Unraveling Functional and Structural Interactions between Transmembrane Domains IV and XI of NhaA Na\(^{+}/\)H\(^{+}\) Antiporter of *Escherichia coli*

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A functionally important, interface domain between transmembrane segments (TMSs) IV and XI of the NhaA Na\(^{+}/\)H\(^{+}\) antiporter of *Escherichia coli* has been unraveled. Scanning by single Cys replacements identified new mutations (F136C, G125C, and A137C) that cluster in one face of TMS IV and increase dramatically the \(K_m\) of the antiporter. Whereas G125C, in addition, causes a drastic alkaline shift to the pH dependence of the antiporter, G338C alleviates the pH control of NhaA. Scanning by double Cys replacements (21 pairs of one replacement per TMS) identified genetically eight pairs of residues that showed very strong negative complementation. Cross-linking of the double mutants identified six double mutants (T132C/G338C, D133C/G338C, F136C/S342C, T132C/S342C, A137C/S342C, and A137C/G338C) of which pronounced intramolecular cross-linking defined an interface domain between the two TMSs. Remarkably, cross-linking by a short and rigid reagent (N,N'-o-phenylenedimaleimide) revived the Li\(^{+}/\)H\(^{+}\) antiporter activity, whereas a shorter reagent (1,2-ethanediyl bismethanethiosulfonate) revived both Na\(^{+}/\)H\(^{+}\) and Li\(^{+}/\)H\(^{+}\) antiporter activities and even the pH response of the dead mutant T132C/G338C. Hence, cross-linking at this position restores an active conformation of NhaA.

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 (for a recent review, see Ref. 1).

*Escherichia coli* has two antiporters, NhaA (2) and NhaB (3), which specifically exchange Na\(^{+}\) or Li\(^{+}\) for H\(^{+}\). Only NhaA is indispensable for adaptation to high salinity, for challenging toxicity, and for growth at alkaline pH (17). The suppressor mutations were found clustered in helix IV (A127T, P129L, and A127V) (17). These results have suggested that residues in TMSs IV and XI are in close proximity and functionally and/or structurally interact (18). We therefore scanned genetically by Cys replacements conserved residues in TMSs IV and XI and identified new residues that participate in the activity and/or pH regulation of the antiporter. We then constructed double Cys replacements, one in TMS IV and the other in TMS XI, and found that many of the pairs of the Cys replacements exhibited a negative interaction, suggesting functional and/or structural interactions between TMSs IV and XI. However, long range effects can explain interaction between pairs of mutants.

Site-directed thiol cross-linking studies have provided considerable insight into the structure of membrane proteins by providing estimates of distances between certain positions in proteins (19–23). We have used this approach to probe proximities between periplasmic and cytoplasmic loops of NhaA and found very strong cross-linking within the pairs of Cys replacements S146C/L316C and A118C/S352C (18) (Fig. 1), suggesting close proximity between the neighboring TMSs IV and XI. However, since loops can be flexible, the distances obtained are not conclusive. Therefore, in the present work, in addition to the genetic approach, we measured distances between the Cys replacements in TMSs IV and XI by intramolecular site-directed cross-linking and identified several double mutants that strongly cross-linked. Together with the pheno-

Similar to many other Na\(^{+}/\)H\(^{+}\) antiporters, both prokaryotic (1) and eukaryotic (9–11), one of the most interesting functional characteristics of NhaA is its dramatic dependence on pH. The rate of activity of NhaA changes over 3 orders of magnitude between pH 7 and 8 (4, 5). This activation is accompanied by a conformational change that involves the N terminus as probed by a monoclonal antibody (1F6 (12)) and loop VIII-IX (Fig. 1) as probed by accessibility of NhaA to trypsin (13).

Amino acid residues that affect the transport activity can be identical to or different from those affecting the pH regulation of NhaA. They are clustered in various domains along the protein (14–17), implying the need of atomic structure for understanding the mechanism of activity and pH regulation of NhaA. Since atomic resolution of NhaA is not yet available, indirect approaches for structure analysis have been applied. Second site suppressor mutations were isolated to mutation G338S, a pH-conditional lethal mutant that is located in TMS XI and grows on high Na\(^{+}\)-selective medium at neutral pH but not at alkaline pH (17). The suppressor mutations were found clustered in helix IV (A127T, P129L, and A127V) (Fig. 1) (17). These results have suggested that residues in TMSs IV and XI are in close proximity and functionally and/or structurally interact (18). We therefore scanned genetically by Cys replacements conserved residues in TMSs IV and XI and identified new residues that participate in the activity and/or pH regulation of the antiporter. We then constructed double Cys replacements, one in TMS IV and the other in TMS XI, and found that many of the pairs of the Cys replacements exhibited a negative interaction, suggesting functional and/or structural interactions between TMSs IV and XI. However, long range effects can explain interaction between pairs of mutants.

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\(^1\) The abbreviations used are: TMS, transmembrane segment; BMI, bismethanethiosulfonate; DMF, dimethylformamide; CL-NhaA, His-tagged NhaA.

