β-Sheet-dependent Dimerization Is Essential for the Stability of NhaA Na⁺/H⁺ Antiporter

Katia Herz, Abraham Rimon, Gunnar Jeschke, and Etana Padan

From the Department of Biological Chemistry, Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel and the Department of Physical Chemistry, ETH, Zurich, 8093 Zurich, Switzerland

A structural model of the NhaA dimer showed that a β-hairpin of each monomer combines to form a β-sheet at the periplasmic side of the membrane. By Cys scanning the entire β-hairpin and testing each Cys replacement for functionality and intermolecular cross-linking, we found that Gln⁴⁷ and Arg⁴⁹ are critical for the NhaA dimer and that K⁵⁷C causes an acidic shift of 1 pH unit to the pH dependence of NhaA. Comparing the growth of the NhaA variants with the previously isolated β-hairpin deleted mutant (Δ(P45-N58)) and the wild type validated that NhaA dimers have an advantage over monomers in growth under extreme stress conditions and unraveled that during this growth the apparent Km of Na⁺ of Δ(P45-N58) was increased 50-fold as compared with the wild type. Remarkably, the effect of the extreme stress on the NhaA variants is reversible. Testing the temperature stability (4–55 °C) of the NhaA variants in dodecyl maltoside micelles showed that the mutants impaired in dimerization were much less temperature-stable than the wild type. We suggest that NhaA dimers are crucial for the stability of the antiporter under extreme stress conditions.

Regulation of intracellular pH, cellular Na⁺ content, and cell volume are processes that are crucial for survival of all living cells. Sodium proton antiporters play primary roles in these processes (1–3). E. coli-NhaA, the main Na⁺/H⁺ antiporter of Escherichia coli (henceforth, NhaA), is indispensable for adaptation to high salinity, for challenging Li⁺ toxicity, and for growth at alkaline pH in the presence of Na⁺ (2, 4). It is widely spread in Enterobacteria (1) and has orthologs throughout the biological kingdom including humans (5).

NhaA is an electrogenic antiporter with a stoichiometry of 2H⁺/Na⁺ (2, 6) and is drastically dependent on pH. Its activity changes over 3 orders of magnitude, between pH 7.0 and 8.5 (2, 7, 8). This pH activation is accompanied by conformational changes (8–10).

The determined crystal structure of the acid (pH 4)-down-regulated NhaA (11) has provided the first structural insights into the relationship between the structure and function of NhaA (3). NhaA consists of 12 transmembrane segments (TMSs)² connected by extramembrane loops with the N and C termini pointing into the cytoplasm. At the periplasmic side, loop I–II, containing a β-hairpin, forms together with the other loops a rigid periplasmic face parallel to the membrane. At the cytoplasmic side, many helices protrude into the cytoplasm forming a rough cytoplasmic face. A negatively charged ion funnel opens from the cytoplasmic face and ends in the middle of the membrane at the putative ion-binding site.

NhaA is a dimer in the native membrane as revealed by genetic complementation data, biochemical pull-down experiments, intermolecular cross-linking (12), electron spin resonance studies (13, 14), and cryoelectron microscopy of two-dimensional crystals (15, 16). A comparison between the three-dimensional crystal structure of NhaA monomers and the electron density map obtained by cryoelectron microscopy of the two-dimensional crystals (17) showed a very good fitting of the monomers in the two crystal types. These results implied that the three-dimensional crystal structure of NhaA is native and allowed to get a high resolution structural model of the NhaA dimer (14). This model shows that the main contact between the monomers is built by the two β-hairpins of the monomers, forming a four-stranded β-sheet at the periplasmic side of the membrane. At the cytoplasmic side, only few residues in the cytoplasmic ends of TMS IX and TMS VII participate in the dimer interface.

Recently, we have constructed a NhaA mutant, Δ(P45-N58), deleted of the β-hairpin (18). The Δ(P45-N58) protein exists exclusively in a monomeric form in the native membrane, proteoliposomes, and β-dodecyl-β-maltoside (DDM) micelles. The NhaA monomers are fully active in isolated membrane vesicles and support growth of cells under the routinely stress conditions of growth, implying that the functional unit of NhaA is the monomer. However, under extreme stress conditions of growth, the wild type dimers were much more beneficial than the mutant monomers in conferring growth resistance (18). These results have suggested that the β-hairpin is needed for NhaA dimerization and that whereas the NhaA monomer is the functional unit of NhaA, the dimers are crucial for growth under extreme stress conditions.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Text S1, Tables S1 and S2, and Fig. S1.

