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 cysteineless 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, Glu346 and Ser351 were inhibitory and targeted correctly but had defective NHE1 expression; whereas others 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, Glu354 and Ser355 were inhibited >70% by positively charged [2-(trimethylammonium)ethyl]methanethiosulfonate but not by anionic [2-sulfonatoethyl]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 Met340–Ser344 and Ile353–Ser359) kinked with a large bend angle around a pivot point at amino acid Ser351. The results suggest that transmembrane IX is critical with pore-lining residues and a kink at the functionally important residue Ser351.

The mammalian Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1) 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-scanning 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 Phe161 was shown to be a pore-lining residue involved in transport using cysteine-scanning mutagenesis (16). The peptide was composed of an N-terminal β-turn region, followed by an extended (nonhelical) structure...
Characterization of TM IX of the Na\(^{+}/\)H\(^{+}\) Exchanger

### Table 1

Oligonucleotides used for site-directed mutagenesis of TM IX

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Oligonucleotide sequence</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y393C</td>
<td>5'-CTTCCCTCTGATTACGGGCA-3'</td>
<td>PvuII</td>
</tr>
<tr>
<td>M340C</td>
<td>5'-CTTTATGTGCCTACTCTGCAC-3'</td>
<td>AflIII</td>
</tr>
<tr>
<td>A341C</td>
<td>5'-CTTTATGTGCCTACTCTGCAC-3'</td>
<td>SphI</td>
</tr>
<tr>
<td>Y342C</td>
<td>5'-CTTTATGTGCCTACTCTGCAC-3'</td>
<td>SphI</td>
</tr>
<tr>
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<td>NdeI</td>
</tr>
<tr>
<td>A345C</td>
<td>5'-CTTTATGTGCCTACTCTGCAC-3'</td>
<td>NsiI</td>
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<tr>
<td>E346C</td>
<td>5'-CTTTATGTGCCTACTCTGCAC-3'</td>
<td>NsiI</td>
</tr>
<tr>
<td>L347C</td>
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<td>BstXI</td>
</tr>
<tr>
<td>A341C</td>
<td>5'-CTTTATGTGCCTACTCTGCAC-3'</td>
<td>BstXI</td>
</tr>
</tbody>
</table>

### Experimental Procedures

**Materials**—Lipofectamine™ 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 MTSET and MTSES were from Toronto Research Chemicals, Toronto, Ontario, Canada. Deuterated MTSES was also obtained from Toronto Research Chemicals, Toronto, Ontario, Canada. Deuterated MTSET and MTSES were from Toronto Research Chemicals, Toronto, Ontario, Canada.

**Site-directed Mutagenesis**—Mutations were made to an expression plasmid containing a tagged human NHE1 isoform of the Na\(^{+}/\)H\(^{+}\) exchanger. The plasmid (pYN4+) contained the cDNA for the entire coding region of the Na\(^{+}/\)H\(^{+}\) 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™ 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™ 2000 reagent (Invitrogen). AP-1 cells were used that lack an endogenous Na\(^{+}/\)H\(^{+}\) exchanger (16). Selection was done using 80 μg/ml genetin (G418), and stable cell lines for experiments were regularly re-established from frozen stocks at passage numbers between 5 and 9.
Characterization of TM IX of the Na\(^+\)/H\(^+\) Exchanger

**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 \(\mu\)g 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 x-ray films. 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 acid load, and the initial rate of Na\(^+\)-induced recovery of cytosolic pH (pH\(_i\)) was measured using 2',7'-bis(2-carboxyethyl)-5(6) carboxyfluorescein-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 \(\mu\)M 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,

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

(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 osmolarity with N-methyl-d-glucamine. For these experiments cells were acidified to the same level with 50 mM NH\(_4\)Cl. 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 Sigmplot and fitting lines to a three-parameter sigmoidal fit.

**Peptide Synthesis and Purification**—TM IX peptide (sequence, KSYMAYLSAELHLHSIGMALIASGVMRPPK; 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% H\(_2\)O, 5% D\(_2\)O solution containing ~75 mM deuterated DPC and 0.25 mM (d\(_6\))-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 1\(^H\), natural abundance gradient-enhanced 1\(^H\)-13\(^C\) HSQC, two-dimensional 1\(^H\)-1\(^H\) 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 of fit by Sparky algorithm after contour adjustment. Peak volumes were empirically calibrated to distance ranges of 0–5.0, 1.8–5.0, 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
(freely available upon request from J. Rainey) was used; the script allowed for assessment of violations and iterative refinement of NOE restraints. Refinement gradually increased in stringency, initially violations >0.5 Å in >50% of structures were lengthened, but subsequently violations >0.1 Å in >25% of structures were modified. After 21 cycles of simulated annealing and NOE refinement, calculated XPLOR-NIH structure energies contained minimal contributions from NOE violations, and magnitudes of all observed violations were minimal. All NOE restraints were satisfied without pruning. Six further cycles of simulated annealing were carried out with incorporation of dihedral angle restraints as described above. The lowest 40 energy structures in the final ensemble of 100 were retained for further analysis. The final sets of restraints have been deposited in the Protein Data Bank with this ensemble of 40 structures (Research Collaboratory for Structural Bioinformatics Protein Data Bank code 2k3c).