1,6-bis(maleimido)hexane; o-PDM, N,N'-o-phenylenedimaleimide; MT-S2-MTS, 1,2-ethanediyl bismethanethiosulfonate; 1H, pH difference across the membrane; DMSO, dimethylsulfoxide; NhaA, His-tagged Cys-less NhaA.
types of the double mutants, this cross-linking defined an interface between TMSs IV and XI with functional implications. Remarkably, cross-linking of the lethal double mutant T132C/G338C revived both the Na$^+$ and Li$^+$ antiporter activities of the mutant and even its regulation by pH, as would be expected if the cross-linking restored an active conformation of the antiporter.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—EP432 is an *Escherichia coli* K-12 derivative, which is *melB*::ΔnhaA::kan, *ΔnhaB*::cat, *ΔlasZ*::YFP, thrI (3). TA16 is *nhaA*Δ*,nhaB*Δ* lacUV5* (TA15lacUV5) and otherwise isogenic (pR151-100). Cells were grown either in L broth (LB) or in modified L broth (LBK (2)). Where indicated, the medium was buffered with 60 mM 1,3-bis[tris(hydroxymethyl)methyl]amino propane, 5 mM MgCl$_2$, and the pH was titrated with HCl. Where indicated, 10 mM LiCl was added. A reversal of the fluorescence level (dequenching) indicates that protons are exiting the vesicles in antiport with either Na$^+$ or Li$^+$.

**Site-directed Mutagenesis**—Site-directed mutagenesis was conducted following a polymerase chain reaction-based protocol (27). All mutations were verified by DNA sequencing of the entire gene, through the ligation junctions with the vector plasmid.

For Cys replacement of Gly$^{125}$, Phe$^{136}$, and Ala$^{337}$, pCL-HAH4 was used as a template with the mutagenic primers described in Table I. The resulting plasmids were pCL-HAH4-G125C, pCL-HAH4-F136C, and pCL-HAH4-A137C. For Cys replacement of Leu$^{334}$ and Ser$^{342}$, pCL-BstX was used as a template with the mutagenic primers described in Table I. The resulting plasmids were pCL-BstX-L334C and pCL-BstX-S342C. To generate L334C and S342C mutation in pCL-HAH4, the MunI-MluI fragment (571 bp) of pCL-BstX-L334C or pCL-BstX-S342C was ligated with the MunI-MluI fragment (4.17 kb) of pCL-HAH4, yielding pCL-HAH4-L334C and pCL-HAH4-S342C. For Cys replacement of Gly$^{335}$, pCL-GMAR100 was used as a template with the mutagenic primers described in Table I. The resulting plasmid was pCL-GMAR100-G338C. For construction of this mutation in pCL-AXH (pCL-AXH-G338C), a Nhel-MluI fragment (879 bp) of the resulting PCR product was ligated to Nhel-MluI (4.1-kb) fragment of pCL-HAH4.

To generate all double mutants, the MunI-MluI fragment (571 bp) of single Cys NhaA mutants in Helix XI, on C-less background, were ligated with MunI-MluI fragment (4.17 kb) of plasmid encoding the single Cys NhaA mutants in Helix IV.

**Isolation of Membrane Vesicles, Assay of Na$^+$/*H*+$^+$ Antiporter Activity**—EP432 cells transformed with the respective plasmids were grown and everted vesicles were prepared and used to determine the Na$^+$/*H*+$^+$ or Li$^+$/*H*+$^+$ antiporter activity as described (28–30). The assay of antiporter activity was based upon the measurement of Na$^+$- or Li$^+$-induced changes in the ΔpH as measured by acridine orange, a fluorescent probe of ΔpH. The fluorescence assay was performed with 2.5 ml of reaction mixture containing 50–100 μg of membrane protein, 0.5 μM acridine orange, 150 mM KCl, 50 mM 1,3-bis[tris(hydroxymethyl)methyl]amino propane, 5 mM MgCl$_2$, and the pH was titrated with HCl. When indicated, 10 mM β-mercaptoethanol or 20 mM dithiothreitol (Sigma) was added to the reaction mixture. After energization with either ATP (2 mM) or β-lactate (2 mM), quenching of the fluorescence was allowed to achieve a steady state, and then either Na$^+$ or Li$^+$ (10 or 100 mM each) was added. A reversal of the fluorescence level (dequenching) indicates that protons are exiting the vesicles in antiport with either Na$^+$ or Li$^+$. As indicated (see Figs. 4A and 6A for data of typical experiments). As shown previously, the end level of dequenching is a good estimate of the degree of fluorescence labeling of the protein resolved in SDS-PAGE (nonreducing conditions in the case of treatment with MTS-2-MTS) to identify the proteolytic products according to Ret. 18. Since there is only one tryptophan cleavage site at Lys$^{223}$, located between TMSs IV and XI (Fig. 1), trypsinolysis results in two tryptic peptides of mobility faster than the intact protein. On the other hand, intramolecular cross-linking results in one fragment of mobility equal to that of the intact protein (18).