2 The abbreviations used are: TMS, transmembrane segment; DDM, β-dodecyl-β-maltoside; BMH, 1,6-bismaleimidohexane; o-PDM, N,N-o-phenylenedimaleimide; MT5-2-MTS, 1,2-ethanediy1 bismethanethiosulfonate; BN, Blue Native; MTSES, 2-sulfonatoethyl methanethiosulfonate.
Nevertheless, several main questions have remained open: (i) Which amino acid in the NhaA β-hairpin is crucial for maintaining the NhaA dimers? (ii) How is the dimer constructed? These questions are particularly interesting because whereas β-sheet is a known common aggregation motif of soluble proteins, oligomerization motifs of membrane proteins are unknown. (iii) Why do the NhaA dimers confer upon the cells higher resistance to Na⁺ and Li⁺, as compared with the monomers? To answer these questions, we Cys replaced amino acid residues (each separately and in combination) in the NhaA segment Pro⁴⁵–Asn⁵⁸, which comprises the β-hairpin of NhaA, and studied the mutations with respect to cell growth under various growth conditions, Na⁺/H⁺ antiporter activity at physiological pH, intermolecular cross-linking, aggregation state, and the ability to provide temperature stability to the NhaA molecule in detergent micelles and in situ in the membrane. We found that the NhaA dimers are critically dependent on the β-sheet and are crucial for NhaA stability under extreme stress conditions.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—EP432 is an E. coli K-12 derivative, which is melBΔld, ΔnhaA1::kan, ΔnhaB1::cat, ΔlacZΔY, thr1 (4). TA16 is nhaA⁺ nhaB⁻ lacQ (TA15lacQ) and otherwise isogenic to EP432 (7). Cells were grown either in L broth (LB) or in modified L broth (LBK (19)). Where indicated, the medium was buffered with 60 mM 1,3-bis-[tris(hydroxymethyl)-methylamino] propane. The cells were also grown in minimal medium A without sodium citrate (20) with 0.5% glycerol, 0.01% MgSO₄·7H₂O, and thiamine (2.5 μg/ml). For plates, 1.5% agar was used. The antibiotics used were 100 μg/ml ampicillin and/or 50 μg/ml kanamycin. To test the resistance to Li⁺ and Na⁺, EP432 cells transformed with the respective plasmids were grown on LBK to A₆₆₀ of 0.5. For growth on agar, samples (2 μl) of serial 10-fold dilutions of the cultures were spotted onto agar plates containing the routine selective media (modified LB medium containing 0.6 M NaCl at pH 7 or 8.3 or 0.1 M LiCl at pH 7) and incubated for 2 days. When indicated, more extreme stress conditions (0.1 M LiCl at pH 8.3 or 8.5) were used. For growth in liquid culture, the routine selective medium was as above, and when indicated, more extreme stress conditions were used (0.7 or 0.8 M at pH 8.4).

**Plasmids**—Plasmid pGM36 encodes NhaA (21). pCL-GM100, a derivative of pGM36 encodes Cys-less NhaA (CL-NhaA) (1, 22). pECO, a derivative of pGM100 (with an EcoRI site at position 5319) encodes NhaA (23). Plasmid pAXH (previously called pYG10), a pET20b (Novagen) derivative encodes His-tagged NhaA (22). pCL-AXH, a derivative of pAXH encodes a His-tagged CL-NhaA (22). pCL-AXH2, a derivative of pCL-AXH lacks BglII site at position 3382 (24). pCL-AXH3, a derivative of pCL-AXH2 contains BstXI silent site at codon 248 in nhaA. pCL-HAH4, a derivative of pCL-HAH3, bearing the silent BstXI mutation at codon 248 in nhaA. It expresses from the tac promoter a variant of CL-NhaA of which segment Arg³⁸⁶–Val³⁸⁸ was replaced by hemagglutinin epitope followed by two factor Xa cleavage site and a His₉ tag (23). All of the plasmids carrying mutations are designated by the name of the plasmid followed by the mutation.

**Site-directed Mutagenesis**—Site-directed mutagenesis was conducted following a polymerase chain reaction-based protocol (25) or DpnI-mediated site-directed mutagenesis (26). For Cys replacement of P45C, V46C, Q47C, L48C, R49C, V50C, G51C, L53C, I55C, N56C, K57C, and N58C, pCL-AXH3 was used as a template. For Cys replacement of E54C, pCL-HAH4 was used as a template. Mutant E54C on pCL-AXH3 background was obtained by ligating the EcoRI-BglII fragment (465 bp) with EcoRI-BglII fragment (4.3 kb) of the plasmid pCL-AXH3. To generate the triple mutant Q47A/L48A/R49A pAXH3 was used as a template. Mutant S52C was described previously in Ref. 12. Mutants P247C, A248C, and L255C were described previously in Ref. 27. The double mutant Δ(P45-N58)-L255C was obtained on a pCL-AXH3 background by ligating the BgIII-Mul fragment (682 bp) with BgIII-Mul fragment (4.08 kb) of plasmid pCL-AXH3. All of the mutations were verified by DNA sequencing of the entire gene, through the ligation junction with the vector plasmid.

**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, 29). 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 (or choline chloride), 50 mM 1,3-bis-(tris(hydroxymethyl)-methylamino) propane, and 5 mM MgCl₂, and the pH was titrated with HCl. After energization with either ATP (2 mM) or D-lactate (2 mM), quenching of the fluorescence was allowed to achieve a steady state, and then either Na⁺ or Li⁺ was added. A reversal of the fluorescence level (dequenching) indicates that protons are exiting the vesicles in antiport with either Na⁺ or Li⁺. As shown previously, the end level of dequenching is a good estimate of the antiporter activity (30), and the concentration of the ion that gives half-maximal dequenching is a good estimate of the apparent Kₘ of the antiporter (30, 31). The concentration range of the cations tested was 0.01–100 mM at the indicated pHs, and the apparent Kₘ values were calculated by linear regression of a Lineweaver-Burk plot.

