A Novel Topology Model of the Human Na\(^+\)/H\(^+\) Exchanger Isoform 1*

(Received for publication, August 5, 1999, and in revised form, October 28, 1999)

Shigeo Wakabayashi‡, Tianxiang Pang, Xiaohua Su§, and Munekazu Shigekawa

From the Department of Molecular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan

The membrane topology of the human Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1) was assessed by substituted cysteine accessibility analysis. Eighty-three cysteine residues were individually introduced into a functional cysteineless NHE1, and these mutants were expressed in the exchanger-deficient PS120 cells. The topological disposition of introduced cysteines was determined by labeling with a biotinylated maleimide in the presence or absence of preincubation with the membrane-impermeable sulfhydryl reagent, 2-trimethylammoniumethyl-methanethiosulfonate; SLO, streptolysin O-permeabilized or nonpermeabilized cells. We proposed a new model for the topology of NHE1 that is significantly different from the model derived from hydropathy analysis. In this model, NHE1 is composed of 12 transmembrane segments (TMs) with the N and C termini located in the cytosol. The large, last extracellular loop in the membrane domain of the original model was suggested to comprise an intracellular loop, a new transmembrane segment (TM11), and an extracellular loop in the new model. Interestingly, cysteines at 183 and 184 and at 324 and 325 mapped to intracellular loops connecting TMs 4 and 5 (IL2) and TMs 8 and 9 (IL4), respectively, were accessible to sulfhydryl reagents from the outside. Furthermore, exchange activities of two mutants, R180C and Q181C, within IL2 were markedly inhibited by extrinsic MTSET. These data suggest that part of IL2 or IL4 may be located in a pore-lining region that is accessible from either side of the membrane and involved in ion transport.

The Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1),\(^1\) which catalyzes an electroneutral exchange of Na\(^+\) for H\(^+\) across the plasma membrane, is involved in the regulation of intracellular pH (pHi) and cell volume (1, 2). Until now, six different NHE isoforms were cloned from mammalian tissues (3–8). Although these isoforms differ in tissue localization, sensitivity of inhibitors, and mode of regulation, they have a similar overall structure consisting of an N-terminal membrane domain (∼500 amino acids) and a C-terminal cytoplasmic domain (∼300 amino acids). The former catalyzes an amiloride-sensitive Na\(^+\)/H\(^+\) exchange, while the latter functions as a regulatory domain (9).

Previous biochemical and molecular studies have provided important information about the structure-function of the cytoplasmic domain of NHE1 (2). We have recently found that the cytoplasmic domain consists of at least four distinct functional subdomains in terms of pH sensitivity, some of which are involved in the regulation of the exchanger by cytosolic Ca\(^{2+}\), protein phosphorylation, and cell ATP (10–12). On the other hand, little is known about the structure of the N-terminal membrane domain of NHE1, which precludes the clarification of the mechanism for ion exchange and the interaction between the cytoplasmic domain and the ion transport pathway in the membrane domain. The N-terminal membrane domain is highly conserved (70% overall identity) among NHE isoforms and is predicted to span the lipid bilayer 10–12 times. A 12-transmembrane segment (TM) model predicted by hydropathy analysis of NHE1 according to the Kyte-Doolittle algorithm (13) is shown in Fig. 1. The N-terminal region including TM1 is believed without experimental evidence to be cleaved off as a signal peptide, because its amino acid sequence is highly variable among NHE isoforms. In the human NHE1, evidence was presented showing that N-glycosylation occurs in the first putative extracellular loop (14). On the other hand, a recent chymotryptic cleavage experiment suggested that some other extracellular loops in the membrane domain of NHE1 are not fully exposed on the extracellular surface (15). Clearly, more systematic analysis of the topology of the exchanger molecule is required.