To measure the effect of the cross-linking agents on the activity of the antiporter, everted membrane vesicles were isolated from EP432 expressing the mutations (300 μg of protein); treated with one of the homobifunctional cross-linkers as described above; washed in 1.5 ml of a 140 mM choline chloride, 250 mM sucrose, 10 mM Tris-HCl (pH 7.5); resuspended in 75 μl of the same solution; and used to test antiporter activity. The degree of cross-linking in vesicles derived from EP432 and TA16 is indistinguishable.

In situ site-directed intermolecular cross-linking was tested as above but without treatment with trypsin. When intermolecular cross-linking takes place, a band corresponding in mobility to that of NhaA dimer, appears in SDS-PAGE (15, 18).

**Accessibility of the Cys Replacements to the Cross-linking Reagents**—The procedure was essentially as described in Ref. 36. Following treatment with the cross-linking reagents, the Nt$^+$-NTA affinity-purified protein was left bound to the Nt$^+$-NTA beads, washed with binding buffer (5 mM imidazol, 500 mM NaCl, 20 mM Tris, and 0.1% N-dodecyl-β-D-maltoside, pH 7.4) and exposed to 0.2 mM fluorescein 5-maleimide (Molecular Probes, Inc.; dissolved in MeSO) to titrate any Cys residue left free. The incubation was for 30 min at 25 °C with gentle titling. Then the protein was washed and eluted as described in Ref. 36. The degree of fluorescence labeling of the protein resolved in SDS-PAGE was determined under UV light (290 nm) as described (18, 36).

**Modeling of TMSs IV and XI**—Initially, two ideal poly-Ala α-helices were built using the program Moleman (37). Then the side chains were altered to those of the respective TMS, according to the putative topology model of NhaA (Fig. 1A) (38) by using the rotamer library in the O software (39). Next, the distances estimated by cross-linking of double Cys replacement mutants located in TMSs IV and XI (see "Results") and on neighboring loops (18) were used as constraints to determine the proximity between the two helices. Alternate orientations of side chains were tested, and those with geometric clashes with neighboring residues were ruled out. Energy minimization of the putative model was carried out using the program CNS (40) to eliminate further clashes between neighboring residues.

**RESULTS**

**Construction of Single and Pairs of Cys Replacements in TMSs IV and XI of NhaA**—In this study, site-directed Cys replacements and site-directed thiol cross-linking were used to identify functional interactions and determine proximities between TMSs IV and XI of NhaA. For this purpose, we used the single Cys replacements mutants (D133C, T132C, P129C, and A127C (14)) that had already been constructed in TMS IV and constructed new single Cys replacement mutants: G125C, F136C, and A137C in TMS IV and L334C, G338C, and S342C in TMS XI (Tables I and II and Fig. 1). All old and new mutations replace conserved amino acid residues (Fig. 1). We then constructed double Cys mutants, each containing one replace-
The mutated bases are shown in boldface type. Additional substitutions (*) have been introduced to create silent mutations in Gly-125, Phe-136, Ala-137, Leu-334, Gly-338, and Ser-342 that generate unique restriction sites BsmI, NsbI, ScaI, NdeI, SspI, and BsaBI, respectively, in the sequence of nhaA.

For characterization of the mutations, EP432 cells transformed with the respective plasmids were used. The expression level was expressed as a percentage of the positive control, EP432/pCL-HAH4. EP432/pBR322 served as a negative control. The apparent Km negative control. The apparent activity (end level of dequenching) is expressed as a percentage of the positive control, EP432/pCL-HAH4. EP432/pBR322 served as a negative control. The apparent Km negative control. The apparent activity (end level of dequenching) is expressed as a percentage of the positive control, EP432/pCL-HAH4.

| Mutation       | DNA sequence of mutagenic oligonucleotide | Codon change observed |
|----------------|------------------------------------------|-----------------------|
| G125C          | CCCCGGAGATGCT*GGGCGATTCG                 | GGC → TGC             |
| F136C          | GCTACTGACATTGCTTGCTGCC*GCACTTG          | TTT → TGC             |
| A137C          | GCTTTTGGCTTGGAGCTACTGGG                 | GCA → TGT             |
| L334C          | GGCCA*TAGTTCCGTTATCG                   | CTG → TGT             |
| G338C          | CGGTATCGTCTTCTGATCTGGC                  | TCT → TGT             |
| S342C          | GTTGTTGTTGACGCGACGCGCG                 | None                  |
| End primers for mutations G125C, F136C, A137C, and G338C | TTTAACGATGATTCGTGGCGG (sense primer) | None                  |
| End primers for mutations L334C and S342C | GTTGTTGTTGACGCGACGCGCG (sense primer) | None                  |

**Table II**

Single and double Cys replacements in TMSs IV and XI of nhaA

The activity (end level of dequenching) is expressed as a percentage of the positive control, EP432/pCL-HAH4. Growth experiments were conducted on agar plates with high Na⁺ (0.6 mM) or high Li⁺ (0.2 mM) at the pH values indicated in parentheses. +++, the number and size of the colonies after 24 h of incubation at 37 °C was identical to that of the wild type; + and −, the number of colonies was similar but size was slightly or much smaller than that of the wild type, respectively; −, no growth. Na⁺/H⁺ and Li⁺/H⁺ antipporter activity at pH 8.5 was determined with 10 mM NaCl or LiCl.