**Detection and Quantifying NhaA and Its Mutated Derivatives in the Membrane**—Total membrane protein was determined according to Ref. 32. The expression level of His-tagged NhaA mutants was determined by NhaA purification (22) and resolving the Ni²⁺–nitrotriacetic acid affinity-purified proteins on SDS-PAGE, staining the gels with Coomassie Blue, and quantification of the band densities by Image Gauge (Fuji) software (22).

**In Situ Site-directed Intermolecular Cross-linking**—Site-directed intermolecular cross-linking was conducted in situ on high pressure membrane vesicles isolated from TA16 cells overexpressing the various NhaA mutants (9). Membranes (300 μg of membrane protein) were resuspended in a buffer (0.5 ml) containing 100 mM potassium phosphate (pH 7).
RESULTS

Mutations of Residues in the β-Hairpin of Loop I–II, Expression in the Membrane and Growth Phenotypes—Based on the NhaA crystal structure (11), electron spin resonance study (13), cross-linking data (12), and the two-dimensional density map of NhaA (15, 16), a high resolution model of NhaA dimer was constructed (14). According to this model, β-hairpins of two NhaA monomers form a β-sheet that participates in the dimer interface. To identify specifically which residues in the β-hairpin contribute to the dimer interface in the native membrane, we constructed Cys replacement mutations of all the amino acid residues in the β-hairpin (Pro45–Asn59) (Fig. 1A). The mutants were constructed in Cys-less NhaA (CL-NhaA), a variant that is as expressed and active as the native NhaA (22). We also constructed a triple mutant Q47A/L48A/R49A on a wild type background.

To characterize the mutations with respect to expression, growth, and antiporter activity, the mutated plasmids were transformed into EP432, an E. coli strain that lacks the two Na⁺ specific antiporters (NhaA and NhaB). This strain neither grows on the routine selective medium (0.6 mM NaCl at pH 7 or pH 8.3 or 0.1 mM LiCl at pH 7) nor exhibits any Na⁺/H⁺ antiporter activity in isolated everted membrane vesicles, unless transformed with a plasmid encoding an active antiporter (reviewed in Refs. 1 and 2).

Whereas mutant S52C was expressed only 10% of the control level (Table 1), all other mutants were expressed to above 34%, as compared with the level of expression of the wild type (100%). Notably, because all mutants are expressed from multicopy plasmids, the expression level of the mutants is way above the level expressed from a single chromosomal gene (34). Whereas the Cys replacement mutants, S52C and N58C grew on LBK agar plates similar to the wild type control, their growth was less efficient on the routine Na⁺ selective medium (Table 1 and data not shown). The variants Q47C and L48C grew both on LBK and on the routine Na⁺ selective media less efficiently than the wild type (Fig. 2A and Table 1). On the Li⁺ routine selective plates, only the growth of S52C was impaired, most likely because of its low expression (Table 1). The rest of the Cys replacement mutants grew similar to the wild type on the routine selective media (Table 1). The triple mutant Q47A/L48A/R49A grew on LBK agar plates (Fig. 2A) and on the routine Na⁺ selective plates very similar to the wild type (Table 1).

The Effect of Cys Replacement Mutations in the β-Hairpin on the Na⁺/H⁺ Antiporter Activity and Their Effect on the Apparent $K_m$—The Na⁺/H⁺ antipporter activity was measured in everted membrane vesicles isolated from EP432 transformed with the plasmids encoding the various Cys replacement mutations. The activity was estimated from the change caused by Na⁺ or Li⁺ to the ΔpH maintained across the membrane, as measured by acridine orange, a fluorescent probe of ΔpH. EP432 transformed with plasmid pCL-AXH3 encoding CL-NhaA or the vector plasmid, pBR322, served as positive and negative controls, respectively (Fig. 3A and Table 1). The apparent $K_m$ values for Na⁺ and Li⁺ at pH 8.5 and the extent of activity at pH 8.5 (maximal dequenching) (Table 1) as well as...
the pH dependence of the Na\(^+/\)H\(^+\) or Li\(^+/\)H\(^+\) antiporter activity were determined for each mutant (Fig. 3, B and C).

Mutations S52C and K57C increased by ~4-fold the apparent \(K_m\) of the antiporter to Na\(^+\) (Table 1). The other mutants did not have any effect on the apparent \(K_m\) for either Na\(^+\) or Li\(^+\) (Table 1). Notably, mutant S52C was the only mutant that showed very low antiport activity in the presence of Na\(^+\) or Li\(^+\), most probably because of its low expression (Table 1).

We have previously defined two types of mutants of NhaA (24, 35). When measured at saturating concentrations of Na\(^+\), the first type of mutations, have a pH dependence very similar to that of the wild type. In contrast, the second type of mutations retain an abnormal pH dependence of the Na\(^+/\)H\(^+\) exchange activity, even at saturating concentrations of the ion.