In this study, to map the transmembrane topology of NHE1, we determined the accessibility of 83 cysteine residues introduced into a cysteineless form of NHE1 (designated “Cys-less NHE1”) to cysteine-directed reagents, biotin maleimide and MTSET, in normal and permeabilized cells (16, 17). The former reagent is almost membrane-impermeable and covalently labels cysteine residues with a biotin group that is readily detectable by the use of streptavidin-biotin chemistry. The latter is membrane-impermeable and used to block the biotinylation. Based on the detailed analysis, we proposed a new topology model of NHE1, which consists of 12-transmembrane segments with the N and C termini located in the cytosol. We found that some introduced cysteines within the intracellular loops are also accessible from the outside, suggesting that residues at these positions may be part of the ion transport pathway.

EXPERIMENTAL PROCEDURES

Materials—Biotin maleimide was purchased from Molecular Probes, Inc. (Eugene, OR). MTSET and streptavidin-conjugated agarose were purchased from Toronto Research Chemicals Inc. and Pierce, respec-

---

*This work was supported by Grants-in-aid for Scientific Research 09680642 and 10470013 from the Ministry of Education, Science, and Culture of Japan and grants from Core Research for Evolutionary Science and Technology of Japan Science and Technology Corporation, the Vehicle Racing Commemorative Foundation, and the Uehara Memorial Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby

‡ To whom correspondence should be addressed. Tel.: 81-6-833-5012 (ext. 2566); Fax: 81-6-872-7485.

§ Supported by the Science and Technology Agency Fellowship of Japan.

1 The abbreviations used are: NHE1–3, Na\(^+\)/H\(^+\) exchanger isoforms 1–3; biotin maleimide, 3-([\(\text{N}\)-maleimidylpropionyl]biocytin; MTSET, 2-trimethylammoniumethyl-methanethiosulfonate; SLO, streptolysin O; EIPA, 5-([\(\text{N}\)-ethyl-N-isopropyl]amiloride; PAGE, polyacrylamide gel electrophoresis; TM, transmembrane segment.
The amino acid sequence of NHE1 (2, 3). Asn 75 is N-glycosylated (14). Nine endogenous cysteines mutated to alanines and 89 cysteines newly introduced into Cys-less NHE1 are shown by filled squares and filled circles, respectively. EL1–EL6, putative extracellular loops connecting TMs 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, and 11 and 12, respectively; IL1–IL5, putative intracellular loops connecting TMs 2 and 3, 4 and 5, 6 and 7, 8 and 9, and 10 and 11, respectively.

**Fig. 1. A topological model of NHE1 predicted from hydropathy analysis.** The model is based on the hydropathy analysis of the primary amino acid sequence of NHE1 (2, 3). Asn 75 is N-glycosylated (14). Nine endogenous cysteines mutated to alanines and 89 cysteines newly introduced into Cys-less NHE1 are shown by filled squares and filled circles, respectively. EL1–EL6, putative extracellular loops connecting TMs 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, and 11 and 12, respectively; IL1–IL5, putative intracellular loops connecting TMs 2 and 3, 4 and 5, 6 and 7, 8 and 9, and 10 and 11, respectively.

Streptolysin O (SLO) was obtained from Sigma. The amiloride derivative, 5-(2-ethyl-N-isopropyl)amiloride (EIPA) was a gift from New Drug Research Laboratories of Kanebo, Ltd. (Osaka, Japan). "Calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37 °C in the presence of 5% CO2. PS120 cells (5 × 105 cells/100-mm dish) were transfected with each plasmid construct using a calcium phosphate co-precipitation technique. Cell populations that stably express mutant NHE1 were selected by a "Hilling" procedure as described (9). Among 89 substituted cysteine mutants, stable transfectants for six mutants (see Fig. 9) were not obtained. Streptolysin O (SLO) was purchased from NEN Life Science Products. A NHE1-exchanger-deficient cell line (PS120) (18) kindly provided by Dr. J. Pouyssegur (Nice, France) and the corresponding transfectants were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Pouyssegur (Nice, France) and the corresponding transfectants were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 5% NaHCO3 and supplemented with 7.5% (v/v) fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37 °C in the presence of 5% CO2. PS120 cells (5 × 105 cells/100-mm dish) were transfected with each plasmid construct using a calcium phosphate co-precipitation technique. Cell populations that stably express mutant NHE1 were selected by a "Hilling" procedure as described (9). Among 89 substituted cysteine mutants, stable transfectants for six mutants (see Fig. 9) were not obtained.

