Monomers of the NhaA Na⁺/H⁺ Antiporter of Escherichia coli Are Fully Functional yet Dimers Are Beneficial under Extreme Stress Conditions at Alkaline pH in the Presence of Na⁺ or Li⁺

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NhaA, the Na⁺/H⁺ antiporter of Escherichia coli, exists in the native membrane as a homodimer of which two monomers have been suggested to be attached by a β-hairpin at the periplasmic side of the membrane. Constructing a mutant deleted of the β-hairpin, NhaA/(Pro45-Asn58), revealed that in contrast to the dimeric mobility of native NhaA, the mutant has the mobility of a monomer in a blue native gel. Intermolecular cross-linking that monitors dimers showed that the mutant exists only as monomers in the native membrane, proteoliposomes, and when purified in β-dodecyl maltoside micelles. Furthermore, pull-down experiments revealed that, whereas as expected for a dimer, hemagglutinin-tagged wild-type NhaA co-purified with His-tagged NhaA on a Ni²⁺-NTA affinity column, a similar version of the mutant did not. Remarkably, under routine stress conditions (0.1 M LiCl, pH 7 or 0.6 M NaCl, pH 8.3), the monomeric form of NhaA is fully functional. It conferred salt resistance to NhaA- and NhaB-deleted cells, and whether in isolated membrane vesicles or reconstituted into proteoliposomes exhibited Na⁺/H⁺ antiporter activity and pH regulation very similar to wild-type dimers. Remarkably, under extreme stress conditions (0.1 M LiCl or 0.7 M NaCl at pH 8.5), the dimeric native NhaA was much more efficient than the monomeric mutant in conferring extreme stress resistance.

Homeostasis of Na⁺ and H⁺ is crucial for survival of all living cells. Sodium/proton antiporters have a major role in maintaining and regulating the cytosolic pH and Na⁺ concentration in prokaryotes (1, 2), plants (3), and animals (4–7). The main Na⁺/H⁺ antiporter in the cytoplasmic membrane of Escherichia coli, NhaA, the family prototype, is widely spread in enterobacteria and has orthologs throughout the biological kingdom including humans (2, 8).

NhaA is a 42-kDa integral membrane protein. It enables E. coli to survive at alkaline pH in the presence of Na⁺ using the respiration-dependent proton gradient to maintain a constant intracellular pH of 7.5 and extrude Na⁺ from the cytoplasm (reviewed in Ref. 9). It is an electrogenic antiporter with a stoichiometry of 2H⁺/Na⁺ (10). Similar to many other both prokaryotic (9) and eukaryotic (4–6) Na⁺/H⁺ antiporters, NhaA is strongly dependent on pH. Its rate of activity changes over three orders of magnitude between pH 7.0 and 8.5 (1, 9, 11). This pH activation, underpinning the mechanism of pH homeostasis, is accompanied by conformational changes (12–14).

The recently determined three-dimensional crystal structure of the acid pH down-regulated NhaA (15) reveals a novel fold, providing insights into the relationship between the structure and function of NhaA. A cytoplasmic funnel opens to the cytoplasm and ends in the middle of the membrane at the putative cation binding site. A cluster of negatively charged amino acids at the cytoplasmic orifice of the funnel have been assigned a role in the pH sensor, transmitting pH signals to regulate the activity of the antiporter.

Many transporters and channels exist in the native membrane as oligomers. In most cases, the functional/structural role of the oligomeric state is still an unknown (16). Several experimental approaches have strongly suggested that NhaA is a dimer in the native membrane; genetic complementation (17), biochemical pull-down experiments (17), intermolecular cross-linking (17), ESR² studies (18, 19), and cryo-electron microscopy of two-dimensional crystals (20, 21).

The dimeric state has been suggested to affect the pH response of NhaA. Intermolecular cross-linking between two NhaA monomers at the dimer interface changes the pH profile of the antiporter (17). Nevertheless, the question of whether the dimeric state of NhaA is essential for its structure and/or function in Na⁺/H⁺ exchange and/or pH regulation has remained elusive.