We therefore measured the effect of Na\(^+\) concentrations above saturation (up to 100 mM) on the maximal extent of dequenching of the mutants between pH 6.5 and 8.5. Mutant K57C has an acidic shift of ~1 pH unit in the pH profile of the Na\(^+/\)H\(^+\) antiporter activity (Fig. 3, A and B), whereas at pH 6.5 and 7, the wild type antiporter is not active or hardly active (10% dequenching) in Na\(^+/\)H\(^+\) exchange, respectively, K57C shows 30 or 50% activity in the respective pH values. All of the other mutants had a pH profile of Na\(^+/\)H\(^+\) exchange very similar to that of the wild type (Fig. 3B shows P45C as an example). Many mutants (P45C, V46C, Q47C, L48C, G51C, L53C, E54C, I55C, and K57C) had a slight but significant acidic shift in their pH profile of the Li\(^+/\)H\(^+\) antiporter activity (Fig. 3C shows mutants G51C as an example). The other variants had a pH profile similar to the wild type (Fig. 3C shows R49C as an example).

**In Situ Intermolecular Cross-linking between β-Hairpins**

Given that two β-hairpins of NhaA form the β-sheet comprising the NhaA dimer interface at the periplasmic side of the membrane, a single Cys replacement in the β-hairpin of one monomer might be able to perform intermolecular cross-linking with its twin in the β-hairpin of the other monomer. Therefore, the Cys replacements in the β-hairpin were scanned in the membrane for intermolecular cross-linking.
membranes were exposed to pH 7.5, to homo-bifunctional, thiol-specific, cross-linking reagents: BMH, o-PDM, and MTS-2-MTS, spanning different distances (~15, 10, and 5.2 Å maximal distances, respectively) (36). The proteins were affinity-purified on Ni\(^{2+}\)-NTA columns, resolved on SDS-PAGE, and Coomassie blue-stained. Mutants that cross-linked had a gel mobility of dimers, whereas the non-cross-linked mutants appeared as monomers. After normalization for the amount of the protein in the bands, dimerization levels were estimated from the bands densities (100% = the sum of the bands densities of the monomers and the dimers for each mutant). Untreated membranes served as a negative control to assess the level of spontaneous dimerization (Fig. 4A, lanes \(a\)), which in most cases was very low (≤10%).

To rule out the possibility that the mutants that did not cross-link were not accessible to the cross-linker, we used fluorescein-5-maleimide to titrate the Cys left free following cross-linking, as described (24). All of the mutants showed very low fluorescein-5-maleimide fluorescence following treatment with the cross-linking reagents (data not shown), implying that these Cys replacements were accessible to all cross-linkers. Therefore, positive or negative cross-linking implies that the distance between the twin Cys replacements is either equal/smaller or larger than the distance spanned by the cross-linker, respectively. Nevertheless, it should be noted that cross-linking is dependent on dynamic collisions, chemical reactivity, and stereochemistry of the residues and not only on their proximities.

The results summarized in Fig. 4 demonstrate that mutants Q47C and R49C perform the strongest intermolecular cross-linking (95–98%) with each of the cross-linking reagents. Cys replacement L48C showed very poor cross-linking with BMH but 90% dimerization in the presence of o-PDM and 80% dimerization with MTS-2-MTS. E54C and N56C preformed intermolecular cross-linking with the longest cross-linker BMH (90 and 65%, respectively). The other Cys replacements in the β-hairpin did not show intermolecular cross-linking above the spontaneous level (Fig. 4A shows P45C as an example).

**The Role of the β-Hairpin in Maintaining NhaA Dimers**—Recently, we have shown that whereas wild type NhaA has a mobility of a dimer in BN-PAGE (Ref. 18 and Fig. 5B, lane \(a\)), the mutant deleted of the β-hairpin, Δ(P45-N58), has mainly a mobility of a monomer (Ref. 18 and Fig. 5A, lane \(b\)). CL-NhaA, similar to wild type NhaA, is resolved on BN-PAGE as a dimer (Fig. 5A, lane \(a\)). Remarkably, whereas mutants that did not cross-link had mainly a mobility of a dimer similar to the wild type (Fig. 5A, lane \(c\), K57C, and data not shown), the mutant that cross-linked showed a mixed mobility; mutant Q47C showed a low level (20%) of monomers, and mutants L48C and R49C showed a higher level of monomers (40 and 50%, respectively) in addition to the dimeric form. These results suggest that amino acids Gln\(^{37}\), Leu\(^{48}\), and Arg\(^{49}\) in the β-hairpin are important for maintaining the NhaA dimers in DDM, and the latter two are most critical.

**The Effect of Chemical Modification by MTSES on the Mobility of Mutants Q47C, L48C, and R49C on BN-PAGE**—It could be argued that the mixed monomeric and dimeric mobility on BN-PAGE observed in mutants Q47C, L48C, and R49C is due to the SH groups that can undergo spontaneous cross-linking under oxidizing conditions. To test whether introduction of a negative charge into the interface will affect the mobility on BN-PAGE, we modified chemically the Cys replacements by MTSES, a negatively charged SH reagent. High pressure membranes were incubated with or without MTSES at pH 7.5, and