**Construction of Na+/H+ Exchanger Mutants**—The plasmid carrying the Na+/H+ exchanger-deficient cell line (PS120) (18) kindly provided by Dr. J. Pouyssegur (Nice, France) and the corresponding transfectants were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 5% NaHCO3 and supplemented with 7.5% (v/v) fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37 °C in the presence of 5% CO2. PS120 cells (5 × 105 cells/100-mm dish) were transfected with each plasmid construct using a calcium phosphate co-precipitation technique. Cell populations that stably express mutant NHE1 were selected by a "Hilling" procedure as described (9). Among 89 substituted cysteine mutants, stable transfectants for six mutants (see Fig. 9) were not obtained.

**Cells and Culture Conditions and Stable Expression**—The Na+/H+ exchanger-deficient cell line (PS120) (18) kindly provided by Dr. J. Pouyssegur (Nice, France) and the corresponding transfectants were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 5% NaHCO3 and supplemented with 7.5% (v/v) fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37 °C in the presence of 5% CO2. PS120 cells (5 × 105 cells/100-mm dish) were transfected with each plasmid construct using a calcium phosphate co-precipitation technique. Cell populations that stably express mutant NHE1 were selected by a “Hilling” procedure as described (9). Among 89 substituted cysteine mutants, stable transfectants for six mutants (see Fig. 9) were not obtained.

**Measurement of 22Na+ Uptake**—22Na+ uptake activity and its pH,
Fig. 2. Characterization of Cys-less NHE1. A, pH$_i$ dependence of EIPA-sensitive $^{22}$Na$^+$ uptake into cells expressing the wild-type or Cys-less NHE1 was measured using the K$^+$/nigericin method. B, pH$_i$ of cells expressing the wild-type or Cys-less NHE1 was clamped at 5.6 by the use of K$^+$/nigericin, and $^{22}$Na$^+$ uptake into these cells was measured as a function of EIPA concentration. C and D, cells expressing the wild-type or NHE1 mutants were incubated with 0.05 or 0.5 mM biotin maleimide, solubilized with the lysis buffer, and then treated with streptavidin-agarose as described under “Experimental Procedures.” Total cell lysate (40 µg) or the proteins recovered with streptavidin-agarose (D) were analyzed by SDS-PAGE. Wild-type or mutant NHE1 proteins were visualized by immunoblot analysis with the NHE1 antibody.

Protein Determination—Protein concentration was measured by the bichinchoninic acid assay (Pierce Chemical Co.) using bovine serum albumin as a standard.

RESULTS

Characterization of Cys-less NHE1—In this study, we first characterized Cys-less NHE1 expressed in the exchanger-deficient PS120 cells. Fig. 2A shows pH$_i$ dependence of $^{22}$Na$^+$ uptake in cells expressing the wild-type and Cys-less mutant exchangers. Cys-less NHE1 exhibited a high $^{22}$Na$^+$ uptake activity (>40 nmol/mg/min) similar to the wild-type, and the pK values for both exchangers were found to be similar (~6.7). Hill coefficients were also similar (1.22 or 1.25 for the wild-type or Cys-less NHE1). Fig. 2B shows the concentration dependence of EIPA on $^{22}$Na$^+$ uptake by both exchangers, which gave similar IC$_{50}$ values (60–80 nM). The amounts of the exchange proteins expressed were also similar (Fig. 2C). Thus, Cys-less NHE1 is fully functional and has almost the same basic properties as the wild-type NHE1.