In the present work, we constructed a deletion mutant of NhaA that encodes monomeric NhaA. This mutant allowed, for the first time, a comparison between the functionality of monomeric and dimeric NhaA. The crystal structure of NhaA revealed a β-hairpin at the periplasmic face of the molecule.
(15). A recent comparison of the x-ray structure of NhaA monomers to NhaA dimers observed in two-dimensional crystals (22) predicted that the main contact between the monomers is built by two β-hairpins forming a 4-stranded β-sheet. In line with this prediction, strong intermolecular cross-linking in the native membrane was identified between two Cys replacements Ser359, each localized in one β-hairpin (17). ESR studies fully supported these results (18) and yielded a structural model of the native NhaA dimer (19).

We therefore constructed the NhaA mutant, NhaA/Δ(Pro45-Asn58), deleted of the β-hairpin. We found that the NhaA/Δ(Pro45-Asn58) protein exists exclusively in a monomeric form both in the native membrane, proteoliposomes, and in DDM micelles. Most importantly, even under routine stress conditions (0.1 M LiCl or 0.6 M NaCl at pH 7 and 0.6 M NaCl at pH 8.3), the monomeric mutant conferred growth resistance and exhibited pH-regulated Na+/H+ antiport activity very similar to the wild-type dimeric NhaA. Strikingly, under extreme stress conditions (pH 8.5 in the presence of 0.7 M NaCl or 0.1 M LiCl), monomeric NhaA/Δ(Pro45-Asn58) is much less efficient than wild-type dimeric NhaA in conferring growth resistance.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—EP432 is an *E. coli* K-12 derivative, which is melBlid, ΔnhaA1::kan, ΔnhaB1::cat, ΔlacZY, thr1 (23). TA16 is *nhaA*::nhaB::lacI2 (TAl5lacI2) and otherwise isogenic to EP432 (11). Cells were grown in either L broth (LB) or modified (24) L broth (LBK). Where indicated, the medium was buffered with 60 mM BTP. Cells were also grown in minimal medium A without sodium citrate (25) with 0.5% glycerol, 0.01% MgSO4 7H2O and thiamine (2.5 μg/ml). For plates, 1.5% agar was used. Antibiotics were 100 μg/ml ampicillin and/or 50 μg/ml kanamycin and/or 12.5 μg/ml tetracyclin. The resistance to Li+ on agar (0.1 M LiCl, pH 7) and Na+ (0.6 M NaCl at pH 7 or pH 8.3) was routinely tested as previously described (24). When indicated, more extreme stress conditions (0.1 M LiCl or 0.7 M NaCl at pH 8.5) were used for growth on agar or in liquid culture.

**Plasmids**—pAXH (previously called pYG10), a pET20b (Novagen) derivative (26), encodes His-tagged NhaA (if not otherwise stated, henceforth denoted NhaA). pCL-AXH, a pAXH derivative, encodes Cys-less NhaA (CL-NhaA (26)). pAXH2 and pCL-AXH2, derivatives of pAXH and pCL-AXH, respectively, lack a BglII site at position 3382 of the plasmid (27). pAXH3 and pCL-AXH3, derivatives of pAXH2 and pCL-AXH2, respectively, contain the BstXI silent site at position 248 of *nhaA* (28). The compatible plasmids, p100HA (a pBR322 derivative) and p184AXH (a pACYC184 derivative) encode differentially tagged NhaA at the C-terminal end; the former with the hemagglutinin epitope (NhaA-(HA)), the latter with a His tag (NhaA-(His)6 (17). Plasmids encoding mutation in NhaA are designated by the name of the plasmid followed by the mutation.

**Construction of Mutants**—Site-directed mutagenesis was conducted following a polymerase chain reaction-based protocol (29). For the Cys replacement mutant, CL-NhaA/S246C, the plasmid pCL-AXH3/S246C was constructed using pCL-AXH3 as a template.

