Cysteine residues were found nonessential in the mechanism of the NhaA antiporter activity of Escherichia coli. The functional C-less NhaA has provided the groundwork to study further histidine 225 of NhaA which has previously been suggested to play an important role in the activation of NhaA at alkaline pH (Rimon, A., Gerchman, Y., Olami, Y., Schuldiner, S. and Padan, E. (1995) J. Biol. Chem. 270, 26813–26817). C-less H225C was constructed and shown to possess an antiporter activity 60% of that of C-less antiporter and a pH profile similar to that of both the C-less or wild-type antiporters. Remarkably, whereas neither the wild-type nor the C-less antiporters were affected by N-ethylmaleimide, C-less H225C was inhibited by this reagent. To determine the degree of alkylaion of the antiporter protein by N-ethylmaleimide, antiporter derivatives tagged at their C termini with six histidines residues were constructed. Alkylation of C-less H225C was measured by labeling of everted membrane vesicles with [14C]-N-ethylmaleimide, affinity purification of the His-tagged antiporter, and determination of the radioactivity of the purified protein. This assay showed that H225C is alkylated to a much higher level than any of the native cysteinyl residues of NhaA reaching saturation at alkyl/Na stoichiometry of 1. The wild-type derivative showed at least 10-fold less alkylaion even at higher concentrations, suggesting that H225C resides in a domain that is much more exposed to N-ethylmaleimide than the native cysteinyl residues of NhaA.

Since H225C residues both in right-side out and inside-out membrane vesicles were quantitatively alkylated by N-ethylmaleimide, this assay was used to determine the accessibility of H225C to other SH reagents by titrating the H225C left free to react with N-ethylmaleimide, following exposure of the membranes to the reagents. Furthermore, since membrane-impermeant probes can react with residues in membrane-embedded agents. Furthermore, since membrane-impermeant reagents can react with residues in membrane-embedded agents.

As expected for a membrane-permeant probe, p-chloromercuribenzoate reacted with H225C as efficiently as N-ethylmaleimide in both membrane orientations. Similar results were obtained with methanethiosulfonate ethylammonium supporting the recent observations that this probe is membrane-permeant. On the other hand, both membrane-impermeant reagents p-chloromercuribenzoate and methanethiosulfonate ethyl-trimethyl ammonium bromide reacted with H225C 10-fold more in right-side out than in inside-out vesicles, and p-chloromercuribenzoate also blocked completely the H225C in intact cells. These results strongly suggest that H225C is exposed to the periplasmic face of the membrane.

Sodium proton antiporters are ubiquitous membrane proteins found in the cytoplasmic and organelle membranes of cells of many different origins, including plants, animals, and microorganisms. They are involved in cell energetics and play primary roles in the regulation of intracellular pH, cellular Na+ content, and cell volume (reviewed in Refs. 1–4).

Escherichia coli has two antiporters, NhaA (5) and NhaB (6), that specifically exchange Na+ or Li+ for H+. nhaA is indispensable for adaptation to high salinity, for challenging Li+ toxicity, and for growth at alkaline pH (in the presence of Na+) (7). Accordingly, expression of nhaA which is dependent on NhaA, a positive regulator, is induced by Na+ in a pH-dependent manner (8–10). NhaB by itself confers a limited sodium tolerance to the cells but becomes essential when the lack of NhaA activity limits growth (11).

Both the NhaA and NhaB are electrogenic antiporters that have been purified to homogeneity and reconstituted in a functional form in proteoliposomes (12–14). With the purified antiporters it was found that the H+/Na+ stoichiometry of NhaA is 2H+/Na+ and that of NhaB is 3H+/2Na+. NhaB but not NhaA is sensitive to amiloride derivatives, and the rate of NhaA but not of NhaB is drastically dependent on pH, changing its Vmax over 3 orders of magnitude from pH 7 to pH 8 (12).