We compared the biotinylation efficiency among the wild-type, Cys-less, and other mutant exchangers that were well expressed in PS120 cells (Fig. 2C). Cells were incubated with 0.05 or 0.5 mM biotin maleimide, and biotylated proteins were subsequently recovered with streptavidin-agarose. After SDS-PAGE, the biotinylated exchangers were visualized by immunoblot analysis with an anti-NHE1 antibody (Fig. 2D). As expected, recovery of the Cys-less NHE1 protein was minimal, indicating that the mutant was only slightly labeled with externally applied biotin maleimide. In contrast, heavy antibody staining was observed for the cysteine-substituted mutant E151C, indicating its strong biotinylation (Fig. 2D). We observed very weak labeling of the wild-type and M-Cys-less NHE1. In the latter mutant, we replaced 6 endogenous cysteine residues in the N-terminal membrane domain with alanine while retaining 3 endogenous cysteines in the C-terminal cytoplasmic domain. Thus, the endogenous cysteine residues in the N-terminal membrane domain are not accessible from the external surface, and a relatively low concentration (0.5 mM) of biotin maleimide is virtually membrane-impermeable under the conditions used.

Biotinylation of Substituted Cysteines in Putative Extracellular Loops—We introduced 15 cysteine residues individually into the putative first extracellular loop of NHE1. Cells expressing Cys-less NHE1 or single cysteine mutants were treated with or without 5 mM MTSET. Cells were then incubated with 0.5 mM biotin maleimide, solubilized with the lysis buffer, and treated with streptavidin-agarose as described under “Experimental Procedures.” Total cell lysate (20 µg) (A and C) or the proteins recovered with streptavidin-agarose (B and D) were separated by SDS-PAGE, and biotinylated NHE1 proteins were visualized by immunoblot analysis with the NHE1-antibody.
these mutant exchangers were biotinylated with variable efficiency. For example, H35C, S56C, T79C, and H81C were strongly biotinylated, while S40C, T68C, R72C, and H76C were only weakly labeled. Preincubation of cells with the membrane-impermeable SH modifier MTSET significantly reduced the biotinylation of all these mutants, indicating that substituted cysteines are accessible to external MTSET. However, the variable efficiency of biotinylation suggests that the first extracellular loop may form a structure in which some amino acid residues are not readily accessible to biotin maleimide.

We introduced 2, 5, 4, 6, and 3 cysteine residues into the putative second, third, fourth, and fifth extracellular loops, respectively (see Fig. 1). These mutants were well expressed in PS120 cells (data not shown). The mutants for the putative second, third, and fourth extracellular loops were all strongly labeled with biotin maleimide, which was significantly reduced by preincubation with MTSET (Fig. 4, A–C). In the putative fifth loop, all of the mutants were strongly biotinylated in a MTSET-sensitive manner (Fig. 4D). However, some mutants (E368C, S375C, H376C, and T377C) were less sensitive to MTSET preincubation (Fig. 4, D). MTSET-sensitive manner (Fig. 4D). However, some mutants (E368C, S375C, H376C, and T377C) were less sensitive to MTSET preincubation (Fig. 4, D). The other mutants, R180C, Q181C, S320C, R321C, and F322C, were slightly labeled without change with MTSET. Thus, these cysteines are not readily accessible to biotin maleimide applied from the outside. In contrast, cysteine mutants for IL5 (H407C, H408C, and W409C) were typically labeled with externally applied biotin maleimide in a MTSET-sensitive manner (Fig. 5E), indicating that these three positions are accessible from external medium.

In order to test if substituted cysteines not readily accessible from the outside in Fig. 5 are accessible from the cytosol, we permeabilized cells with streptolysin O. As shown in Fig. 6A, cell permeabilization extensively increased the biotinylation of M-Cys-less NHE1 that contains three endogenous cysteines in the cytoplasmic domain. However, the same treatment did not enhance the biotinylation of Cys-less NHE1 and E151C (data not shown). The streptolysin O treatment also increased biotinylation of R180C, Q181C, F182C, E248C, I249C, H250C, H251C, R321C, or R323C, each of which was inhibited by preincubation with MTSET (Fig. 6, B–D; data not shown for some mutants). The data suggest that these substituted cysteines are accessible from the cytosol. In contrast, two mutants, S126C (data not shown) and S127C (Fig. 6A), were not labeled even in permeabilized cells, suggesting that these residues may be embedded in the lipid bilayer and thus inaccessible from either side of the membrane.

