutants expressed to reasonable levels, greater than 60% of the values of the controls. It was notable that Y339C and S344C were mainly present as a lower band. Most notable was the Y339C mutant that was now mostly intracellular. Most of the other mutants had reduced targeting to the cell surface by between 15 and 30%, although this still left approximately half of the protein targeting to the cell surface. Non-specific binding of NHE1 protein to streptavidin agarose beads was ~11%, so the values shown overestimate the level of surface protein.

As part of our analysis of the effect of the mutations, we determined how the mutations of the protein affected NHE1 activity. The rate of recovery from an acute acid load induced by ammonium chloride was determined as described earlier (44). Fig. 3 illustrates an example (Fig. 3A) and a summary (Fig. 3B) of
the rates of recovery from stable cell lines transfected with cysteineless or wild type Na\(^+\)/H\(^+\) exchanger or mutants of TM IX. We also corrected the rate of recovery for both the level of expression and surface targeting so that we could determine whether the mutations affect the activity of the protein directly or indirectly through effects on trafficking and expression. Five of the mutants were severely affected by mutation to cysteine, Tyr\(^{339}\), Leu\(^{343}\), Ile\(^{353}\), Ala\(^{355}\), and Val\(^{361}\). Correction for surface targeting and expression improved the relative activity of all these mutants slightly, although not to the level of controls. All five had less than 20% uncorrected activity compared with the cysteineless NHE1 protein. Because their low activity made further experiments impractical, these mutants were not used for any further analysis.

**Sulfhydryl Modification of Cysteine Mutants**—We examined the sensitivity to MTSET or MTSES of the 20 mutant Na\(^+\)/H\(^+\) exchangers of TM IX that had greater than 20% residual activity of the cysteineless NHE1 (Fig. 4). MTSES caused slight, but not significant, stimulation of activity of both the wild type NHE1 and many of the mutant NHE1s (Fig. 4, A and C). Of the mutant Na\(^+\)/H\(^+\) exchangers, the E346C and S351C mutants were significantly affected by treatment with the positively charged MTSET (Fig. 4, B and C). In both cases the reduction of activity was approximately 30% of the control activity. Treatment of these mutants with the negatively charged reagent MTSES did not result in significant reductions in activity. No other mutants were greatly affected by either MTSET or MTSES. However, there were minor but significant reductions in activity in two residues adjacent to the affected residues. The A345C and the L350C mutants were reduced in activity 20–30%. The V362C mutant also showed a minor, significant reduction in activity.

To characterize the effect of mutation of Glu\(^{346}\) and Ser\(^{351}\) in more detail, we examined the effect of the change to these amino acids on the kinetics of Na\(^+\)/H\(^+\) exchanger activity. Extracellular sodium and intracellular pH values were varied, and the activity of the protein was measured, and estimates of proton affinity and \(V_{\text{max}}\) were calculated. With either mutation, both the \(V_{\text{max}}\) and the \(K_{\text{m}}\) values of transport were reduced (Fig. 4).
5). When we determined the rate of proton transport of cNHE1 and the S351C and E346C mutants, we found that proton transport was greatly impaired in the mutants. The maximal rates of transport of cNHE1 and of the S351C and E346C mutants were 1.5, 1.07, and 0.12 mM/s, respectively. The activation of the mutant Na$^+/H^+$ exchanger proteins by H$^+$ was also greatly impaired. Wild type NHE1 was half-maximally activated at approximately pH 7.2, whereas the S351C mutant was activated at pH 5.7 (Fig. 5A), about a 32-fold increase in H$^+$ concentration. The H$^+$ affinity of the E346C mutant was so poor it was not measurable by the techniques used in our study.