None of the eight histidines of NhaA were found essential for the Na+/H+ antiporter activity of NhaA (15). However, replacement of histidine 225 (His-225, previously mistakenly numbered His-226) by Arg (H225R) suggested that His-225 has an important role in the pH sensitivity of the antiporter (15). Whereas the activation of the wild-type NhaA occurs between pH 7.5 and pH 8, that of H225R antiporter occurs between pH 6.5 and pH 7.5. In addition, while the wild-type antiporter remains almost fully active, at least up to pH 8.5, H225R is reversibly inactivated above pH 7.5, retaining only 10–20% of the maximal activity at pH 8.5 (15). Furthermore, we have recently shown that replacement of His-225 with either cysteine (H225C) or serine (H225S) but not alanine (H225A) yielded an antiporter with a wild-type pH-sensitive phenotype, implying that polarity and/or hydrogen bonding, the common properties shared by His, Cys, and Ser, are essential at position 225 for pH regulation of NhaA (16).

In the present work we found that the three cysteine residues of NhaA are not essential in the mechanism of the antiporter, confirming previous findings on the lack of inhibition by
Membrane Topology of H225C in NhaA

### TABLE I

Primers used for constructing the Cys-mutations in nhaA

| Mutation | Mutagenic primera | Locationb | Codon change observed |
|----------|-------------------|-----------|----------------------|
| C200S    | TATTGAACTGTCTGGTGACGCGG | 693–717   | TGT → TCT            |
|          | (586–610)         |           |                      |
| C308S    | TTAGTCTGTCCAGCCTGGTTGGCCT | 1017–1041 | TGC → AGC            |
|          | (910–934)         |           |                      |
| C335S    | TGGGAGTCTCGGATCCTGTTTT | 1098–1122 | TGC → TCC            |
|          | (662–685)         |           |                      |
| H225C    | ATCGGGGGGTTTCGGCAACTCT | 769–792   | CAC → TGC            |
|          | (−38–10)          |           |                      |
| End primer | ATCGCTCTCTTTAACCAC | −318–300f | None                 |
|          | (−425–407)        |           |                      |
| End primer | CAGCATTTTGCGGTTGGATTGCA | 1706–1740d | None                 |
|          | GCAGCGGAAGAT       | (1599–1633) |                      |

a The mutated codons are shown in boldface.
b Location numbers are on the sequence as appeared in GenBank access number J03879.
c This primer is upstream to the sequence of nhaA and can be found in access number X17311, complementary to 237–217.
d This primer is downstream to the sequence of nhaA and can be found in access number L24072 complementary to 408–374.

SH reagents (17). The functional antiporter devoid of cysteiny1 residues has provided the basis to design mutants of NhaA in which a suggested important residue such as His-225 is replaced with cysteyln1 residue which can then be reacted specifically with sulfhydryl reagents. Analysis of these site-directed chemical modifications can bear upon both the importance as well as the topology of the original residue (Cys scanning). For this purpose, C-less H225C was constructed and shown to possess an antiporter activity 60% of that of C-less antiporter and a pH profile similar to that of both the C-less or wild-type antiporters. Remarkably, whereas neither the wild-type nor the C-less antiporters were affected by NEM,1 C-less H225C was inhibited by this reagent. Furthermore, H225C was alkylated to a much higher level than any of the native cysteiny1 residues of NhaA suggesting that H225C resides in the protein in a domain which is much more exposed to NEM than the native cysteiny1 residues of NhaA. However, since NEM is membrane-permeable, it was impossible to deduce from these experiments the membrane topology of H225C with respect to neither the periplasmic nor the cytoplasmic face of the membrane.

Various SH reagents have been suggested to be membrane-impermeant and react only with residues exposed to the medium containing the reagents. Thus periplasmic exposed residues are expected to be affected only in intact cells or RSO membrane vesicles and cytoplasmic facing residues only in ISO membrane vesicles. On the other hand, membrane-permeant reagents are expected to react irrespective of the membrane orientation. On the basis of these differences various SH reagents were exploited to assay the cross-membrane topology of native or strategically placed Cys residues in membrane-embedded proteins. This approach was of value to the structural work of bacteriorhodopsin (18). A similar approach is now being used to probe functional regions with many membrane proteins including the acetylcholine receptor ion channel (19, 20) as well as LacY, the H+/lactose symporter (21), and UhpT, the anion exchange protein (22) both in E. coli.