Intriguingly, we found that T183C was strongly biotinylated in a MTSET-sensitive manner in nonpermeabilized cells (Fig. 5B), although biotinylation of H34C and Q181C was strongly enhanced by the streptolysin O treatment as stated above (Fig. 5D). Similar aberrant biotinylation occurred in nonpermeabilized cells expressing T323C, S324C, or H325C (Fig. 5D), although the labeling is not so strong. The data suggest that these cysteines are accessible from outside, despite their suggested intracellular localization.

We next determined the localization of the N terminus by utilizing a cysteine mutant designated C8*, in which 8 endog-
Membrane Topology of NHE1

FIG. 6. Effect of cell permeabilization on biotinylation of the putative intracellular loops of NHE1. Cells expressing Cys-less NHE1, single cysteine mutants for the putative intracellular loops (A, IL1; B, IL2; C, IL3; D, IL4), or C8+ mutant (E) were treated with or without 350 units/ml SLO and then incubated with biotin maleimide in the presence or absence of preincubation with MTSET as described in the legend to Fig. 3. E151C was used as a positive control.

FIG. 7. Biotinylation of single cysteine mutants for the last putative extracellular loop. A–C, nonpermeabilized cells expressing Cys-less NHE1 or single cysteine mutants were treated with biotin maleimide in the presence or absence of preincubation with MTSET as described in the legend to Fig. 3. Total cell lysate (40 μg) (A) or the proteins recovered with streptavidin-agarose (B and C) were separated by SDS-PAGE, and mutant NHE1 proteins were visualized by immunoblot analysis with the NHE1 antibody. D, cells expressing indicated mutants were treated with or without 350 units/ml of SLO and then incubated with biotin maleimide in the presence or absence of preincubation with MTSET as described in the legend to Fig. 3.

DISCUSSION

In this study, we have utilized the reactivity of introduced cysteines toward biotinylating reagent to determine the membrane topology of NHE1. This approach has been used to determine the topology of human P-glycoprotein (16) and several other transporters (17, 20, 21). We found that externally applied biotin maleimide did not virtually label the wild-type, Cys-less, and M-Cys-less NHE1 (Figs. 2, C and D) but strongly labeled M-Cys-less NHE1, in which 3 native cysteines were retained in the C-terminal cytoplasmic domain, when cells were permeabilized (Fig. 6A). These results confirmed the pre-
Membrane Topology of NHE1

has been proposed for a Na⁺/H⁺ antiporter of Escherichia coli (22). The assignment of TM1 to TM9 of NHE1 in the new model is different from that in the original hydropathy model (Fig. 1). We could not obtain evidence for the existence of the intracellular loop connecting TM2 and TM3 (IL1), because the introduced cysteines at positions 126 (data not shown) and 127 (Fig. 6A) were not labeled in permeabilized cells. However, since the hydrophobic region of amino acids 105–150 is long enough to span the lipid bilayer twice, we consider this hydrophobic region to form TM2 and TM3.

The transmembrane disposition of TM10 to TM12 of the original model is extensively modified in the new model. We found that introduced cysteines at positions 373–377, 381, and 407–409 were accessible from the outside. The intervening hydrophobic stretch of amino acids 385–406, which mostly corresponds to TM10 of the original model, is not long enough to span the lipid bilayer twice if it forms an α-helix. Because two mutants, K384C and S388C, were not labeled with biotin maleimide from either side of the membrane (see "Results"; we tentatively placed most of this hydrophobic segment within the lipid bilayer. Cysteines at positions 471–474 in the last extracellular loop of the original model were labeled with biotin maleimide from the outside in a MTSET-sensitive manner, while cysteines at positions 443, 447, and 448 were labeled from the inside. Thus, the hydrophobic stretch of amino acids 449–470 appears to cross the lipid bilayer with an inside to outside orientation. Such an interpretation is consistent with our finding that introduced cysteines in this region were not readily biotinylated from either side of the membrane (Fig. 7, B and C). Since cysteine at position 448, but not that at position 449, was biotinylated from the inside, the new TM11 could start from Gln449 (Fig. 7D).