Effects on sodium transport were also quite large (Fig. 5B). The $V_{\text{max}}$ values for Na$^+$ of cNHE1, E346C, and S351C were 0.0198, 0.0089, and 0.0109 (ΔpH/S), respectively, indicating a
Characterization of TM IX of the Na\(^+/H^+\) Exchanger

**FIGURE 5.** Cation affinity of wild type and mutant Na\(^+/H^+\) exchangers. Na\(^+/H^+\) exchanger activity of cells expressing either wild type NHE1 or NHE1 with the E346C or S351C mutations was determined as described under "Experimental Procedures." A, dependence of proton efflux on intracellular proton concentration. Cells were preincubated with varying concentrations of ammonium chloride for various times. Proton efflux was calculated from proton concentration. Cells were preincubated with varying concentrations of Na\(^+\) for various times. Proton efflux was calculated from the initial rate of recovery times the buffering capacity. B, dependence of Na\(^+/H^+\) exchanger activity on external sodium concentration. Na\(^+\) concentrations were varied while maintaining osmolarity with N\(-\)methyl-o-glucamine. Cells were acidified to the same degree as described under "Experimental Procedures." Results are mean ± S.E. of at least six experiments. Where not shown S.E. was too small to be displayed.

A large decrease for the mutants in the maximal rate of transport. The \(K_m\) values for Na\(^+\) for cNHE1, E346C, and S351C were 39.3, 99.2, and 77 mM, respectively, demonstrating greatly reduced affinity for Na\(^+\) in both mutants.

**Peptide Design and Conditions for NMR Spectroscopy**—The synthetic peptide contains three lysine residues, one at the N terminus and two at the C terminus, which are not present in the endogenous sequence of the NHE1 protein. Cationic residues at the termini of peptides have been shown to facilitate both peptide purification and maintenance of transbilayer orientation (45). For convenience, the lysine residues are numbered relative to the endogenous sequence. All structural studies were conducted on the 31-residue peptide produced by chemical synthesis with no isotope labels.

Although a methanol/chloroform/water (4/4/1, v/v) mixture and dimethyl sulfoxide were both shown to solubilize the TM IV segment of NHE1 (16), the TM VII segment was not soluble under these or other organic solvent conditions (17). Thus, DPC micelles were employed in the latter case (17) and are also employed in this work, acting simultaneously as a solubilization medium for the peptide and as a membrane mimetic environment. Sample components were 0.9 ± 0.1 mM peptide, ~75 mM deuterated DPC, and 0.25 mM (\(d_2\) 2,2-dimethyl-2-silapentane-5-sulfonic acid (as a chemical shift standard) in 95% H\(_2\)O, 5% D\(_2\)O adjusted to pH ~5.05 and studied at 30 °C. The peptide concentration was estimated based on \(^1\)H one-dimensional NMR spectrum integrations relative to the internal DSS standard using assigned resonances. This temperature provided good NMR spectral characteristics, prevented precipitation for extended periods of spectroscopic study, and allows for use of the cryogenically cooled triple-resonance probe on the 800-MHz Canadian National High Field NMR Centre spectrometer. This combination of factors allowed determination of the structure of the TM IX segment in DPC micelles.

**Resonance Assignment and Structure Calculation**—Sequential chemical shift assignments were carried out using two-dimensional TOCSY, natural abundance \(^1\)H-\(^15\)N HSQC, and NOESY experiments (17, 46). Natural abundance \(^1\)H-\(^15\)N HSQC was not feasible because of low signal-to-noise arising from the tumbling rate of the peptide-containing micelles. Resonance assignments for \(^1\)H were complete except for Met-\(^1\)H\(^d\) and Phe-\(^4\)H\(^d\). In some cases there were missing or ambiguous \(^13\)C assignments for Tyr-\(^1\)C\(^\alpha\), Met-\(^1\)C\(^\alpha\)/\(^1\)C\(^\beta\), Leu-\(^1\)C\(^\alpha\)/\(^1\)C\(^\beta\)/\(^1\)C\(^\gamma\), Ser-\(^1\)C\(^\gamma\)/\(^1\)C\(^\delta\), Glu-\(^1\)C\(^\gamma\), Phe-\(^1\)C\(^\gamma\), His-\(^1\)C\(^\gamma\)/\(^1\)C\(^\delta\), and Lys-\(^1\)C\(^\gamma\). Assessment of unambiguous assignments was made as described previously (17). A total of 1231 unique NOE restraints (Table 2) were used for calculation of the TM IX structure. These are summarized graphically in terms of the standard connectivities examined for secondary structure characterization and in terms of the number of unique restraints per residue in Fig. 6.