| Mutation       | Expression | Growth | Activity* | Apparent K<sub>m</sub> |
|----------------|------------|--------|-----------|------------------------|
|                |            | Na⁺ (pH 7) | Na⁺ (pH 8.3) | Li⁺ (pH 7) | Na⁺ | Li⁺ |
| Helix IV       |            | %       | mM        | mM        | mM |
| G125C          | 30         | +++     | −         | +++       | 16  | 79 | 12 | 0.56 |
| A127C          | 92         | +++     | +++       | +++       | 100 | 100 | 0.24 | 0.02 |
| P129C          | 99         | +++     | +++       | +++       | 100 | 100 | 0.4 | 0.028 |
| T132C          | 95         | ++      | +++       | +++       | 54  | 91 | 12.4 | 0.7 |
| D133C          | 10         | ++      | +++       | +++       | 87  | 70 | 3.6 | 1.24 |
| F136C          | 100        | +       | −         | −         | 1   | 0 | 102 |
| A137C          | 100        | +++     | +++       | +++       | 42  | 84 | 7.2 | 0.8 |
| Helix XI       |            | %       | mM        | mM        | mM |
| L334C          | 70         | +++     | +++       | +++       | 100 | 83 | 0.84 | 0.16 |
| G338C          | 100        | +++     | −         | −         | 39  | 79 | 0.68 | 0.5 |
| S342C          | 80         | +++     | +++       | +++       | 94  | 70 | 0.76 | 0.12 |
| Double mutants |            | %       | mM        | mM        | mM |
| G125C/L334C    | 80         | −       | −         | −         | 2   | 4 | >100 | >100 |
| G125C/G338C    | 40         | −       | −         | −         | 0   | 0 |
| G125C/S342C    | 30         | −       | −         | −         | 0   | 0 |
| A127C/L334C    | 100        | +++     | +         | +         | 78  | 100 | 0.68 | 0.023 |
| A127C/G338C    | 30         | +++     | +++       | +++       | 30  | 30 | 36 | 10.5 |
| A127C/S342C    | 40         | +++     | +++       | +++       | 65  | 88 | 0.5 | 0.08 |
| P129C/L334C    | 100        | +++     | +         | +         | 100 | 100 | 0.56 | 0.046 |
| P129C/G338C    | 100        | +++     | +++       | +++       | 8   | 77 | 31.6 | 2.6 |
| P129C/S342C    | 20         | +++     | +++       | +++       | 48  | 23 | 0.05 | 0.69 |
| T132C/L334C    | 70         | +++     | −         | −         | 24  | 100 | 110 | 1.26 |
| T132C/G338C    | 50         | −       | −         | −         | 0   | 0 |
| T132C/S342C    | 30         | +++     | −         | −         | 49  | 100 | 3.7 | 3.4 |
| D133C/L334C    | 100        | +++     | +         | +         | 100 | 100 | 0.23 | 0.19 |
| D133C/G338C    | 40         | −       | −         | −         | 0   | 0 |
| D133C/S342C    | 80         | +++     | +++       | +++       | 38  | 49 | 0.21 | 0.2 |
| F136C/L334C    | 40         | −       | −         | −         | 0   | 0 |
| F136C/G338C    | 30         | −       | −         | −         | 0   | 0 |
| F136C/S342C    | 100        | −       | −         | −         | 0   | 0 |
| A137C/L334C    | 50         | +++     | −         | −         | 29  | 44 | 13.3 | 0.062 |
| A137C/G338C    | 20         | +++     | −         | −         | 5   | 5 | 56 | 7.0 |
| A137C/S342C    | 50         | +++     | −         | −         | 45  | 21 | 5.8 | 15 |
| Controls       |            | %       | mM        | mM        | mM |
| pCL-HAH4       | 100        | +++     | +++       | +++       | 100 | 100 | 0.2 | 0.02 |
| pBR322         | −          | −       | −         | −         | −   | − | − | − |

* The activity (end level of dequenching) is expressed as a percentage of the positive control, EP432/pCL-HAH4. EP432/pBR322 served as a negative control. The apparent K<sub>m</sub> for the ions was determined at pH 8.5, as described under “Experimental Procedures.”

Table I

Oligonucleotides used for constructing the Cys mutations in nhaA

The mutated bases are shown in boldface type. Additional substitutions (*) have been introduced to create silent mutations in Gly-125, Phe-136, Ala-137, Leu-334, Gly-338, and Ser-342 that generate unique restriction sites BsmI, NsbI, ScaI, NdeI, SspI, and BsaBI, respectively, in the sequence of nhaA.

Table II

Interactions between TMSs IV and XI of NhaA
readily detected by Western analysis, and way above the level expressed from a single chromosomal gene that is hardly detected by Western analysis (17, 18).