The N terminus has been predicted to be cleaved off as a signal peptide at a site somewhere before the glycosylation sites in the first extracellular loop. A typical structure of the signal peptide has three distinct domains, i.e. an N-terminal positively charged region (1–5 residues long); a central, hydro-


ducts from the cell surface, while similar labeling with biotin maleimide only in permeabilized cells defines the cysteines as being localized intracellularly.

Using Cys-less NHE1, we introduced single cysteine residues into the regions of the exchanger modeled to be localized extracellularly or intracellularly from hydropathy analysis. Cys-less NHE1 has basic properties similar to the wild-type NHE1 in that it was highly expressed in the transfectants and exhibited Na⁺/H⁺ exchange activity comparable with that of the wild-type exchanger (Figs. 2A and 8). In addition, both Cys-less and wild-type exchangers exhibited essentially the same pH and EIPA concentration dependences of exchange activity (Fig. 2, A and B). We found that most of single cysteine mutants exhibited relatively high exchange activity (Fig. 8), although stable transfectants could not be obtained with some mutants. We observed two bands reactive with the NHE1-antibody in the transfectant cells (Figs. 3A and 7A), i.e. high and low molecular weight forms of the exchanger that are thought to be the mature proteins with N- and O-linked glycosylation or the immature proteins only containing high mannose oligosaccharide, respectively (14). As expected, in most of the cases, the cysteine residues introduced into the putative extracellular loops were strongly labeled with biotin maleimide only in the high molecular weight form of the exchanger. However, the low molecular weight form of NHE1 was also biotinylated in some cases (e.g. see Fig. 4D). Although we do not know the precise reason for that, it is possible that part of the lower form is expressed in the plasma membrane under some conditions.

Fig. 9 shows a new topological model of NHE1 predicted on the basis of the present results. The model consists of 12 TMs and intracellular and extracellular loops connecting TMs with both the N and C termini located in the cytoplasm. Interestingly, a 12-TM model with the intracellular N and C termini
phobic region (7–15 residues); and a more polar C-terminal region (3–7 residues long) (23). Although NHE1 was indeed predicted to possess a signal peptide,2 cleavage of the N terminus of NHE1 has not yet been experimentally proven. We observed that all of the introduced cysteines in the first extracellular loop were biotinylated in a MTSET-sensitive manner (Fig. 3, B and D), suggesting that these residues including cysteine at position 35 near the extracellular interface of TM1 were retained in the mature NHE1 protein. Furthermore, mutant C8* was labeled only after cells were permeabilized (Fig. 6E), suggesting that Cys8 is also retained in the mature protein and localized in the cytosol. It is therefore most likely that NHE1 is not cleaved during protein processing, unless introduced individual cysteines somehow prevent the cleavage of protein by signal peptidases. Thus, the central hydrophobic stretch in the N-terminal signal peptide-like structure is likely to form the first transmembrane α-helix.

As described above, we observed that M-Cys-less NHE1 containing three endogenous cysteines (amino acids 538, 561, and 794) were labeled with biotin maleimide only after cells were permeabilized (Fig. 6A). This result is consistent with much previous biochemical data showing that most of the C-terminal domain of NHE1 (~300 amino acids) is located intracellularly (10, 24–26). Recently, Shrode et al. (15) have shown that hemagglutinin epitope tagged at the C-terminal end of NHE1 can be recognized by the hemagglutinin-specific antibody only after cells expressing NHE1-hemagglutinin were permeabilized. All these results strongly suggest that the C-terminal tail (amino acids 500–815) of NHE1 is entirely located intracellularly, which is in contrast to the recent report that part of the C terminus of NHE3 is exposed on the extracellular side (27).

We found that cysteines at positions 180–182 were readily biotinylated from the inside but not from the outside (Figs. 5B and 6B). This suggests that these positions are accessible from the inside. Intriguingly, however, 22Na+ uptake by two mutants R180C and Q181C was strongly inhibited by externally applied MTSET (Fig. 8), suggesting that these residues are accessible from the outside. We also found that cysteine at position 183 was strongly biotinylated from the outside in a MTSET-sensitive manner (Fig. 5B). These data suggest that at least a portion of the loop connecting TM4 and TM5 (IL2) is localized within the membrane, raising an intriguing possibility that it could form a structure lining the aqueous pore that is accessible from either side of the membrane and involved in the ion transport. As described above, cysteines at positions 180 and 181 are accessible to MTSET from the outside as evidenced by its inhibition of 22Na+ uptake but were not readily accessible to biotin maleimide from the same side. Such a difference in the accessibility may simply reflect the difference in the reagent size, because biotin maleimide is much bulkier than MTSET.