To generate the deletion mutant NhaA/Δ(Pro45-Asn58), plasmid pAXH3/Δ(Pro45-Asn58) was constructed using pAXH3 as a template, and the DNA sequence encoding amino acid residues Pro45-Asn58 of *nhaA* was deleted. The mutagenic primers contained mutagenic bases (designated in bold) that introduced a unique restriction site (Xhol) into the mutated DNA as a silent mutation: CGACCTTCTCGAGCATGCTTTATGGAT-AAATGACGC and GCGTCATTATTCTCATACAGCATC-GTCTCGAGAAGTGGC. The end primers were: GTTGGTA-GGGTTAACACCTGCGG and CAACCTGCTTCTCCTTCCGG. The mutations were verified by DNA sequencing of the entire gene, through the ligation junction with the vector plasmid.

To generate the deletion mutant in a Cys-less background (NhaA-CL/Δ(Pro45-Asn58)), the BglII-MluI fragment of pCL-AXH3 (682 bp) was ligated with the BglII-MluI fragment (402 bp) of pAXH3/Δ(Pro45-Asn58).

To generate the mutant NhaA-CL/Δ(Pro45-Asn58)/S246C, the BglII-MluI fragment of pCL-AXH3/Δ(Pro45-Asn58) (402 bp) was ligated with the BglII-MluI fragment of pCL-AXH3/S246C (682 bp). To construct the (His)6 or HA-tagged versions of NhaA/Δ(Pro45-Asn58) mutant (plasmids p184AXH/Δ(Pro45-Asn58) and p100HA/Δ(Pro45-Asn58), respectively), an EcoRI-MunI fragment (576 bp) of p184AXH was replaced with the EcoRI-MunI fragment (534 bp) of pAXH3/Δ(Pro45-Asn58) or the Van91I-MunI fragment (468 bp) of p100HA was replaced with the Van91I-MunI fragment (426 bp) of pAXH3/Δ(Pro45-Asn58), respectively. Plasmids expressing the wild type and the NhaA/Δ(Pro45-Asn58) variants from the native promoter of *nhaA* were pGMAR100 (30) and pGMAR100/Δ(Pro45-Asn58), respectively.

**Overexpression and Affinity Purification of His-tagged Antiporters by Ni2+-NTA Chromatography**—To overexpress the plasmids encoding the His-tagged antiporters, TA16 transformed with the respective plasmids was used as described (26). Miniscale purification was performed basically as described (26, 31). Membranes (0.5–2 mg of membrane protein/ml) were extracted with 1% DDM, and the His-tagged NhaA was affinity-purified on a Ni2+-NTA agarose column (Qiagen, Hilden, Germany) and, if not otherwise stated, eluted by acid elution (25 mM potassium citrate, pH 4, 100 mM KCl, 5 mM MgCl2, and if not otherwise stated 0.03% DDM) and stored as described (22, 31). When indicated, the protein was precipitated in 10% trichloroacetic acid for 0.5 h at 4 °C, centrifuged (21,000 × g, 30 min, 4 °C), resuspended in sampling buffer, and loaded on the gel.

**Detection and Quantifying of NhaA and Its Mutated Derivatives in the Membrane**—NhaA and its mutated derivatives were quantified by Western analysis using an anti-NhaA monoclonal antibody (mAb176) (32) or anti-hemagglutinin mAb (Babco, Berkeley, CA), as indicated. The total membrane protein was determined according to Ref. 33. The expression level of His-tagged NhaA mutants was determined by resolving the Ni2+-NTA affinity-purified proteins on SDS-PAGE, staining the gels
by Coomassie Blue and quantifying the band densities (Image Gauge, Fuji).

SDS-PAGE was as described (26, 34). In cases where the protein was labeled with fluorescein-5-maleimide (Molecular Probes), the extent of the fluorescence labeling was estimated from photographed gels under UV light (260 nm) as described (35). The standard deviation was between 5 and 10%.