**Structural Analysis of TM IX Peptide**—An ensemble of the 40 lowest energy structures from 100 calculated peptide structures was obtained that satisfy the 1231 observed unique NOE

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**TABLE 2**

NMR structural statistics for the final ensemble of 40 structures retained out of 100 calculated

| Dihedral restraints | No dihedral restraints |
|--------------------|------------------------|
| Unique NOE restraints | 1231 | 44.8 ± 3.1 |
| Total | 54.2 ± 5.6 | 8.69 ± 2.3 |
| Intra-residue | 432 | |
| Sequential | 536 | |
| Medium range | 238 | |
| Long range | 2 | |
| Ambiguous | 23 | |

Ramachandran plot statistics

| Core | 54.3% |
| Allowed | 34.6% |
| Generously allowed | 7.0% |
| Disallowed | 4.0% |

XPLOR-NIH energies (kcal/mol)

| Total | 54.2 ± 5.6 | 44.8 ± 3.1 |
| NOE | 9.95 ± 2.4 | 8.69 ± 2.3 |

NOE violations

| Violations >0.5 Å | 0 | 0 |
| Violations of 0.3–0.5 Å | 13 | 15 |
| Violations of 0.2–0.3 Å | 25 | 33 |
restraints with minimal violations (Table 2). Dihedral angle restraints ($\phi = -60 \pm 30; \psi = -40 \pm 40$) consistent with $\alpha$-helical structure were imposed over residues Met$^{340}$–Ser$^{344}$ and Ile$^{353}$–Ser$^{359}$. The dihedral restraints resulted in a significant but reasonable ($\sim 17\%$) inflation in total energy relative to an ensemble restrained only by NOEs (Table 2). The break in helicity over residues 345–352 is also consistent with secondary chemical shift data (Fig. 7A).

A 9-replicate averaged CD spectrum was analyzed using the DICHROWEB (42, 43) interface by applying several secondary structure deconvolution algorithms (results summarized in Table 3). A 190 nm low-wavelength cutoff was used for meaningful signals as detailed under “Experimental Procedures.” The normalized standard deviation parameter was used to filter those algorithms that did not produce a good fit to the data (normalized r.m.s.d. $>0.2$) (47). The calculated average secondary structure contributions are $26 \pm 5\%$ helix, $31 \pm 6\%$ sheet, $16 \pm 4\%$ turn, and $29 \pm 10\%$ random (Table 3). In light of the relatively high average deviations across algorithms, a qualitative inspection of the CD spectra is relevant (Fig. 8). To reflect the 12/31 ($\sim 39\%)$ helical residues predicted based on NMR dihedral restraints, a (theoretical) 39% helix CD spectrum from 200 to 240 nm was generated by K2D (41). The curves are qualitatively consistent with a strong helical contribution (Fig. 8).

Superposition of all members of the ensemble over the full length of the peptide was not possible. However, based on r.m.s.d. analysis as detailed in Ref. 46, Lys$^{337}$–Leu$^{350}$ and Gly$^{352}$–Val$^{362}$ were respective N- and C-terminal segments consistent with a relatively invariant structural fold (Figs. 9 and 10). The intervening Ser$^{351}$ serves as a pivot point between the N- and C-terminal portions of the peptide, although its dihedral angles across the ensemble of structures are well clustered (Fig. 10, triangles), and its dihedral angle order parameters are close to unity, although in close proximity to a flexible region (Fig. 7B). Although residues assigned to the two helical segments were also well clustered by dihedral angle (Fig. 10, $\times$), there was more variability for those residues falling into superimposable regions outside of the helices proper (Fig. 10, hollow gray circles). C-terminal residues Arg$^{364}$ and Pro$^{365}$, which were not favored for inclusion in a C-terminal superposition, had reasonably well clustered dihedral angles in the ensemble (Fig. 10, squares). This is interesting because the preceding Met$^{363}$, which was also not superimposable, had a large dispersion of backbone dihedral angles over the ensemble (Fig. 10, gray circles with dark fill).