It is important to note that TM4 has been suggested to contain part of the amiloride-binding site (28). Single amino acid substitutions at Phe161 and Leu163 within the 160VF-FLFPPP169 segment of TM4 were initially found to cause reduction of the amiloride sensitivity (29), while more recently the G174S mutation in the adjacent region in TM4 was also found to cause reduction in the amiloride affinity (29). Double mutant L163F/G174S exhibited markedly reduced affinity not only for amiloride (or its derivatives) but also for Na+ (29). These previous results fit nicely with the present results and together support the view that TM4 and the adjacent loop (IL2) may form part of an ion transport pathway in the exchanger. As pointed out previously (29), TM4 is a proline-rich segment

---

2 Prediction of the signal peptide was made on the World Wide Web.
containing three highly conservative proline residues (Pro\textsuperscript{167}, Pro\textsuperscript{168}, and Pro\textsuperscript{178}). It is likely that these proline residues produce a kink in α-helix, which could create a space to accommodate part of I2L in the membrane.

Cysteines introduced into the putative intracellular loop connecting TM8 and TM9 (IL4) exhibited topological dispositions similar to those in the loop connecting TM4 and TM5 (IL2) in that positions 321 and 323 were accessible from the inside, while positions 324 and 325 were accessible from the outside (Figs. 5D and 6D). However, \[^{22}\text{Na}^+\] uptake by six cysteine mutants of IL4 was not significantly influenced by external MTSET (Fig. 8). On the other hand, recent molecular studies including one with chimeric exchangers between NHE1 and NHE3 revealed the functional importance of a 66-amino acid segment encompassing IL4, TM9, and EL5 for the interaction of the exchanger with inhibitors such as amiloride derivatives and HOE694 (30) as well as of His\textsuperscript{349} in TM9 for the amiloride sensitivity (31). These previous results, together with our data showing that at least a portion of IL4 is localized within the membrane, raise a possibility that this loop may also be involved in ion transport. Clearly, more detailed analysis is required to clarify the functional importance of the region encompassing IL4 and TM9.

In summary, based on the results from cysteine accessibility analysis, we proposed a novel 12-TM model with the N and C termini located in the cytoplasm. This new topology model would provide a basis for the further study of the structure-function relationship of NHE1.