**The Effect of the New Single Mutations in TMSs IV or XI on the Growth Phenotype and the Na+/H+ Antiporter Activity**—As summarized in Table II, all new single mutants retained certain growth capacity on the selective media, implying that none of the replaced residues are essential for the Na+/H+ antiporter activity of NhaA. However, the results also show that residues Gly^{125}, Phe^{136}, and Gly^{338} are essential for certain properties of NhaA that support growth in the presence of high Na+, at alkaline pH, whereas residues Phe^{136} and Gly^{338} are, in addition, important for growth on high Li+ at neutral pH (Table II).

The Na+/H+ antiporter activity, at various pH values, was monitored in everted membrane vesicles of EP432 expressing the various single mutants, as recovery (dequenching) from the respiration-dependent fluorescence quenching of acridine or-
The apparent $K_{m}$ values for Na$^{+}$ and Li$^{+}$ were determined for each mutant at pH 8.5 (Table II), whereas the pH dependence was monitored at low (10 mM) and high (100 mM) concentrations of NaCl. The latter procedure allowed us to differentiate between three types of mutations in NhaA (14) as follows. (a) Mutations that affect solely the apparent $K_{m}$ of the antiporter have a pH profile of activity that is similar to that of the wild type when measured at saturating Na$^{+}$ concentrations. (b) Mutations that shift the pH dependence with hardly any effect on the apparent $K_{m}$ exhibit the pH shift, whether tested at saturating Na$^{+}$ concentrations or not. (c) Mutations that affect both the apparent $K_{m}$ and the pH dependence of NhaA.

As expected from the high sensitivity of mutant F136C to Na$^{+}$ and Li$^{+}$ (Table II), its apparent $K_{m}$ for Na$^{+}$ increased dramatically (510-fold), its rate decreased, and it lost the Li$^{+}$/H$^{+}$ antiporter activity (Table II and Fig. 2B). Due to its very low activity, it was impossible to determine accurately the pH profile of this mutant. Although to a lesser extent, the other two new mutations in TMS IV, G125C and A137C, also strongly increase the apparent $K_{m}$ of the antiporter for both Na$^{+}$ (60- and 36-fold, respectively) and Li$^{+}$ (28- and 40-fold, respectively) (Table II). However, whereas the pH dependence of A137C was very similar to that of CL-NhaA at saturating concentration of Na$^{+}$ (Fig. 2A), G125C caused a drastic alkaline shift (of one pH unit) to the pH dependence of NhaA, which was independent of the concentration of Na$^{+}$ (Fig. 2B). Hence, whereas A137C affects the apparent $K_{m}$ of NhaA to the ions, G125C affects both the apparent $K_{m}$ and the pH dependence of NhaA.

Mutants L334C and S342C of TMS XI show a maximal end level of fluorescence dequenching (Fig. 2C) and kinetic parameters that are similar to those of the wild type (Table II). However, whereas the pH dependence of L334C is identical to that of CL-NhaA, the pH profile of S342C is drastically shifted to the acidic range by one pH unit (Fig. 2C) in a Na$^{+}$ concentration-independent fashion.

Similar to the previously described mutant G338S in TMS XI (17), G338C alleviates the pH control of NhaA and has a small effect on the kinetic parameters of the antiporter (Table II and Fig. 2D).

The Effect of the Double Mutations (One in TMS IV and the Other in TMS XI) on the Growth Phenotype and the Na$^{+}$/H$^{+}$ Antiporter Activity—We used the single mutants to construct double Cys replacements, one replacement in TMS IV and the other in TMS XI. By comparing the growth and transport phenotypes of the single mutants with those of the 21 new double mutants, two patterns of phenotypes were identified as follows. (a) Some growth and transport phenotypes were similar to that of both respective single mutants (Table II and Fig. 3). Thus, the growth and transport of the double mutants A127C/L334C, A127C/S342C, P129C/L334C, P129C/S342C, D133C/L334C, and D133C/S342C were very similar to the respective single mutants and the control. (b) Surprisingly, in many of the double mutants, a drastic negative interaction between the double mutations was found (Fig. 3 and Table II), and the mutants G125C/L334C, G125C/G338C, G125C/S342C, T132C/G338C, D133C/G338C, F136C/L334C, F136C/G338C, and F136C/S342C completely lost antiporter activity and growth capacity (Fig. 3B and Table II). The negative interaction between residues in TMSs IV and XI can be due to a direct physical interaction with functional consequences. Alternatively, it is also possible that the two TMSs are wide apart in the NhaA molecule and that long range effects, mediated through the protein, exert the phenotypes of the double mutants. Since the atomic structure of NhaA has not yet been solved, we applied intramolecular cross-linking to estimate proximities between TMSs IV and XI.