**CD Spectroscopy and Analysis**—Samples for CD spectroscopy were diluted from the previously described NMR sample to obtain samples of ∼10 μM peptide, ∼3 mM deuterated DPC, and the unbuffered pH was adjusted to ∼4.8 with H₂SO₄/NaOH. Nine replicate measurements were collected over 3 separate days on a Jasco J-810 spectropolarimeter (Easton, MD) at 30°C in a 0.1-cm path length (Hellma, Müllheim, Germany) water-jacketed cell (20 nm/min scan rate). Data were collected in a wavelength range of 260-180 nm, but the signal to noise ratio was considered reasonable only at a detector (photomultiplier tube) voltage under 700 (based on previous instrumental observation). Averaged data were analyzed by several algorithms (41) using the DICHROWEB interface (42, 43).

**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 shown below each mutant. Most mutants expressed to reasonable levels, greater than 60% of the values of the controls. It was apparent 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.

Identification of transmembrane amino acids can affect surface targeting of the Na⁺/H⁺ exchanger (16). Therefore, we examined intracellular targeting of the NHE1-expressing cell lines. After cells were treated with sulfonamide-SS-biotin, labeled proteins of lysates were bound to streptavidin-agarose beads. To identify NHE1 protein we used Western blotting with anti-HA antibody and examined equal amounts of total cell lysates and unbound lysates. This revealed the relative amounts of tagged intracellular NHE1 protein. Fig. 2B illustrates examples of the results and a summary of at least six experiments. Both the 110- and 95-kDa bands were included in the analysis. The results revealed that, in several cases, mutation of the amino acids to cysteine caused decreases in targeting of the protein to the plasma membrane. 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. Nonspecific 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, Tyr₃₃⁹, Leu₃₄³, Ile₃₅₃, Ala₃₅₅, and Val₃₆₁. 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 Glu₃⁴⁶ and Ser₃⁵¹ 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 $V_{max}$ were calculated. With either mutation, both the $V_{max}$ and the $K_m$ values of transport were reduced (Fig. 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 (\(\Delta pH/S\)), respectively, indicating a
Characterization of TM IX of the Na\textsuperscript{+}/H\textsuperscript{+} Exchanger

![Graph A](Image 9x-15 to 34x815)

**FIGURE 5. Cation affinity of wild type and mutant Na\textsuperscript{+}/H\textsuperscript{+} exchangers.** Na\textsuperscript{+}/H\textsuperscript{+} 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 Na\textsuperscript{+} 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\textsuperscript{+}, as described under “Experimental Procedures.” B, dependence of Na\textsuperscript{+}/H\textsuperscript{+} exchanger activity on external sodium concentration. Na\textsuperscript{+} concentrations were varied while maintaining osmolarity with Na\textsuperscript{+} acetate. This combination of factors allowed determination of the dependence of proton efflux on intracellular Na\textsuperscript{+} 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\textsuperscript{+}, as described under “Experimental Procedures.”

A large decrease for the mutants in the maximal rate of transport. The $K_m$ values for Na\textsuperscript{+} for cNHE1, E346C, and S351C were 39.3, 99.2, and 77 mM, respectively, demonstrating greatly reduced affinity for Na\textsuperscript{+} 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$_4$) 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.

**Table 2: NMR structural statistics for the final ensemble of 40 structures retained out of 100 calculated**

<table>
<thead>
<tr>
<th>Restrains</th>
<th>Dihedral restraints</th>
<th>No dihedral restraints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unique NOE restraints</td>
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</tr>
<tr>
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<tr>
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<tr>
<td>Medium range $</td>
<td>i-j</td>
<td>24</td>
</tr>
<tr>
<td>Long range $</td>
<td>i-j</td>
<td>15</td>
</tr>
<tr>
<td>Disallowed</td>
<td>4.0%</td>
<td></td>
</tr>
</tbody>
</table>

Average deviations are shown for XPLOR-NIH energies.