REFERENCES

1. Grinstein, S., Rotin, D., and Mason, M. J. (1989) \textit{Biochim. Biophys. Acta} \textbf{988}, 73–97
2. Wakabayashi, S., Shigekawa M., and Pouyssegur, J. (1997) \textit{Physiol. Rev.} \textbf{77}, 51–74
3. Sardet, C., Franchi, A., and Pouyssegur, J. (1989) \textit{Cell} \textbf{56}, 271–280
4. Orlowski, J., Kandasamy, R. A., and Shull, G. E. (1992) \textit{J. Biol. Chem.} \textbf{267}, 9331–9339
5. Tsé, C.-M., Brant, S. R., Walker, M. S., Pouyssegur, J., and Donowitz, M. (1992) \textit{J. Biol. Chem.} \textbf{267}, 9340–9346
6. Tsé, C.-M., Levine, S. A., Yun, C. H. C., Montrose, M. H., Little, P. J., Pouyssegur, J., and Donowitz, M. (1993) \textit{J. Biol. Chem.} \textbf{268}, 11917–11924
7. Numata, M., Recrecia, K., Lake, N., and Orlowski, J. (1998) \textit{J. Biol. Chem.} \textbf{273}, 6951–6959
8. Baird, N., Orlowski, J., Szabo, E. Z., Zaub, H. C., Schultheiss, P. J., Menon, A. G., and Shull, G. (1999) \textit{J. Biol. Chem.} \textbf{274}, 4377–4382
9. Wakabayashi, S., Fafournoux, P., Sardet, C., and Pouyssegur, J. (1992) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{89}, 2424–2428
10. Bertrand, B., Wakabayashi, S., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994) \textit{J. Biol. Chem.} \textbf{269}, 13703–13709
11. Wakabayashi, S., Bertrand, B., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994) \textit{J. Biol. Chem.} \textbf{269}, 13710–13715
12. Ikeda T., Schmitt, B., Pouyssegur, J., Wakabayashi, S., Shigekawa, M. (1997) \textit{J. Biochem. (Tokyo)} \textbf{121}, 295–303
13. Kyte, J., and Dodolittle, R. F. (1982) \textit{J. Mol. Biol.} \textbf{157}, 105–132
14. Counillon, L., Pouyssegur, J., and Reithmeier, R. A. F. (1994) \textit{Biochemistry} \textbf{33}, 10463–10469
15. Shrode, L. D., Gan, B. S., D'Souza, S. J. A., Orlowski, J., and Grinstein, S. (1998) \textit{Am. J. Physiol.} \textbf{275}, C431–C439
16. Loo, T. W., and Clarke, D. M. (1995) \textit{J. Biol. Chem.} \textbf{270}, 843–848
17. Grunewald, M., Bendahan, A., and Kanner, B. I. (1998) \textit{Neuron} \textbf{21}, 623–632
18. Pouyssegur, J., Sardet, C., Franchi, A., L’Allemain, G., and Paris, S. (1984) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{81}, 4833–4837
19. Wakabayashi, S., Ikeda, T., Iwamoto, T., Pouyssegur, J., and Shigekawa, M. (1997) \textit{Biochemistry} \textbf{36}, 12854–12861
20. Fujinaga, J., Tang, X.-B., and Casey, J. R. (1999) \textit{J. Biol. Chem.} \textbf{274}, 6626–6633
21. Chen, J.-G., Liu-Chen, S., and Rudnick G. (1998) \textit{J. Biol. Chem.} \textbf{273}, 12675–12681
22. Rothman, A., Padan, E., and Shuldiner, S. (1996) \textit{J. Biol. Chem.} \textbf{271}, 32288–32292
23. von Heijne, G. (1990) \textit{J. Membr. Biol.} \textbf{115}, 195–201
24. Sardet, C., Counillon, L., Franchi, A., and Pouyssegur, J. (1999) \textit{Science} \textbf{274}, 723–726
25. Wakabayashi, S., Bertrand, B., Shigekawa, M., Fafournoux, P., and Pouyssegur, J. (1994) \textit{J. Biol. Chem.} \textbf{269}, 5583–5588
26. Lin, X., and Barber, D. L. (1996) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{93}, 12631–12636
27. Biemesderfer, D., DeGray, B., and Aronson, P. S. (1998) \textit{J. Biol. Chem.} \textbf{273}, 12391–12396
28. Counillon, L., Franchi, A., and Pouyssegur, J. (1993) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{90}, 4508–4512
29. Counillon, L., Noel, J., Reithmeier, R. A. F., Pouyssegur, J. (1997) \textit{Biochemistry} \textbf{36}, 2951–2959
30. Orlowski, J., and Kandasamy, R. A. (1996) \textit{J. Biol. Chem.} \textbf{271}, 19922–19927
31. Wang, D., Balkovetz, D. F., and Warnock, D. G. (1995) \textit{Am. J. Physiol.} \textbf{269}, C392–C402
A Novel Topology Model of the Human Na\(^+\)/H\(^+\) Exchanger Isoform 1
Shigeo Wakabayashi, Tianxiang Pang, Xiaohua Su and Munekazu Shigekawa

*J. Biol. Chem. 2000, 275:7942-7949.*
doi: 10.1074/jbc.275.11.7942

Access the most updated version of this article at http://www.jbc.org/content/275/11/7942

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 19 of which can be accessed free at http://www.jbc.org/content/275/11/7942.full.html#ref-list-1