**TABLE 1**

**Mutations in loop I-II of NhaA**

For characterization of the mutations, EP432 cells transformed with the plasmids carrying the indicated mutations were used. The expression level was expressed as a percentage of the control cells (EP432/pCL-AXH3). Growth experiments were conducted on agar plates with high Na\(^+\) (0.6 M) or high Li\(^+\) (0.1 M) at the pH values indicated. +++, control: number and size of colonies after 48 h of incubation at 37 °C is similar to that of wild type on LBK; +, number of colonies after 48 h of incubation at 37 °C at alkaline pH is similar to that of the control but the colony size was smaller; +, both size and number (1 order of magnitude) smaller than the control; −, no growth. Na\(^+\)/H\(^+\) and Li\(^+\)/H\(^+\) antiporter activity at pH 8.5 was determined with 10 mM NaCl or LiCl. The activity (level of dequenching) is expressed as a percentage of the positive control (EP432/pCL-AXH3). EP432/pBR322 served as a negative control. The apparent \(K_m\) for the ions was determined at pH 8.5, as described under "Experimental Procedures."

| Mutation | Expression | Growth | Activity | Apparent \(K_m\) |
|----------|------------|--------|----------|-----------------|
|          |            | 0.6 M Na\(^+\) | 0.1 M Li\(^+\) | Na\(^+\) | Li\(^+\) |
|          |            | pH 7    | pH 8.3   | pH 7    | mm |
| Loop I-II|            |         |          |         |     |
| P45C     | 155        | ++++    | ++       | ++++    | 67  | 83  | 0.11 | 0.01 |
| V46C     | 106        | ++++    | +        | ++++    | 71  | 70  | 0.43 | 0.08 |
| Q47C     | 34         | ++++    | +        | ++++    | 81  | 79  | 0.30 | 0.05 |
| L48C     | 79         | ++++    | +        | ++++    | 68  | 71  | 0.14 | 0.03 |
| R49C     | 132        | ++++    | +        | ++++    | 61  | 100 | 0.51 | 0.04 |
| V50C     | 96         | ++++    | +        | ++++    | 95  | 93  | 0.14 | 0.01 |
| G51C     | 122        | ++++    | +        | ++++    | 100 | 94  | 0.17 | 0.02 |
| S52C     | 10         | ++++    | +        | ++++    | 30  | 29  | 0.80 | 0.11 |
| L53C     | 178        | ++++    | +        | ++++    | 93  | 93  | 0.10 | 0.04 |
| E54C     | 109        | ++++    | +        | ++++    | 85  | 90  | 0.24 | 0.03 |
| I55C     | 188        | ++++    | +        | ++++    | 86  | 67  | 0.18 | 0.02 |
| N56C     | 207        | ++++    | +        | ++++    | 72  | 78  | 0.13 | 0.02 |
| K57C     | 190        | ++++    | +        | ++++    | 82  | 100 | 0.07 | 0.02 |
| N58C     | 81         | ++++    | +        | ++++    | 81  | 77  | 0.14 | 0.03 |
| Q47A-L48A-R49A | ND       | ++++    | +        | ND      | ND  | ND  | ND  | ND  |

Controls

|         |            |         |          |         |     |
|---------|------------|--------|----------|---------|
| pCL-AXH3| 100        | ++++    | +        | +++     | 88  | 75  | 0.19 | 0.07 |
| pBR322  |            | −       | −        | −       | −    | −   | −    | −    |
NhaA Dimers Are Crucial for Stability

**A.**

![Figure 2](image1)

**B.**

![Figure 2](image2)

**FIGURE 2.** Growth characteristics of wild type and mutants Q47C, L48C, R49C, and Q47A/L48A/R49A under extreme stress conditions with respect to concentration of Li⁺, Na⁺, and pH. Cells of EP432 expressing the different NhaA variants were grown on LBK (A₀₀₀ of 0.5), A, samples (2 μl) of serial 10-fold dilutions of the cultures were spotted onto agar plates containing LBK (left panel) or LBK containing 0.1 M LiCl at pH 8.5 or 8.3 (right panel top or bottom, respectively) and incubated for 2 days. Similar results were obtained in three independent experiments. B, growth in liquid medium. The cells of NhaA and Q47C/L48C/R49C were grown on LBK (A₀₀₀ of 1), diluted 10-fold into LB containing different NaCl concentrations at different pH values obtained with 60 mM Bis-Tris-Propane and incubated under growth conditions. ● LBK, ○ 0.6 M NaCl at pH 7; ▲ 0.7 M NaCl at pH 8.4; ▣ 0.8 M NaCl at pH 8.4. Open and closed symbols represent the triple mutant Q47C/L48C/R49C and wild type strains, respectively. The standard deviation was between 5 and 10%.

then the affinity-purified proteins were resolved on BN-PAGE. This treatment did not affect the mobility of mutants Q47C and R49C (data not shown) but increased the fraction (2-fold) of L48C monomers that resolved on BN-PAGE (Fig. 5A, compare lanes f and g).

These results led us to construct the triple Ala replacement mutant Q47A/L48A/R49A. Ala instead of Cys replacement avoids dimerization caused by SH groups. Unfortunately, it was impossible to resolve mutant Q47A/L48A/R49A on BN-PAGE because of its instability when purified from the membrane (data not shown).