**Structural Superposition of TM IX—**Peptide segments between 4 and 19 residues long were iteratively superimposed to provide a minimum r.m.s.d. relative to the backbone of the lowest energy structure (Fig. 9). Those superpositions producing the largest contiguous segment of residues with r.m.s.d. values $<1.0$ were additionally filtered based on average r.m.s.d. across the entire segment. The following permutations were assessed: N-terminal segments between residues 337–342 and 345–354 and C-terminal segments between residues 349–354 and 357–367 were superimposed by the method of Kabsch (48) as implemented in the LSQKAB software of the CCP4 suite (49). Lys$^{337}$–Leu$^{350}$ and Gly$^{352}$–Val$^{362}$ were the respective N- and C-terminal regions with r.m.s.d. values most consistent with a fixed region of structure (Fig. 9, A and B). The dihedral angle order parameter (Fig. 7B) was consistent with a region of structural flexibility (e.g. a pivot point at Ser$^{351}$) between the determined segments.
DISCUSSION

When we replaced amino acids of TM IX with cysteines, we found that TM IX was less sensitive than TM IV to these alterations. For TM IV, 11 of the 23 cysteine mutants had less than 20% of the activity of the control cNHE1 (16). For TM IX this only occurred in 5 instances out of 25. These results confirm our suggestion (16) that the susceptibility to mutation appears to vary not only between proteins but also within different transmembrane segments of the same protein. This was noted earlier with the lactose permease (50).

The 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. Some but not all of the amino acids of TM IX were strictly required. All the mutants of TM IX resulted in some decreases in activity that were usually caused by effects on targeting and expression levels. This suggests an important, but not essential, function of these side chains.

**Structural Analysis of TM IX**

Where the full-length structure of proteins is available, studies have shown that isolated transmembrane segments both reflect the structure of intact proteins and often retain their functional characteristics. Specific examples include bacteriorhodopsin (31, 32), rhodopsin (33), the cystic fibrosis TM conductance regulator (34, 35), and the fungal G-protein-coupled receptor Ste2p (51). Stabilization of the physiologi-

**TABLE 3**

**DICROWEB algorithm analysis of NHE1 TM IX circular dichroism spectra**

A 9-replicate data set collected over 3 days was averaged in the range of 190–260 nm and used for the analysis. Only sum total contributions from each type of secondary structure are shown, even for those algorithms that estimate the number of segments of a given secondary structure (63). Average deviations are shown for average values.

| Algorithm  | Protein reference data base | Helix | Sheet | Turn | Random | Total | Normalized r.m.s.d. |
|------------|-----------------------------|-------|-------|------|--------|-------|---------------------|
| K2D        | None*                       | 30    | 14    | N/A  | 55     | 99    | 0.167               |
| CDSSTR     | 4                           | 21    | 34    | 20   | 25     | 100   | 0.018               |
| CDSSTR     | 7                           | 22    | 38    | 20   | 19     | 99    | 0.016               |
| CONTIN-LL  | SP175 (64)                  | 19    | 39    | 8    | 33     | 99    | 0.021               |
| CONTIN-LL  | 4                           | 31.2  | 29.9  | 15.0 | 23.9   | 100.0 | 0.196               |
| CONTIN-LL  | 7                           | 31.7  | 31.5  | 16.9 | 20.0   | 100.1 | 0.196               |
| Average    |                             | 26 ± 5| 31 ± 6| 16 ± 4| 29 ± 10| 102   |                     |