**Ramachandran plot statistics**

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

**XPLOR-NIH energies (kcal/mol)**

<table>
<thead>
<tr>
<th>NOE violations</th>
<th>Total</th>
<th>44.8 ± 3.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Violations &gt;0.5 Å</td>
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<td>44.8 ± 3.1</td>
</tr>
<tr>
<td>Violations of 0.3–0.5 Å</td>
<td>13</td>
<td>44.8 ± 3.1</td>
</tr>
<tr>
<td>Violations of 0.2–0.3 Å</td>
<td>25</td>
<td>44.8 ± 3.1</td>
</tr>
</tbody>
</table>

Resonance assignments for $^1$H were complete except for Met-H$\alpha$ and Phe-H$\alpha$. In some cases there were missing or ambiguous $^{13}$C assignments for Tyr-C$\beta$, Met-C$\beta$/C$\gamma$/C$\delta$, Ser-C$\gamma$/C$\delta$, Glu-C$\alpha$, Phe-C$\beta$, His-C$\alpha$ and Lys-C$\alpha$. 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...
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 ± 5% helix, 31 ± 6% sheet, 16 ± 4% turn, and 29 ± 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 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). Other superimpositions 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 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 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 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).

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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-
Characterization of TM IX of the Na\textsuperscript{+}/H\textsuperscript{+} Exchanger

Cysteine-scanning accessibility analysis and led to the present widely used model with TM IX including amino acids 338–360. More recently a model was developed based on fold recognition using the E. coli NhaA crystal structure as a template for phylogenetic analysis and homology modeling (13). In this model, amino acids 338–360 of TM IX correspond to part of TM VII, a subsequent extracellular loop, and all of TM VIII. Landau et al. (13) suggest that the previously described TM IX is an artifact of the window size used for hydropathy analysis. However, the TM VIII arising from their model has a membrane-spanning length of ~19 Å, which is short for a eukaryotic membrane (53).

**Structure-Function Correlation**—In all cases, we were able to express an intact Na\textsuperscript{+}/H\textsuperscript{+} exchanger protein. However, five of the mutants had decreased NHE1 protein activity. For Leu\textsuperscript{346} and Ser\textsuperscript{351} this was due, at least in part, to decreased expression and improper targeting. For Tyr\textsuperscript{339}, Ile\textsuperscript{355}, and Val\textsuperscript{361}, significant defects in protein function were introduced by mutation.

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. 9C). 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 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 translocation pore (54). There were minor inhibitory effects of MTSET on the adjacent amino acids Ala\textsuperscript{345} and Leu\textsuperscript{350} (Fig. 9C). 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 et al. (13).

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-adsorbing 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 $^1H$ NMR spectrum relative to the DSS internal standard.

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-adsorbing 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 $^1H$ NMR spectrum relative to the DSS internal standard.
been shown to affect accessibility to sulfhydryl-reactive reagents in both K⁺/H⁺11001 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 Ser351 being pore-lining in both cases and MTSET obstructing the pore. In the first topology model (11), Glu346 is also in the intramembrane region and should be pore-lining, potentially on the same face of a TM segment as Ser351. 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 Glu346, as proposed by Landau et al. (13). MTSET modification could then obstruct cation transport by preventing entry to the pore (Glu346).

The observed increase in $K_{m(\text{Na}^+)}$ 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 Ser351 as suggested by Landau et al. (13). The decrease in $V_{\text{max(\text{Na}^+)}}$ may imply a measurable contribution to inhibition by a perturbation in overall pore structure or that ion coordination by Ser351 is rate-limiting (i.e. that we are not simply observing reduced electrostatic interaction that can be overcome by ligand saturation). Finally, the reduced $V_{\text{max}}$ 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 Met340–Ser344 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
second major helical segment (Ile\textsuperscript{353}–Ser\textsuperscript{359}) would be consistent with the extracellular loop assignment of residues 345–352 (13). The observed pivot at Ser\textsuperscript{351} (Fig. 9C) also supports the possibility of an adjacent flexible extracellular loop at the location suggested by Landau et al. (13). Glu\textsuperscript{346} and Gly\textsuperscript{352} 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 kinked helical peptide, is similar to that previously reported for TM VII (17), but with a larger bend angle at the pivot point, Ser\textsuperscript{351}. Amino acids Met\textsuperscript{340}–Ser\textsuperscript{344} and Ile\textsuperscript{353}–Ser\textsuperscript{359} 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 detailed analysis of this protein may reveal its topology.

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\textsuperscript{+}/H\textsuperscript{+} exchanger. We demonstrate that 5 of 25 residues are very sensitive to mutation to cysteine, and that Glu\textsuperscript{346} and Ser\textsuperscript{351} 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, Ser\textsuperscript{351}. Amino acids Met\textsuperscript{340}–Ser\textsuperscript{344} and Ile\textsuperscript{353}–Ser\textsuperscript{359} 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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REFERENCES