In the present work, we have used the same approach to probe the reactivity of C-less H225C to various SH reagents, both in intact cells as well as in membrane vesicles of known orientation. The differential reactivity of the membrane-impermeant reagents with C-less H225C in the different experimental systems showed that H225C is exposed to the periplasmic face of the membrane.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains and Culture Conditions**—EP432 is an E. coli K12 derivative, which is melBld, ΔnhaA1::kan, ΔnhaB1::cat, ΔlacZ, thr1 (11). TA16 is nhaA nhaB lacI and is otherwise isogenic to EP432 (12). DH5α (U.S. Biochemical Corp.) was used as a host for construction of plasmids. Cells were grown either in L broth (LB) or in modified L broth (LKB) in which NaCl was replaced by KCl (17) 87 mm, pH 7.5. Where indicated, the medium was buffered with 50 mM BTP, and pH was titrated with HCl. Cells were also grown in minimal medium A without sodium citrate (23). Thiamine (2.5 μg/ml) was added to all minimal media. When required, threonine (0.1 mg/ml) was added. For plates, 1.6% agar was used. Antibiotics were 100 μg/ml ampicillin and/or 50 μg/ml kanamycin and/or 12 μg/ml chloramphenicol. Resistance to Li+ and Na+ was tested as described previously (15).

**Plasmids**—All plasmids are pBR322 derivatives. pGM36 carries wild-type nhaA (24). Plasmids encoding His-tagged antiporters are pET20b (Novagen) derivatives as described below. pEP3T is a plasmid overproducing nhaA (12).

**Site-directed Mutagenesis of Cysteines of NhaA and Construction of C-less NhaA and C-less H225C NhaA**—Site-directed Cys replacements were achieved by serine, C200S, C335S and C308S (Fig. 1), were obtained following a polymerase chain reaction-based protocol (25). DNA of pGM36 was utilized as the template. The end primers and the mutagenic primers are described in Table I. In all cases, the resulting mutagenized DNA was utilized as the template. The end primers and the mutagenic primers as described in Table I (1762). Primers used for constructing the Cys-mutations in nhaA

1 The abbreviations used are: NEM, N-ethylmaleimide; BTP, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; DM, N-dodecyl β-d-maltoside; MOPS, 3-(3-N-morpholino)propane sulfonic acid; PCMB, p-chloromercuribenzoic acid; PCMBs, p-chloromercuribenzoatesulfonate; MTSEA, methanethiosulfonateethylammonium; MTSET, methanethiosulfonateethyl-trimethyl ammonium bromide; RSO, right-side-out membrane vesicles; ISO, inside-out membrane vesicles; TMS, trans-membrane segment; bp, base pair(s); kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl] glycine.

2 Location numbers are on the sequence as appeared in GenBank access number J03879.

3 This primer is upstream to the sequence of nhaA and can be found in access number X17311, complementary to 237–217.

4 This primer is downstream to the sequence of nhaA and can be found in access number L24072 complementary to 408–374.
The BglII-MluI fragment containing the mutation was cloned instead of BglII-MluI of pGM36.

**Construction of His-tagged NhaA Derivatives**—To construct a plasmid overproducing His-tagged NhaA we used pEP3T (12) and amplified by polymerase chain reaction a fragment (1.5 kb) using two mutagenic end primers (CTAGTCTAGAGGATCCGGAGCTTAT) and (TTTTCCTTTGGCAGCATACTGTAGGGACCGCAAGCA) introducing XhoI in the 5' and NotI in the 3' ends. The fragment was cloned into TA vector (Invitrogen, San Diego, CA), and the XhoI-NotI fragment of the recombinant plasmid was cloned into XhoI-NotI fragment (3.6 kb) of modified pET20b (Novagen, Madison, WI). The latter plasmid contains between NotI and XhoI sequences encoding two factor Xa cleavage sites in frame with the sequences encoding the 6 histidines. The resulting recombinant plasmid, designated pYG10 (Fig. 1), encodes for NhaA fused in frame in its C terminus to the two factor Xa cleavage sites followed by 6 histidines. To construct His-tagged derivatives of the C-less NhaA and C-less H225C the NheI-MluI (880 bp) fragment of pC-less or pC-less-H225C was cloned into NheI-MluI (4.2 kb) of pYG10 yielding plasmid His-tag C-less and His-tag C-less-H225C, respectively. All constructs were verified by sequencing using the Sequenase kit (Version 2.0, U.S. Biochemical Corp.).