* K2D is a neural network algorithm that does not directly require a reference protein data base.
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FIGURE 8. Circular dichroism spectra for the TM IX peptide in DPC micelles. CD samples were prepared by serial dilution of the NMR sample while maintaining DPC at a concentration of 3 mM, which is considerably greater than the critical micelle concentration of DPC (CMC\textsubscript{DPC} = 1 mM) (62). No additional UV-absorptive ions were added to the mixture. Data were collected between 260 and 180 nm and are plotted where the signal/noise was significant (“Experimental Procedures”). For comparison, the empirical data are plotted relative to a theoretical 39\% helix spectrum generated using the K2D algorithm (41). Protein concentrations used for mean residue ellipticity (MRE) calculations are estimations based on integration of the one-dimensional \textsuperscript{1}H NMR spectrum relative to the DSS internal standard.

The Glu\textsuperscript{346}, Ile\textsuperscript{353}, and V\textsuperscript{361} side chains face the opposite direction of Tyr\textsuperscript{339}, Ser\textsuperscript{351}, and Ala\textsuperscript{355} side chains (Fig. 9C) in our structure. This would seem to conflict with their importance in function; however, Landau \textit{et al.} (13) suggest that Ser\textsuperscript{351} faces the lipid bilayer despite its functional importance and that it can rotate by 180° to face the pore for ion coordination. If true, the segment may sometimes face the pore and sometimes the lipid bilayer.

Only two mutants, Glu\textsuperscript{346} and Ser\textsuperscript{351}, were strongly inhibited by reaction with MTSET, and no mutants were inhibited by MTSES. The most likely explanation for their reactivity with MTSET is an interaction at a site that lines and blocks the ion translocation pore (54). There were minor inhibitory effects of MTSET on the adjacent amino acids Ala\textsuperscript{345} and Leu\textsuperscript{350} (Fig. 4C). Their mutation could cause a minor perturbation of the structure of TM IX in this region that affects Glu\textsuperscript{346} and Ser\textsuperscript{351} but is also consistent with these residues having at least partial exposure to the pore. In support of this concept, Ala\textsuperscript{345}, Glu\textsuperscript{346}, and Leu\textsuperscript{350} all cluster with side chains on the same face of the segment throughout the ensemble of structures. Although Ser\textsuperscript{351} is consistently (37/40 ensemble members) on the opposite face of the peptide, this observation may be explained by the rotation mechanism suggested by Landau \textit{et al.} (13).

Positively charged MTSET but not negatively charged MTSES inhibited NHE1 activity in the E346C and S351C mutants. This contrasts with our results for the F161C mutant of TM IV that was inhibited by both compounds (16) but mirrors results with TM VII (55). MTSET may disrupt cation transport by direct electrostatic repulsion, whereas negative MTSES would not. Alternatively, MTSES may not be able to react with these amino acids because of repulsion on the protein surface from negatively charged amino acids important in ion coordination (56). Local protein conformation and chemistry have
been shown to affect accessibility to sulfhydryl-reactive reagents in both K$^+$/H$^+$ channels (57) and in the FMRF-amide-activated sodium channel (58). The E346C cNHE1 mutant exhibited a higher degree of kinetic inhibition for the same measured parameters as S351C (Fig. 5) and was similarly affected by MTSET (inhibition) and MTSES (no inhibition). In both topology models (11, 13), Ser351 is located in the intramembrane region of the protein. Therefore, our results are consistent with Ser$^{351}$ being pore-lining in both cases and MTSET obstructing the pore. In the first topology model (11), Glu$^{346}$ is also in the intramembrane region and should be pore-lining, potentially on the same face of a TM segment as Ser$^{351}$. However, our structural analysis did not place these residues on the same face, only 3/40 ensemble members show this positioning. This may suggest an extracellular loop position for Glu$^{346}$, as proposed by Landau et al. (13). MTSET modification could then obstruct cation transport by preventing entry to the pore (Glu$^{346}$).