**Blue Native-PAGE**—Blue native gel electrophoresis was carried out as previously described (36). The main gel and the overlay were made of 10 or 4% polyacrylamide, respectively in 0.015% DDM. The sample buffer contained 50 mM NaCl, 1 mM EDTA, 50 mM imidazole/HCl (pH 7), 0.015% DDM, and 10% glycerol. The cathode buffer contained Coomassie Blue 0.02% (G250, Merck), and 0.015% DDM was added to both the anode and cathode buffers. The electrophoresis was conducted at 15 mA for 1 h. The gel was stained by Coomassie Blue or silver-stained, dried, and the band densities were determined as above.

**Site-directed Intermolecular Cross-linking**—Site-directed intermolecular cross-linking was conducted in situ on membrane vesicles or on NhaA proteoliposomes or on purified protein in detergent solution. Membranes were isolated from TA16 cells expressing the various NhaA mutants. They were resuspended (0.3 mg of protein) in a buffer (0.5 ml) containing 100 mM potassium phosphate, 5 mM MgSO4 (pH 7.4), and one of the freshly prepared homo-bifunctional cross-linkers: 2 mM BMH (Pierce), 1 mM o-PDM, (Sigma), or 2 mM MTS-2-MTS (Toronto Research Chemicals). The stock solutions of the cross-linkers were prepared in DMF at 200 mM, so that the amount of DMF in the reaction mixture did not exceed 1%, a concentration that did not affect the antiporter activity. The suspension was incubated at 26 °C with gentle rotation for 60 min. The reaction with BMH or o-PDM was terminated by the addition of 10 mM β-mercaptoethanol, and the reaction with MTS-2-MTS was terminated by dilution (15-fold) and centrifugation (Beckman, TLA 100.4, 265,000 × g for 20 min at 4 °C). Following extraction of the membranes with 1% DDM and centrifugation, the supernatants were added to 50 μl of Ni2+-NTA-agarose beads, and the protein was affinity-purified, resolved on SDS-PAGE (nonreducing conditions in the case of treatment with MTS-2-MTS). The gel was stained by Coomassie Blue, dried, and the band densities were determined as above. When intermolecular cross-linking takes place, a band, corresponding in mobility to that of the NhaA dimer, appears in SDS-PAGE (17, 35). Intermolecular cross-linking was conducted on NhaA-proteoliposomes (30 μl containing 2 μg of protein) practically as described above.

For intermolecular cross-linking of the protein in DDM micelles, the affinity-purified protein (from 2 mg of membrane protein), was eluted (2 ml) at pH 4 in the presence of the indicated DDM concentration and dialyzed twice for 1 h in 100 ml containing 5 mM MgSO4, 100 mM potassium phosphate (pH 7.4), and the indicated DDM concentration. The reaction mixture for cross-linking contained 0.5 ml of the dialyzed protein, the indicated DDM concentrations and the cross-linking proceeded and terminated as above. Then, the proteins precipitated by 10% trichloroacetic acid were resuspended in sampling buffer and resolved on SDS-PAGE. Each of the cross-linking experiments was repeated at least twice with practically identical results.

**Determination of the Accessibility to the Cross-linking Reagent**—When no cross-linking was observed, it was crucial to ascertain that the Cys replacement was accessible to the reagent. For this purpose, following cross-linking in the membranes, the beads with the affinity-purified protein were resuspended in 100 μl of binding buffer (in the presence of the indicated DDM concentrations), containing 0.2 mM fluorescein-5-maleimide (Molecular Probes) and further incubated for 30 min at 25 °C to determine the free Cys (35). The protein was eluted by incubation for 30 min at 4 °C in 20 μl of SDS-PAGE sampling buffer supplemented with 300 mM imidazole and centrifuged (Eppendorf, 20, 800 × g, 2 min, 4 °C). The affinity-purified protein was separated on SDS-PAGE. For evaluation of the fluorescence labeling, SDS-PAGE gels were photographed under UV light (260 nm) as described (35). Then, the gels were stained by Coomassie Blue to identify the bands with mobility corresponding to that of NhaA monomer and dimer (17, 35). The magnitude of fluorescence normalized per protein reflects inversely the accessibility to the cross-linking reagent. When the accessibility of the purified NhaA variant was tested in the presence of the indicated concentrations of DDM, following cross-linking, 0.2 mM fluorescein 5-maleimide was added to the reaction mixture, and incubation continued for 30 min as above. Then, the proteins were trichloroacetic acid-precipitated and processed as above.