Effect of Reducing Conditions on the Na$^{+}$/H$^{+}$ and Li$^{+}$/H$^{+}$ Antiporter Activity of the Double Cys Replacement Mutants—Since eight of the double Cys replacement mutants were inac-

![Fig. 2. Na$^{+}$/H$^{+}$ antiporter activity in everted membrane vesicles of the single NhaA mutants in TMSs IV or XI.](image-url)
tive (Fig. 3B and Table II), we considered the possibility that spontaneous cross-linking by oxidation occurs between the two vicinal SH groups, forming S–S bonds. Therefore, the antiporter activity of the inactive double mutants was tested in the presence of β-mercaptoethanol (reducing conditions). The double mutant F136C/S342C was the only mutant that was activated significantly by reduction; following the addition of β-mercaptoethanol, the rate of fluorescence dequenching reflects the restored antiporter activity, which reaches a fluorescence dequenching end level of 45% (Fig. 4, compare B and C). Furthermore, the subsequent addition of diamide (20 mM) that impose oxidative conditions restored the inactivation (Fig. 4 C). Following the addition of diamide, the drastic drop in fluorescence reflects the fast rate of activity of the respiratory proton pumps that restore the pH. No activity was observed with LiCl, or at different pH values. Either in the presence or absence of β-mercaptoethanol, diamide had no effect on Cys-less NhaA (data not shown). The formation of an S–S bond between F136C and S342C, in the presence of diamide, was verified by SDS-PAGE (under nonreducing conditions) as described below (data not shown). Hence, an S–S bond exists between F136C and S342C and inhibits the activity of the double mutant. These results imply the close proximity between positions F136C and S342C and thus the utility of thiol-specific homobifunctional cross-linking reagents.

**In Situ Intramolecular Cross-linking between TMSs IV and XI of NhaA**—Using the cross-linking reagents described below, we did not find any intermolecular cross-linking between monomers of the single or double mutants (data not shown).

We have previously shown that site-specific intramolecular cross-linking by thiol-specific homobifunctional cross-linking reagents, is a very powerful tool to measure proximities between double Cys replacements introduced into the CL-NhaA molecule (18). In this protocol, the cross-linkers are applied, in situ, on the membrane and the unique trypsin cleavable site of NhaA at Lys45 (Fig. 1A) (13, 18) is exploited to detect changes in the mobility of cross-linked products of double Cys replace-
The results presented in Fig. 5 show that the double Cys replacement mutations, T132C/S432C (Fig. 5B, lanes e–c) and T132C/G338C (Fig. 5C, lanes e–c) were cross-linked by the three cross-linking reagents BMH, o-PDM, and MTS-2-MTS. Following cross-linking, instead of the two tryptic products (Fig. 5, B and C, lanes b), one prominent band of mobility similar to that of the intact untreated protein appeared on SDS-PAGE (Fig. 5, B and C, compare lanes e–c with lane a). The double mutants A137C/S342C (Fig. 5D) and A137C/G338C (Fig. 5E) were cross-linked only by MTS-2-MTS. The double mutant D133C/G338C was cross-linked significantly by o-PDM (Fig. 5F, lane d) and partially by BMH (Fig. 5F, lane c) but not by MTS-2-MTS (2 mM).

It was previously shown (41) that cross-linking might be overlooked when the concentration of the reagent is too high. This could cause separate labeling of both cysteine residues rather than cross-linking. In line with these results in one mutant, D133C/G338C, treated with MTS-2-MTS, cross-linking appeared by reducing the concentration of the reagent from 2 mM to 50 μM (Fig. 5F, lane f) and even to 50 μM (Fig. 5F, lane g). We therefore applied various concentrations of the reagents (2 to 0.05 mM) in all cross-linking attempts, but no further improvement was obtained.

The positive cross-linking results clearly imply very close proximity between TMSs IV and XI of NhaA. Figure 5, A–F, membrane vesicles were prepared from TA16 expressing the indicated double Cys replacement mutants as described under “Experimental Procedures.” The membranes were cross-linked, in situ, by BMH (lanes c), o-PDM (lanes d), or MTS-2-MTS (lanes e) or not cross-linked (lanes b). For analysis of the cross-linked products, the Ni2+-NTA affinity-purified protein was treated with trypsin and resolved by SDS-PAGE as described under “Experimental Procedures.” Control samples that were treated with neither cross-linker nor trypsin are shown in lanes a. Lanes f and g in F, the cross-linking was conducted with 50 and 500 μM MTS-2-MTS, respectively. The results presented are representative of experiments performed at least twice. HF and LF, the tryptic fragments of CL-NhaA-His6, open arrow, intact CL-NhaA-His6, G and H, accessibility of the double Cys replacement mutants, P129C/L334C and T132C/S342C, to the cross-linking reagents (lanes b–d, respectively). Following cross-linking, the Ni2+-NTA affinity-purified protein bound to the beads was subjected to fluorescein maleimide (0.2 mM), eluted, and resolved by SDS-PAGE. Control samples untreated with cross-linkers are shown in lane a. For further details, see “Experimental Procedures.” The gels were photographed under UV light (lower panel) to determine the level of labeling by the fluorescent maleimide and then stained with Coomassie Blue and photographed in ordinary light to assess the protein concentration (upper panel).