The observed increase in $K_{m(Na^+)}$ for S351C mutants is consistent with at least moderately reduced extracellular coordination efficacy (Fig. 5), and may therefore be supportive of a role in the extracellular cation funnel for Ser$^{351}$ as suggested by Landau et al. (13). The decrease in $V_{max(Na^+)}$ may imply a measurable contribution to inhibition by a perturbation in overall pore structure or that ion coordination by Ser$^{351}$ is rate-limiting (i.e. that we are not simply observing reduced electrostatic interaction that can be overcome by ligand saturation). Finally, the reduced $V_{max}$ values for H$^+$ transport and especially the reduced intracellular (i.e. allosteric) activation of S351C cNHE1 by H$^+$ are in agreement with an overall perturbation in tertiary structure (Fig. 5).

Based on dihedral restraints and r.m.s.d. analysis, our data suggest that Met$^{340}$–Ser$^{344}$ is helical, and although this is consistent with these residues lining a TM segment as they do in both models, the lack of contiguous $\alpha$-helical character observed in the ensemble C-terminal to this segment until the
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second major helical segment (Ile³⁵³–Ser³⁵⁹) would be consistent with the extracellular loop assignment of residues 345–352 (13). The observed pivot at Ser³⁵¹ (Fig. 9C) also supports the possibility of an adjacent flexible extracellular loop at the location suggested by Landau et al. (13). Glu³⁴⁶ and Gly³⁵² have been implicated as being critical in NHE1 inhibitor binding (20, 21). Placement of these residues at or near the extracellular surface is consistent with a site of drug interaction. A distorted and interrupted helix with a bent TM L-shape would result if the peptide NMR structure were applied as a single transmembrane segment (11). The bent structure we observe is consistent with a eukaryotic membrane span, the distance between Cα of Ser³³⁸ and Ser³⁵⁹ is 28 ± 2 Å in our structures. Conversely, if the Landau et al. (13) topology is correct, residues His³⁴⁹ and Val³⁶² would be at the extracellular and intracellular faces, giving a transbilayer length of 19 ± 1 Å, a length typically assumed to be insufficient to span a eukaryotic membrane (53). To attempt to resolve these topological issues, a series of experiments were done with amino acids 345–348 to determine the accessibility to the extracellular surface. However, it was not possible to get consistent results that either verified or disproved their extracellular or transmembrane location (not shown). Even when externally accessible residues are detected, they are sometimes assigned intracellular locations using the argument that they represent pore-lining residues (11), making assignment of location somewhat arbitrary. Our functional studies showing inhibition by MTSET or our structural studies on the isolated TM IX peptide provided valuable data on the importance of these residues in function, but they are unable to shed light on their precise topology.

We found that amino acid Met³⁶³ displayed an elevated dispersion of backbone dihedral angles, and this may suggest functionally important flexibility at this position (Fig. 10). However, our results also showed that the M363C mutation did not eliminate activity (Fig. 3B) and that it did not react with MTSET or MTSES, which is somewhat contradictory to the idea that it is critical to function. Wakabayashi et al. (11) suggested that M363C is in an extracellular loop. Landau et al. (13) placed Met³⁶³ in an intracellular loop. Our results strongly support Met³⁶³ as a loop residue displaying a high degree of flexibility, but they cannot localize this residue to either face of the membrane nor assign a critical pore-lining role to this amino acid.

Overall, our study gives a detailed structural and functional picture of the functionally critical amino acids 339–363 of the NHE1 isoform of the Na⁺/H⁺ exchanger. We demonstrate that 5 of 25 residues are very sensitive to mutation to cysteine, and that Glu³⁴⁶ and Ser³⁵¹ are involved in cation transport and likely line the cation pore. The ensemble of structures, representing a kinked helical peptide, is similar to that previously reported for TM VII (17), but with a larger bend angle at the pivot point, Ser³⁵¹. Amino acids Met³⁴⁰–Ser³⁴⁴ and Ile³⁵³–Ser³⁵⁹ are helical. To resolve whether amino acids 339–363 represent one transmembrane segment or parts of two, a full-length structure of NHE1 is most likely necessary. We recently overexpressed and purified the full-length NHE1 protein, and a low resolution structure has been obtained (52). Further detailed analysis of this protein may reveal its topology.

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