**Isolation of Everted Membrane Vesicles, Assay of Na⁺/H⁺ Antiporter Activity, and Quantitation of NhaA in the Membranes**—Assays of Na⁺/H⁺ antiporter activity were conducted on everted membrane vesicles (26). The assay of antiporter activity was based upon the measurement of Na⁺ (or Li⁺) induced changes in the ΔpH as described (5, 27). NhaA in everted membrane vesicles (1 mg) was quantitated by Western analysis as described previously (15). Protein was determined according to Bradford (28).

**Isolation of His-tagged Antiporters**—The procedure was performed at 4°C. Everted membrane vesicles (100–300 μg protein) were suspended in 1.15 ml containing 60 mM choline chloride, 4.5 mM Tris-Cl, pH 7.9, 0.1% DM, 10% glycerol. Forelution the washed loaded resin was resuspended in 500 μl of binding buffer, mixed for 10 min, centrifuged, and washed again in wash buffer (30 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9, 0.1% DM). For loading of the resin with His-tagged NhaA the mixture was incubated for 1 h with gentle mixing and then centrifuged.

The loaded resin was resuspended in 500 μl of binding buffer, mixed for 10 min, centrifuged, and washed again in wash buffer (30 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9, 0.02% DM, 10% glycerol). For elution the washed loaded resin was resuspended in 90 μl of elution buffer (300 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9, 0.02% DM, 10% glycerol) and incubated for 10 min as above. The supernatant devoid of the resin was collected by two consecutive centrifugations. The proteins in the supernatant were precipitated in 10% trichloroacetic acid for 30 min and collected by centrifugation (20,000 × g, 15 min), resuspended in sample buffer, and resolved by SDS-PAGE (29).

**Treatment with NEM**—Everted membrane vesicles were treated with NEM in 50 μl of reaction mixture containing, in final concentrations, membrane vesicles (100 μg protein), 100 mM potassium phosphate (at the indicated pH), 10 mM MgSO₄, and 1 mM NEM. The reaction (28°C, 20 min) was stopped by addition of 20 μl dithiothreitol. Antiporter activity was assayed as described above using K-ATP instead of D-lactate to establish ΔpH since NEM inactivates electron transport. If not otherwise indicated for treatment with [14C]NEM, membrane vesicles (100–300 μg of protein) were resuspended in 500 μl of solution, pH 7.5, and incubated as above in the presence of 25 μM [14C]NEM (DuPont NEN, 40 mCi/mmol). Solubilization and purification of His-tagged NhaA protein was as above.

**RESULTS AND DISCUSSION**

**Replacement Mutations of the Cysteines of NhaA**—NhaA, the Na⁺/H⁺ antiporter of E. coli, has three cysteinyl residues (Fig. 1). To study the role of the cysteinyl residues in the activity of the antiporter, we replaced each cysteine of NhaA separately to obtain mutations encoding serine instead of cysteine 200 (C200S), 308 (C308S), or 335 (C335S). In addition, both a cysteine-less (C-less) NhaA was constructed with all cysteine residues replaced by serines, as well as C-less with a Cys replacement of His-225 (C-less H225C).

**Growth Phenotype and NhaA Expression of the Cysteine Replacement Mutants**—To study the growth phenotype of the mutants, each mutated plasmid was introduced into a strain lacking both antiporters NhaA and NhaB (EP432) (11). This strain does not have Na⁺/H⁺ activity and thus is highly sensitive to Na⁺ but regains antiporter activity and becomes resistant to the ion upon transformation with multicopy plasmid bearing wild-type nhaA. It was therefore found most suitable to explore plasmidic antiporter mutations with respect to the capacity to confer Na⁺ resistance and antiporter activity (30). The results of the growth experiments show that at pH 7.5 in the presence of up to 0.7 mM NaCl, the growth rate of all cysteine mutants was very similar to that of the wild-type (75–85 min doubling time). Also at pH 8.5 in the presence of 0.6 mM NaCl, all mutants grew, but C200S, C-less, and the C-less H225C mutants were slightly slower (doubling time of 95, 90, and 95 min, respectively) compared with the wild-type and the other cysteine mutants (doubling time of 75 min). There was no significant difference in colony formation of the various strains on agar plates.