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identical to the untreated control (Fig. 5G, lower panel, lane a). In marked contrast, as expected, the mutant T132C/S342C is accessible to all three cross-linking reagents, since pretreatment with these cross-linkers inhibited the subsequent labeling by the fluorescent probe (Fig. 5H, lower panel, lanes b–d). Accordingly, mutants that cross-linked only with MTS-2-MTS (A137C/S342C and A137G/G338C) (Fig. 5, D and E) or partially with BMH (D133C/G338C) (Fig. 5F) were found accessible only to the respective cross-linkers (data not shown). The mutants A127C/L334C and P129C/L334C were not accessible to all three cross-linking reagents and in agreement did not perform any cross-linking (data not shown). This inaccessibility can be due to a hydrophobic environment surrounding the Cys replacements in the TMS in which Cys residues are less reactive. Similar to the metal-tetracycline/H⁺ antiporter (42), many Cys replacements in NhaA (14, 25) are not accessible to SH reagents (reagents that react with Cys residues in proteins). Most interestingly, four double mutants G125C/S342C, G125C/L334C, G125C/G338C, and A127C/G338C were accessible to all cross-linkers and yet were not cross-linked by them (data not shown). Most probably, these double mutants are separated by more than 15.6 Å, the span of BMH, the longest cross-linker used. The double mutants A127C/S342C, P129C/S342C, P129C/G338C, T129C/L334C, D133C/L334C, F136C/G338C, F136C/S342C, and A137C/L334C, were all accessible to MTS-2-MTS but were not cross-linked by this reagent. The double mutant D133C/S342C was accessible to BMH and slightly to MTS-2-MTS but was not cross-linked by either. Hence, the lack of cross-linking of these double Cys replacements cannot be ascribed to inaccessibility to the cross-linking reagents. Most probably, these double Cys replacements are not close enough to be cross-linked by these short reagents (23).

It should be noted that we optimized the cross-linking conditions only with respect to concentrations of the three reagents (see above) but not with respect to other conditions such as pH, temperature, and prolonged incubation time. Therefore, the number of the residues that cross-linked may be underestimated.

Effect of the Bifunctional Cross-linking on the Na⁺/H⁺ and Li⁺/H⁺ Antiporter Activity—Cross-linking of the Cys replacement mutant A137C/G338C by MTS-2-MTS (Fig. 5E) inhibited totally (data not shown) the very low Na⁺/H⁺ antiporter activity (Fig. 3C) of the mutant. Cross-linking of the double Cys replacement mutant T132C/S342C (Fig. 5B) that had a significant Na⁺/H⁺ antiporter activity (Fig. 3B) was 80% inhibited by treatment with the three cross-linking reagents (data not shown). Although the double mutants A137C/S342C and D133C/G338C were cross-linked by certain of the cross-linkers (Fig. 5, D and F, respectively), their activity (Fig. 3B) was not affected by the cross-linking (data not shown).

Most surprising was the behavior of the double mutant T132C/G338C that cross-linked by all three cross-linking reagents (Fig. 5C). This mutant has neither Na⁺/H⁺ nor Li⁺/H⁺ antiporter activity (Figs. 3B and 6A and Table II), yet cross-linking of the dead mutant with o-PDM restored Li⁺/H⁺ activity only at pH 8.5 and at high Li⁺ concentrations (50–100 mM; Fig. 6B). Most importantly, BMH (that also cross-linked T132C/G338C (Fig. 5C)) did not restore activity (Fig. 6B). Hence, it is mere cross-linking, rather than the type of cross-linker, that determines whether the cross-linked sample is active or not.

Strikingly, MTS-2-MTS, a shorter (5.2-Å) cross-linking reagent compared with o-PDM (7.7–10.5 Å (23, 43)), was much more potent in activating T132C/G338C. Cross-linking by MTS-2-MTS restored the Li⁺/H⁺ antiporter activity at all pH values with a pH dependence between pH 6.5 and 8.5 very similar to the wild type control (Fig. 6C). Furthermore, cross-linking by MTS-2-MTS also restored the Na⁺/H⁺ antiporter activity (about 50% dequenching) and its activation by pH between 7 and 8 (Fig. 6C). We therefore suggest that cross-linking between TMSs IV and XI, at positions Thr132 and Gly338 by the short cross-linking reagents, restores an active conformation of the antiporter that is crucial for antiporter activity and pH regulation.

**DISCUSSION**

Using Cys-scanning mutagenesis of either single conserved residues in TMS IV or XI or double residues, one in each TMS, of the NhaA antiporter, we identified new mutations that affect the transport activity and/or pH regulation of NhaA. Furthermore, we revealed positions that, when replaced simultaneously in the two TMSs, showed negative interaction in trans, implying functional and/or structural interactions between the two TMSs. By scanning the double Cys replacement mutants with cross-linking reagents of various length and flexibility, we have proven that many of these positions in TMSs IV and XI are indeed within very close proximity. Strikingly, for the first
time, cross-linking revived activity and even the pH dependence of a completely inactive double mutant (T132C/G338C) of NhaA. These results highly suggest that the cross-linking restores an active conformation of NhaA in which both TMSs IV and XI participate.