**Isolation of Membrane Vesicles, Assay of Na+/Li+/H+ Antiport Activity**—EP432 cells transformed with the respective plasmids were grown in LBK, and everted membrane vesicles were prepared and used to determine the Na+/H+ or Li+/H+ antiport activity (37, 38). The assay of antiport activity was based on the measurement of Na+ or Li+-induced changes in ΔpH measured by acridine orange, a fluorescent probe of ΔpH maintained across the membrane. The fluorescence assay was performed with 2.5 ml of reaction mixture containing 50–100 μg of membrane protein, 0.5 mM acridine orange, 150 mM KCl, 50 mM BTP, and 5 mM MgCl2, and the pH was titrated with HCl. After energization (down arrow in Fig. 6A) with either ATP (2 mM) or d-lactate (2 mM), quenching of the fluorescence was allowed to achieve a steady state and then Na+ (10 mM) was added (up arrow in Fig. 6A). A reversal of the fluorescence level (dequenching) indicates that protons are exiting the vesicles in antiport with Na+. As shown previously, the magnitude of dequenching is a good estimate of the antiport activity (39), and the concentration of the ion that gives half-maximal dequenching is a good estimate of the apparent Km of the antiporter (39, 40). The concentration range of the cations tested was 0.01–100 mM at the indicated pH values, and the apparent Km values were calculated by linear regression of a Lineweaver-Burk plot. High pressure membranes were prepared as previously described (13).

**Reconstitution of NhaA into Proteoliposomes and Measurement of ΔpH-driven 22Na Uptake**—NhaA proteoliposomes were reconstituted and ΔpH-driven 22Na uptake was determined as previously described (11, 30). All experiments were repeated at least twice with practically identical results.
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RESULTS

Construction of the β-Hairpin Deletion Mutant NhaA/Δ(Pro<sup>45</sup>-Asn<sup>58</sup>)—Constructing a monomeric NhaA can be most helpful in allowing a straightforward comparison between NhaA dimers and monomers with respect to functional and structural properties, and deduction thereof of the functional/structural role of the dimeric state. We could apply this approach because of the structural data available for NhaA. The crystal structure of NhaA revealed a β-hairpin formed by amino acid residues from Pro<sup>45</sup> to Asn<sup>58</sup> (15) in the loop between helices Ia and II at the periplasmic side of the NhaA monomer (Ref. 15 and Fig. 1A). Recently (22), the three-dimensional crystal structure of two NhaA monomers were fitted to the three-dimensional reconstructed map of NhaA dimer obtained by cryo-electron microscopy of two-dimensional crystals (20). It has been suggested that two β-hairpins form an anti-parallel β-sheet that hold the monomers together in the native dimer at the periplasmic side of the membrane. Furthermore, based on the crystal structure and measuring of distance distribution between spin labels (bound to Cys replacements in NhaA) by a pulsed electron paramagnetic resonance (DEER), a model of the physiological NhaA dimer has been obtained. It revealed that the NhaA dimer interface is formed by very few contacts additional to the β-sheet. These include Val<sup>254</sup> and Trp<sup>258</sup> in the N terminus of TMS IX at the cytoplasmic face of the membrane (19). Based on this structural analysis, we assumed that deleting the DNA sequence encoding the β-hairpin (Δ(Pro<sup>45</sup>-Asn<sup>58</sup>)) would allow us to explore the structural-functional role of the β-hairpin and possibly also the role of the dimerization state of NhaA.