The level of the NhaA-Cys mutated proteins in the membranes was determined immunologically using polyclonal antibody produced against the C terminus of NhaA (15). The mutants C308S, C-less, and C-less-H225C were expressed at a level similar to that of the wild-type protein. The level of C335S and C200S in the membrane was 60 and 30% that of the wild-type, respectively.

**Na⁺/H⁺ Exchange Activity in Everted Membrane Vesicles**—To determine whether any of the mutations affect antiporter activity, we isolated everted membrane vesicles from EP432 transformed with each of the mutant plasmids and assayed the specific Na⁺/H⁺ antiporter activity of these membranes as a function of pH. Again this host proves very useful since it has no background Na⁺/H⁺ antiporter activity when transformed with the vector plasmid (pBR322, Fig. 2A). The data obtained from the mutants at pH 8.5 are shown in Fig. 2A, and the pH profile of the Na⁺/H⁺ antiporter activity throughout the pH range from 7 to 8.5 is summarized in Fig. 2B. For comparison, the Na⁺/H⁺ antiporter activity versus pH of wild-type plasmid transformants is also shown.

The Na⁺/H⁺ antiporter activity of the C308S mutant is very similar to that of the wild-type NhaA both in maximal activity...
and pH sensitivity. Below pH 8 C335S and C200S show similar activity which is between 40 and 50% that of the wild-type. Above pH 8, whereas C335S reaches the steady state level of the wild-type, C200S remains at 60%.

The activity of the C-less mutant of NhaA is very similar to C200S and reaches a steady state level of about 60% of the wild-type at alkaline pH (Fig. 2A and B). Thus, it is apparent that replacement of all three Cys residues, whether each separately or all together, in the same antiporter molecule does not markedly impair the antiporter activity, implying that neither of the cysteinyl residues of NhaA are mandatory to the \( \text{Na}^{+}/\text{H}^{+} \) antiporter activity of NhaA.

It is obvious that high resolution structure of the transporter is required to determine the role of any residue in the mechanism. Nevertheless, it has recently become apparent (31, 32) that functional Cys replacements in transporters can be very useful since they allow site-specific labeling of the protein, at the reactive SH groups, with probes that can examine various aspects of structure and function relationship of the protein (Cys scanning). A principal difficulty with this general approach, however, is the complexity resulting from the presence of multiple cysteinyl residues in most proteins, three in the case of the NhaA antiporter. Thus, in addition to the important conclusion reached in this paper that cysteinyl residues do not play a critical role in the mechanism of the \( \text{Na}^{+}/\text{H}^{+} \) antiporter, the construction of a functional antiporter, devoid of cysteinyl residues, provides the basis for Cys-scanning analysis of NhaA.

Since His-225 has been shown to play an important role in the pH response of NhaA, we have replaced His-225 of the C-less antiporter with Cys. The \( \text{Na}^{+}/\text{H}^{+} \) antiporter activity of Cless H225C at pH 8.5 in inverted membrane vesicles was about 60% that of the C-less antiporter and 30–40% that of the wild-type (Figs. 3 and 2B). Most interestingly, the pH profile of C-less H225C was similar to the wild-type or the C-less NhaA (Fig. 2B). Taken together these results show that C-less H225C NhaA antiporter although at a decreased level is still func-
tional and exhibits a pH dependence similar to that of wild-type and the C-less antiporter. These results are consistent with our previous data (16) showing that an antiporter harboring an H225C mutation in otherwise wild-type antiporter is similar to the latter both in activity and pH sensitivity. The C-less H225C mutant thus affords the possibility to test the effect of SH reagents at position 225 of NhaA.