As described previously, residues in the N terminus of TMS IX and in loop VIII-IX (Ser²⁴⁶, Pro²⁴⁷, Ala²⁴⁸, Val²⁵⁴, and Leu²⁵⁵) form the interface of the NhaA dimer at the cytoplasmic side of the membrane (27). To test whether these residues are important in maintaining the NhaA dimer, we studied the mobility of Cys replacements P247C, A248C, and L255C on BN-PAGE (Fig. 5B). These mutants showed a mobility of a dimer similar to the wild type (Fig. 5B, lanes c, e, and f). In contrast, similar to the deletion mutant Δ(P45-N58) (Fig. 5B, lane b), a double mutant containing both the β-hairpin deletion and L255C (Δ(P45-N58)-L255C) showed a mobility of a monomer (Fig. 5B, lane d). These results suggest that single Cys replacements of amino acid residues in TMS IX and loop VIII-IX that are located at or in very close proximity to the NhaA dimer interface at the cytoplasmic side of the membrane do not impair the NhaA dimer. Hence, the β-hairpin at the periplasmic side of the membrane is the dominant structural element that holds the NhaA dimer.

**Growth Phenotype of Mutants Q47C, L48C, R49C, and Q47A/L48A/R49A under Extreme Stress Conditions**—We have previously shown that under extreme conditions of growth that combine alkaline pH (8.5) with either LiCl (0.1 M) or NaCl (0.7 M) (18), the dimeric wild type NhaA was more efficient than the monomeric mutant Δ(P45-N58) (18). Although these results were consistent with the notion that dimeric NhaA is more beneficial than monomeric NhaA under extreme stress conditions, it was impossible to exclude the possibility that a large deletion had an effect in addition to causing monomerization of NhaA. We therefore, tested the growth on agar of the single Cys replacement mutants, Q47C, L48C, R49C, and Q47A/L48A/R49A, in EP432 strain under the extreme stress conditions (Fig. 2A). A plasmid encoding CL-NhaA or NhaA served as a positive control (Fig. 2A, left panels), and the vector plasmid served as a negative control (data not shown). Whereas variant R49C grew on LBK very similar to the wild type (Fig. 2A, left top panel) the growth of variants Q47C and L48C on LBK was reduced by ~1 order of magnitude. Under the extreme stress conditions pertaining to Li⁺ (Fig. 2A, top right panel), whereas the wild type was hardly affected, the growth of mutants Q47C, L48C, and R49C was drastically impaired. Reductions of 3 and 2 orders of magnitude in the growth resistance were observed with L48C and R49C, respectively, and a reduction of 1 order of magnitude was observed for Q47C (Fig. 2A, top right panel). Hence, mutants L48C and R49C are most sensitive to the extreme stress condi-
To test whether the effect of the extreme stress conditions on the NhaA variant is reversible, mutants, Q47C, R49C, Δ(P45-N58), Q74A/L48A/R49A, and the wild type control were grown under extreme stress conditions (0.7 m NaCl, pH 8.4) for 6 h as described in Fig. 2B, and the growth of all mutants was practically arrested (data not shown). Then the cells were washed, resuspended in LBK, and further incubated for 8 h (Fig. 6A). The protein expression level of all variants was determined before and after application of the extreme stress conditions (Fig. 6B). The protein levels of mutants Q47C, R49C, and Δ(P45-N58), before and after the extreme stress, were very similar to that of the wild type control. The protein level of mutant Q74A/L48A/R49A was 58% of the control before the extreme stress and retained 40% during the extreme stress conditions. Hence, the arrest of growth under the extreme stress conditions cannot be ascribed to degradation of the NhaA variants.

Indeed, all strains resumed growth after restoring normal growth conditions in LBK (Fig. 6A), the first three mutants and the control with a lag of 1 h and Q74A/L48A/R49A with a 3-h lag that can be accounted for by its lower protein concentration. It is concluded that the harm caused to the NhaA mutants during growth under the extreme stress conditions is reversible.

The Effect of Temperature on the Stability of the NhaA Dimers in DDM Micells—The reason for the more resistant wild type over the β-hairpin mutants and the deletion mutant with respect to growth under extreme stress conditions may be due to higher stability of the NhaA dimers as compared with the monomers. To test whether NhaA dimers are more stable compared with monomers with respect to temperature, we used Ni²⁺-NTA affinity-purified proteins of the NhaA variants and incubated them at 4 °C or 55 °C for the indicated times and then separated them on SDS-PAGE. The controls CL-NhaA and NhaA were similarly very stable at 4 °C (Fig. 7, A and C, and supplemental Table S2). Notably, NhaA was slightly more stable than CL-NhaA at 55 °C (supplemental Table S2 and compare Fig. 7, A and C). Whereas NhaA has not shown signs of aggregation even after 60 min of incubation at 55 °C, CL-NhaA was slightly aggregated after 30 min of incubation and significantly aggregated following 60 min of incubation at 55 °C. These results revealed that the native cysteines contribute to the stability of NhaA at high temperature.