We constructed a plasmid, pAXH3/Δ(Pro<sup>45</sup>-Asn<sup>58</sup>), encoding NhaA/Δ(Pro<sup>45</sup>-Asn<sup>58</sup>) (Fig. 1B). Compared with the wild-type control encoded from pAXH3 (100% expression), NhaA/Δ(Pro<sup>45</sup>-Asn<sup>58</sup>) was significantly expressed (40–50%) and efficiently purified on a Ni<sup>2+</sup>-NTA affinity column (see below, Fig. 7C). Equal amounts of the purified proteins were subjected to SDS-PAGE. As expected from a deletion of 14 amino acids, the mobility in the gel of SDS-denatured NhaA/Δ(Pro<sup>45</sup>-Asn<sup>58</sup>) (Fig. 2A, lane b) was slightly, but reproducibly, faster than that of the SDS-denatured monomeric wild-type protein (Fig. 2A, lane a).

NhaA Exists as a Dimer in β-DDM (0.015–0.03%) Solution in Contrast to NhaA/Δ(Pro<sup>45</sup>-Asn<sup>58</sup>) Which Exists as a Monomer—Using gel filtration chromatography, we have previously shown that similar to its dimeric state in the membrane, wild-type NhaA exists as a dimer in β-DDM (0.03%) solution (32). As expected, under similar gel filtra-
tion conditions, the NhaA/Δ(Pro^{45}-Asn^{58}) mutant protein had a larger retention volume compared with the wild-type protein (data not shown). However, because neither the size of the detergent micelle nor the amount of lipids in the different oligomeric states of NhaA is known, we sought for another technique to separate the monomers from the dimers of NhaA.

Here, we demonstrate the conditions for separation of wild-type NhaA dimers and monomers on blue native gels. In the presence of 0.015–0.03% DDM, wild-type NhaA was nicely resolved in the native gel as a single band (Fig. 2, B, lane a, and C, lanes a and b). As a mobility marker for NhaA dimer we used an intermolecular cross-linked two CL-NhaA derivatives, each carrying a single Cys replacement of Ser^{246} (S246C), an amino acid residue at the C terminus end of loop VIII–IX (15). This Cys replacement was chosen on the basis of the model of the NhaA dimer obtained by the ESR study (see above and Ref. 19). This residue is located at or in close proximity to the dimer interface. We Cys-scanned this segment of NhaA for intermolecular cross-linking,4 confirmed the previous cross-linking data obtained with V254C (17), and found that S246C cross-links more even more efficiently than V254C (see below) and in contrast to the latter, does not affect the pH dependence of NhaA (data not shown). Similar to CL-NhaA (26) and many single Cys replacements derived from it (35), CL-NhaA/S246C is as expressed and active as the native NhaA (data not shown), and its mobility in SDS-PAGE is identical to that of the wild-type protein (Fig. 2A, lane a compare with Fig. 4A, lane a). Thus, when cross-linked, it can be used as a marker for the NhaA dimer on denaturing (Fig. 2A, lane c and Figs. 3 and 4) as well as on native gels (Fig. 2B, lane c).

Hence, on the blue native gel, native NhaA has a mobility of a dimer, identical to the cross-linked CL-NhaA/S246C (Fig. 2B, lanes a and c). Before loading to the native gels, incubation of the samples in sample buffer containing 2% SDS for 15 min converted the wild-type protein into monomer (Fig. 2B, lane d) while the cross-linked protein remained a dimer (Fig. 2B, lane f). Incubation of the affinity-purified NhaA for 2 h in the presence of increasing DDM concentrations (above 0.03%), progressively transformed the wild-type NhaA dimers into monomers (Fig. 2C, lanes c–g). At 1.5% DDM, almost all NhaA dimers were converted into monomers. Similar behavior was observed with CL-NhaA/S246C (data not shown).