While NEM Has No Effect on Wild-type or C-less NhaA It Inactivates Both H225C and C-less H225C—In accordance with previous results (17), the Na⁺/H⁺ antiporter activity of the wild-type NhaA protein, like the C-less antiporter, was not affected by treatment of the membranes with NEM (up to 2 mM) (Fig. 3). However, NEM (at 0.5 mM) inhibited, significantly, the C-less H225C antiporter (Fig. 3A). The most significant effect was obtained when the NEM treatment was conducted at pH 7.5 (Fig. 3B). The maximal inhibition (60–70%) was not increased with further increase of the NEM concentration. The presence of Na⁺ had no effect on the degree of NEM inhibition. Similar results (not shown) were obtained with NhaA mutant which harbors H225C in an otherwise wild-type protein (H225C). Interestingly, the residual activity of the NEM-inactivated C-less H225C is not sensitive to pH (not shown). Taken together these results emphasize again the importance of His-225 in the unique response of NhaA to pH (15, 16).

H225C Is Exposed to Alkylation by [¹⁴C]NEM Much More Readily Than the Native Cysteinyl Residues of NhaA—The observation that NEM significantly affects the NhaA antiporter, harboring instead of histidyl a cysteinyl residue at position 225, but has no effect on a wild-type antiporter with its three cysteines (C200, C308, C335), raised two alternatives: a modification of the cysteinylic residues by NEM has no inhibitory effects on the antiporter activity, and the Cys residues, which are in putative transmembrane segment (15), are not accessible to NEM.

To test whether the NhaA cysteinyl residues are NEM-accessible, we constructed His-tagged NhaA (Fig. 1) (and in addition His-tagged C-less and His-tagged C-less H225C). Plasmidic His-tagged NhaA was as effective as plasmidic wild-type antiporter in conferring Na⁺ resistance upon _Salmonella_ΔnahAΔnahB and in promoting Na⁺/H⁺ antiporter activity in isolated membrane vesicles (not shown). The pH sensitivity of the His-tagged NhaA was also similar to that of the wild-type antiporter. The His-tagged NhaA readily bound onto the Ni⁺⁺ resin (Fig. 4A). Out of the many membrane proteins (Fig. 4A, lane 2) exposed to the resin many did not bind (Fig. 4A, lane 3) or were eluted by the washes at low imidazole concentrations (≤30 mM, Fig. 4A, lanes 4 and 5). At 300 mM imidazole the His-tagged NhaA was eluted as a prominent band at 30 kDa (Fig. 4, lane 6). As expected from its longer C terminus His-tagged NhaA was slightly heavier than the native NhaA (Fig. 4A, lane 7). The two additional very weak bands of 51 and 83 kDa shown are most probably aggregates of the antiporter, a property noted before for this protein (12).

This rapid isolation of the His-tagged NhaA formed the basis for an assay designed to probe _in situ_ the reactivity of the NhaA Cys residues to NEM as well as to other SH reagents. In this assay everted membrane vesicles were treated with [¹⁴C]NEM, and the His-tagged NhaA derivatives were isolated on the Ni⁺⁺ resin and subjected to autoradiography (Fig. 4B) to estimate the number of NEM titratable molecules per purified NhaA protein.

These results show that when the [¹⁴C]NEM treatment was conducted at a concentration of 25 μM, His-tagged C-less H225C (Fig. 4B, lane 1) was labeled to a level at least 10-fold higher than the His-tagged wild-type antiporter (Fig. 4B, lane 3), in spite of the three cysteines of the latter. As expected there was no labeling of the His-tagged C-less NhaA (Fig. 4B, lane 2).

The concentration dependence of the alkylation by [¹⁴C]NEM was determined with His-tagged C-less H225C (Fig. 5). The results show that at 0.5 mM NEM saturation of the alkylation reaction was reached. To estimate the maximal number of [¹⁴C]NEM molecules bound per purified His-tagged C-less H225C NhaA molecule, the amount of the radioactivity bound to the antiporter at 0.5 mM NEM was also determined directly by scintillation counting of the purified antiporter fraction. A NEM/NhaA mol ratio of 1.02 was calculated from the amount of radioactivity, specific activity of [¹⁴C]NEM, and the amount of the pure protein in the fraction, suggesting a NEM/NhaA stoichiometry of 1. A similar stoichiometry was found with RSO membrane vesicles. At this NEM concentration maximal inhibition (at pH 7.5) of the Na⁺/H⁺ antiporter activity was observed in everted membrane vesicles isolated from C-less H225C cells (Fig. 3).