Although only conserved residues were replaced, none were found essential for growth in high Na\(^+\)-selective media, at least at neutral pH (Table II). Nevertheless, this mutagenesis identifies new residues that are important for the antiporter activity. In TMS IV, P136C, G125C, and A137C increase the apparent \(K_m\) for Na\(^+\) and Li\(^+\) of the antiporter activity (Table II); in addition to its effect on the apparent \(K_m\), the mutation G125C drastically shifts the pH dependence of the antiporter (by one pH unit) to the alkaline range (Fig. 2B). We have previously isolated mutations in TMS IV (P129L, T132C, and D133C) that affect drastically the apparent \(K_m\) of the antiporter for Na\(^+\) and Li\(^+\) (14). It is remarkable that these mutations together with the new mutations cluster in one face of TMS IV (Fig. 1, B and C), implying its importance in determining the apparent \(K_m\) for the ions of the antiporter. Notably, TMS IV is one of the most conserved TMS in NhaA (14).

The mutations L334C, G338C, and S342C of TMS XI had a minor effect on the apparent \(K_m\) of the antiporter for both ions (Table II). However, whereas L334C had a pH profile very similar to that of the wild type, the pH profile of S342C was shifted to the acidic range by one pH unit (Fig. 2C), and G338C lost entirely its control by pH (Fig. 2D). We have previously found that G338S lacks the pH control of NhaA (17). Taken together, these results imply the importance of TMS XI in the pH response of NhaA and corroborate our previous results, showing that residues affecting the pH dependence of NhaA can be separated from those affecting the apparent \(K_m\) of the antiporter (14).

Comparison of the growth and transport phenotypes of the double mutants with those of the respective single mutants shows a very pronounced difference in many double mutants that cannot be explained by the phenotype of the single mutants or by differences in expression levels between the single and the double mutants (Table II and Figs. 2 and 3). Among the 21 new double mutants, only six (A127C/L334C, A127C/S342C, P129C/L334C, P129C/S342C, D133C/L334C, and D133C/S342C) were very similar to that of the wild type, the pH profile of S342C was shifted to the acidic range by one pH unit (Fig. 2C), and G338C lost entirely its control by pH (Fig. 2D). We have previously found that G338S lacks the pH control of NhaA (17). Taken together, these results imply the importance of TMS XI in the pH response of NhaA and corroborate our previous results, showing that residues affecting the pH dependence of NhaA can be separated from those affecting the apparent \(K_m\) of the antiporter (14).

The cross-linking data were used to construct a distance map between the respective Cys replacements in TMSs IV and XI. We assume that the estimated distances between the double Cys replacements are similar to the respective positions in native NhaA. Our cross-linking data are most consistent with the packing models of TMSs IV and XI shown in Fig. 1, B and C. Most importantly, these putative models are most consistent with the cross-linking, accessibility, and physiological results obtained. Thus, approximately in the middle of TMS IV and TMS XI, all residues that cross-link cluster in one face of each helix (Fig. 1, B and C). We suggest that this is an interface domain where the helices are in very close proximity. The double Cys replacements that are accessible to all three cross-linkers but do not cross-link (data not shown) are located wide apart (pairs of G125C) or on the opposite face of the helix (A127C/G338C). Most interestingly, the double Cys replacements that are active (Table II) are located far apart from the interface domain (all pairs of A127C and P129C). In marked contrast, in many double mutants that show strong negative complementation, at least one member of the double Cys replacements is located in the interface domain (pairs of F136C, D133C/G338C, T132C/G338C, G125C/G338C, and G125C/S342C) (Table II and Fig. 1, A and B), implying that the interface is very sensitive to replacements of its native residues.

In this paper, we also show that testing the effect of cross-linking on the activity of the respective mutants is a very powerful tool to discover positions in two different TMSs where their proximity in trans has most important functional implications. Cross-linking inhibited drastically the mutant T132C/
S342C but not the mutant A137C/S342C. This result may suggest that whereas, at the former position, flexibility between the two helices is important, it is not at the latter position. However, we cannot exclude the possibility that the cross-linking affected the activity of T132C/S342C and A137C/S342C by chemical modification rather than by cross-linking. In marked contrast, cross-linking of the dead mutant T132C/G338C by ω-PDM revived its Li+/H+ antiporter activity, whereas cross-linking by BMH had no effect. Hence, it was the correct proximity between T132C and G338C, rather than the chemical modification, that restored activity. Cross-linking by the shorter reagent, MTS-2-MTS was even more efficient in restoring activity; it revived both Na+/H+ and Li+/H+ antiporter activities and even the pH response of the T132C/G338C mutant.

The revival of the antiporter activity by cross-linking is remarkable. Since NhaA is a dimer with functional implications (15), it is possible that the double Cys replacements T132C/G338C destabilizes NhaA dimer, and the cross-linking brings the two subunits together. However, we believe that this intriguing interpretation is unlikely for the following reasons: (a) we did not find any inter-molecular cross-linking between the single or double mutants; (b) the trypsin digest of the cross-linked double mutants of NhaA (Fig. 5C) shows only the products expected from the cross-linked monomer. We therefore suggest that the cross-linking at the interface between the helices, at position T132C/G338C, restores a conformation of NhaA that is essential for its transport activity and pH regulation.

In summary, we unraveled closed proximity between TMSs IV and XI of NhaA and identified a functionally important interface domain between the two TMSs. In addition, we show that scanning activity of single and double Cys replacements combined with cross-linking provides a most powerful tool for determination of structure and function relationship between pairs of TMSs in polytopic membrane proteins.