The Cys replacement variants and Δ(P45-N58) were stable similar to the controls at 4 °C (Fig. 7, A and B, and supplemental Table S2). However, whereas CL-NhaA and Q47C aggregated significantly only after 60 min of incubation at 55 °C, Δ(P45-N58) aggregates already after 30 min of incubation at 55 °C (Fig. 7A and supplemental Fig. S2), and mutants L48C and R49C, although less than Δ(P45-N58), were already aggregated significantly after 30 min of incubation at 55 °C, and aggregation increased following 60 min of incubation (Fig. 7B and supplemental Fig. S2). Remarkably, the variant Q47L/L48A/R49A showed marked aggregation already at 4 °C (Fig. 7C and supplemental Fig. S2), and incubation at 55 °C completed the aggregation. These results show that the dimer is important for the temperature stability of the protein in DDM micelles; both monomeric mutants impaired in the β-hairpin either by a deletion of the β-hairpin or by mutations in the critical interface of
The Apparent $K_m$ of Monomeric NhaA Variant Increased Dramatically during Growth under Extreme Stress Conditions—

The instability of the monomeric NhaA variants in DDM at increasing temperatures does not necessarily imply instability in the membrane and therefore cannot simply account for the impaired growth of these variants under extreme stress conditions. Furthermore, these growth conditions had no effect on the amount of the proteins in the membrane (Ref. 18, Fig. 6B, and data not shown). We therefore isolated membrane vesicles from cells expressing the NhaA variants grown under the extreme stress conditions and tested their $\text{Na}^+ / \text{H}^+$ antiporter activity. The results summarized in Table 2 show that whereas the apparent $K_m$ of the wild type did not change following 12 h of growth under the extreme stress conditions, the apparent $K_m$ of $\Delta$(P45-N58) was dramatically increased (up to 50-fold).

We have previously shown that NhaA changes its conformation with pH, which can be probed by trypsin digestion, a fluorescent probe attached to E252C, or monoclonal antibody (3). Thus, NhaA is resistant to trypsinolysis below pH 6.5, and between pH 6.5 and 8.5 a unique site (at Lys$^{34}$) becomes progressively sensitive to pH, reflecting the pH-induced activity of NhaA. Interestingly, the pH profile of trypsin digestion of purified NhaA variants following growth under the extreme stress was very similar to that of the wild type (data not shown).

**DISCUSSION**

Recently, measuring the distance distribution between spin labels by pulsed electron paramagnetic resonance (DEER, double electron-electron resonance), a structural model of the physiological NhaA dimer was obtained (14). The model supported by the crystal structure (17) and cross-linking data (12) revealed two points of contacts between the two NhaA monomers; one, at the cytoplasmic side of the membrane, is formed by few hydrophobic interactions between residues of helices VII and IX, and the other, at the periplasmic side of the membrane, is produced by a $\beta$-sheet formed by joined two $\beta$-hairpins, one per each monomer.

In a recent study (27), we used scanning Cys replacements for intermolecular cross-linking and identified amino acid residues that participate in the NhaA dimer interface at the cytoplasmic side of the membrane: (i) Residues Ser$^{246}$ and Pro$^{247}$ at the junction between TMS IX and loop VIII-IX and (ii) L255 inside TMS IX. As predicted by the electron spin resonance based model of the NhaA dimer (14), the residues that cross-link efficiently cluster on one face of TMS IX,
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which is directed away from the cytoplasmic funnel toward the other monomer exactly above the β-sheet (Ref. 18, Fig. 1B, and supplemental Text S1, Table S1, Fig. S1).

Herein, we show that mutants in the cytoplasmic end of TMS IX resolved on BN-PAGE as dimers similar to the wild type dimers (Fig. 5B). Hence, the NhaA dimer is not impaired by single Cys replacements of the residues forming the contacts at the cytoplasmic side of the membrane. These results imply that either multiple mutations in many contacts are needed at this location to affect NhaA dimerization or that the main structural feature maintaining and/or forming the NhaA dimer is the β-sheet at the periplasmic side of the membrane.

The β-sheet is formed (14) by four hydrogen bonds between residues Gln47 and Arg49 and backbone groups of the respective β-hairpin. Remarkably, our results strongly support the latter predictions and also revealed that these are the most important contacts forming and/or maintaining the NhaA dimer. Thus, the two mutants Q47C and R49C performed very strong cross-linking with all three reagents (90–100% dimerization) (Fig. 4). For more details see supplemental Text S1, Table S1, and Fig. S1. Furthermore, in contrast to the wild type NhaA, which had a mobility of a dimer on BN-PAGE, the mutants resolved as two bands, one of monomeric and the other of dimeric mobility (Fig. 5A). This result reveals that a single Cys replacement of either Q47C or R49C destabilizes the dimer.

β-Sheets formed by joint β-hairpins are a classical motif of oligomer formation in soluble proteins (37–39). Our results present the first example of a polytopic membrane protein in which a β-hairpin of two monomers form a β-sheet that is essential for dimerization. Interestingly, the NhaA crystal structure shows that the β-sheet is located parallel to the membrane in the periplasmic space outside the membrane (11). Therefore, our results have revealed how an oligomerization motif of soluble proteins is recruited to form a dimer of a membrane protein.

As many other membrane proteins, many secondary transporters exist in the native membrane in a variety of oligomeric states (3, 40) that tend to be conserved in a given family or subfamily (40). In most cases, the reason for oligomerization of secondary transporters is unknown (40). From a structural point of view, volume exclusion, protein localization, and orientation in the two-dimensional space of the membrane enhance the likelihood of self-association of membrane proteins into oligomers. Multimerization of membrane proteins provides possibilities for scaffolding, interfaces, and allostery. Only in a very few cases, it has been suggested (41) or shown (42) that monomers cooperate with one another during transport, implying that the oligomeric state is essential for function.