The effect of NEM concentration on the alkylation of the native cysteines of the wild-type NhaA was also determined (Fig. 5). It was observed that up to 0.5 mM the alkylation was very low but increased with increasing concentrations. However, even at 2 mM [¹⁴C]NEM there was no saturation of the reaction. It is concluded that H225C is much more exposed to NEM than the native NhaA cysteines.

The fact that H225C is much more exposed to NEM as compared with the native Cys residues of NhaA is intriguing. Since H225C in right-side out membranes has been found as reactive to NEM as in everted membrane vesicles, it is suggested that neither membrane permeability nor the topology of H225C with respect to the face of the membrane is the cause for the preferential reactivity of H225C. Since the native cysteines...
Membrane Topology of H225C—The cross-membrane topology of H225C was investigated by probing in situ the accessibility of H225C to membrane-impermeant SH reagents both in oriented membrane preparation, ISO, or RSO as well as intact cells. Accessibility to the probe implied that H225C is located at or near the face of the membrane exposed to the side of application of the impermeant probe. Inaccessibility of the H225C residue to the impermeant probe but accessibility to a permeant probe suggested to be impermeant to the protein. The error bars are shown.

Small, charged and highly water-soluble sulfhydryl-specific reagents derived from methanethiosulfonate (MTS) have previously been used to probe the cross-membrane topology of cysteines strategically placed in the acetylcholine receptor (19, 20). These include the positively charged MTSEA and MTSET, cysteines strategically placed in the acetylcholine receptor (19, 20). We therefore probed H225C with MTSEA.

A typical experiment is shown in Fig. 6A using MTSEA. Surprisingly, treatment with MTSEA for 1 min whether applied to right-side out or inside-out membrane vesicles reduced completely the number of NEM-reactive H225C residues.

The level of the alkylation was determined by autoradiography of the affinity purified antipporter (Ni²⁺ resin) separated on SDS-PAGE, as shown in Fig. 5, in untreated membranes the alkyl/NhaA stoichiometry was close to one whether right-side out or everted membrane vesicles were used. In addition, C-less NhaA was not labeled under the conditions employed (Fig. 4B). Both these controls implied that alkylation by [¹⁴C]NEM can be used to titrate the number of reactive H225C residues in NhaA. Therefore the difference between the number of NEM alkylatable H225C before and after exposure to the -SH reagent of selective permeability allowed us to estimate the reactivity of H225C to this reagent.

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mercurials that share chemical specificity for the reaction with cysteines. They differ, however, in several properties. PCMBS is lipid-soluble weak acid which when protonated (pK 4) has access to cysteine residues at both membrane surfaces. On the other hand, in PCMBs the strongly acidic sulfonic acid group (pK 1.5) is very soluble in water and is largely membrane-impermeant, which attacks those cysteines exposed to the medium. Therefore a comparison of the response to PCMB and PCMBs could, in principle, give important information concerning the location of reactive cysteines as shown most elegantly with UhpT transporter (22).

Fig. 7 shows the results obtained with PCMB and PCMBs with both RSO and ISO membrane vesicles. In both membrane types PCMB reduced dramatically the titratable NEM residues as expected for membrane-permeable SH reagent (Fig. 7, lanes 1 and 6). On the other hand, only in RSO membrane vesicles PCMBs has a pronounced effect, reducing by 10–15-fold the NEM-titratable residues (compare lanes 1 and 4 in Fig. 7). This is inconsistent with the isoform of NhaA we have previously published a putative secondary structure model with 11 hydrophobic TMS connected with hydrophilic loops (12, 15). In this model H225C was predicted to be located in the loop connecting TMS VII and VIII at the edge of TMS 7. However, a careful study of the topology of NhaA using phoA fusions showed that NhaA consists of 12 TMS, and in this model H225C is located at the edge of TMS 8 facing the periplasmic face of the membrane (37). In line with this model the C terminus was found exposed to the cytoplasm. The results obtained in the present work showing directly that H225C is exposed at the periplasmic face of the membrane strengthens the new 12 TMS model of NhaA.

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