On the basis of positive complementation (12), chemical modification, cross-linking (27), and electron spin resonance study (13), it has been argued that NhaA is a functional dimer, and therefore the integrity of the NhaA dimer is important for the activity of NhaA and/or its regulation by pH and that chemical modification at the NhaA dimer interface disturbs the native conformation of the dimer and causes harmful defects to NhaA (27). Whereas these results are consistent with a role of the dimers in pH regulation and/or activity of NhaA, they do not necessarily mean that the dimeric state is essential for activity and/or pH regulation of the antiporter. Recently, by deleting the β-hairpin at the periplasmic side of NhaA, we revealed that under the routinely used stress conditions, the functional unit of NhaA is a monomer (18). We therefore suggest that mutants of amino acid residues in the dimer interface may show positive genetic complementation and be affected by chemical modification and/or cross-linking not because the dimer is impaired but rather because they participate in additional roles important for NhaA activity and/or pH regulation (27).

Here, we found a novel example of a residue in the dimer interface that has a functional effect. Most Cys replacements in the β-hairpin had very small effect on the antiporter activity or its regulation by pH (Table 1 and Fig. 3). In mark contrast, Cys replacement K57C shifted the pH profile of the antiporter by −1 pH unit to the acidic side (Fig. 3B). In a crystal structure-based computation (multiconformation continuum electrostatic analysis), we have previously shown that Lys57 forms a charge pair with Asp65 of TMS II (43). Furthermore, we have recently found that Cys replacement D65C affects dramatically the antiporter activity by increasing more than 10-fold the apparent $K_m$ and that the negative charge at position 65 is crit-
ical for this effect. We therefore suggest that the charge pair Lys⁵⁷–Asp⁶⁵ is important for the pH-induced conformational change of NhaA.

As suggested previously (18), the integrity of the NhaA dimers are most important for growth under extreme stress conditions, a combination of high Li⁺ (0.1 M) or Na⁺ (0.6–0.8 M) concentrations at alkaline pH (pH 8.3–8.5) (Fig. 2). Thus, single Cys replacements that impair the dimer integrity (Q47C and R49C) as revealed on BN-PAGE (Fig. 5) are much less efficient in growth under the Li⁺ extreme stress conditions compared with the wild type (Fig. 2). The triple alanine replacement Q47A/L48A/R49A could not grow under either Li⁺ or Na⁺ extreme stress conditions (Fig. 2).

Remarkably, the harmful effect on growth of the extreme stress conditions was fully reversible; exposure to 6 h of extreme stress (0.7 M NaCl, pH 8.4) arrested growth of all mutants but

of Table 2. Apparent $K_m$ values of NhaA and Δ(P45-N58) after growth under stress conditions

| Time (h) | NhaA | Δ(P45-N58) |
|---------|------|------------|
| 0       | 0.22 | 0.43       |
| 4       | 0.16 | 0.45       |
| 8       | 0.15 | 1.6        |
| 12      | 0.09 | 5.3        |

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growth resumed within 1 h (Q47C, R49C, and Δ(P45-N58)) or 3 h (Δ(P45-N58)) following transfer back to growth in LBK (Fig. 6A). Accordingly, as compared with the wild type control, the protein levels of the first three NhaA variants were hardly affected, and the protein level of Q47A/L48A/R49A was reduced to only 40% by the extreme stress. Taken together, these results imply that breakdown of the NhaA variants cannot account for the growth arrest induced by the extreme stress on the antiporter mutants.

We further revealed that dimerization of NhaA is crucial for temperature stability of the antiporter. When purified in DDM micelles, the dimeric NhaA can withstand temperature of 55 °C up to 60 min. In mark contrast, the deletion mutant Δ(P45-N58) aggregates very strongly, and the single Cys replacement L48C and R49C aggregate very significantly already after 30 min at 55 °C (Fig. 7, A and B, and supplemental Table S2). Because these results were obtained with purified NhaA variants in DDM micelles, they could not account simply for the impaired growth phenotype under the extreme growth conditions. Remarkably, we found a drastic increase (up to 50-fold) in the apparent $K_m$ of the antiporter for Na$^+$ in everted membrane vesicles isolated from cells grown under the extreme stress conditions (Table 2). The $K_m$ increase cannot be ascribed to either a change in dimer/nomeron ratio or degradation of the protein because Δ(P45-N58) is, from the start, monomeric (95–98%) and is not degraded during growth under the extreme stress condition (Ref. 18 and Fig. 6B). Notably, it is remarkable that the effect of the extreme stress is completely reversible (Fig. 6).

Our results highly suggest that dimerization of NhaA is most important for the stability of the antiporter in the membrane under extreme stress conditions. As yet, we do not know the reason why the dimers are more stable than the monomers under extreme stress conditions. One possibility is the many negatively charged residues at the dimer interface (14, 27), which in the dimer are occluded between the monomers. Under the extreme stress conditions, these residues may be exposed to the high pH and Na$^+$ in the bulk phase, a situation that may induce a harmful but reversible conformational change that impairs the stability of the monomer.

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