It is noteworthy that extraction of the membranes (about 2 mg of membrane protein/ml) in the presence of 1% DDM does not impair the NhaA dimers whereas when purified (about 10 μg NhaA/ml), 1%–1.5% DDM converts the dimers into monomers (Fig. 2C, lanes a–g). It is known that the effect of detergent is dependent on the concentration ratio between proteins (and phospholipids) and detergent. Although we do not know the exact phospholipid concentration in the purified NhaA preparation, it certainly was reduced following purification (22). Clearly, the protein/detergent concentration ratio is reduced at least 200-fold during purification. These changes, most probably, account for the increase in the effective detergent concentration affecting the purified NhaA variants.

4 T. Tzubery, A. Rimon, and E. Padan, unpublished results.
the membrane. Using the three bifunctional cross-linking reagents, BMH, o-PDM, and MTS-2-MTS, 90–95% of the protein appeared as dimers (Fig. 3A, lanes f–h, respectively) compared with 10% of the untreated control (Fig. 3A, lane e) in SDS-PAGE. As expected, the mobility of the cross-linked protein was about twice that of the monomer. CL-NhaA did not cross-link, showing that the cross-linking is specific to the Cys replacement (Ref. 28 and data not shown). Hence, similar to V254C in the N terminus of helix IX (17), S246C, neighboring the N terminus of helix IX (15), is located at or in proximity to the NhaA dimer interface, and its capacity to perform intermolecular cross-linking in the membrane could be used to monitor the native dimeric state of NhaA variants in the membrane.

We next constructed mutant CL-NhaA/Δ(Pro45-Asn58)/S246C and tested its capacity to perform intermolecular cross-linking in the membrane. Remarkably, in marked contrast to CL-NhaA/S246C, CL-NhaA/Δ(Pro45-Asn58)/S246C showed very low cross-linking with any of the cross-linking reagents (Fig. 3A, compare lanes b–d to lanes f–h); similar to the untreated control (Fig. 3A, lane a), it formed mainly monomers (85–90%) in SDS-PAGE. The very low observed level of proteins with a dimer mobility most probably reflects a low level of aggregation, because occasionally, it has been observed in the untreated control (Fig. 3A, lane e). Hence, in the absence of the β-hairpin, S246C in CL-NhaA/Δ(Pro45-Asn58)/S246C can no longer perform intermolecular cross-linking in the membrane.

Cross-linking in Reconstituted Proteoliposomes of CL-NhaA/S246C and CL-NhaA/Δ(Pro45-Asn58)/S246C—Similar to the behavior in the native membrane, when reconstituted into proteoliposomes, CL-NhaA/S246C retained its intermolecular cross-linking capacity (Fig. 3B, lane f–h) whereas CL-NhaA/Δ(Pro45-Asn58)/S246C remained incapable of cross-linking (Fig. 3B, lanes b–d).

In β-DDM Solution (0.015–0.03%), Cys-less NhaA/S246C but Not Cys-less NhaA/S246C/Δ(Pro45-Asn58) Performs Intermolecular Cross-linking—Because intermolecular cross-linking of S246C is indicative of dimerization of NhaA variants in the membrane (see above), it was important to test how pure CL-NhaA/S246C performs intermolecular cross-linking in DDM micelles compared with its behavior in the membrane and compared with CL-NhaA/Δ(Pro45-Asn58)/S246C.

Intermolecular cross-linking experiments, similar to those performed in the membrane, were performed with the purified protein variants CL-NhaA/S246C (Fig. 4, A–C) and CL-NhaA/Δ(Pro45-Asn58)/S246C (Fig. 4D) as a function of DDM concentration. Purified (untreated) CL-NhaA in the presence of 0.03% DDM served as a negative control (Fig. 4, lanes a). CL-NhaA/S246C cross-linked very efficiently with the three cross-linking reagents, BMH, o-PDM, MTS-2-MTS at DDM concentrations between 0.015 and 0.03% (Fig. 4, A–C, lanes b and c, respectively). Increasing further the DDM concentrations to 0.1, 0.3, and 1% (Fig. 4, A–C, lanes d–f, respectively) progressively reduced the intermolecular cross-linking by all cross-linking reagents, and at 1% DDM the capacity of intermolecular cross-linking scanned, after drying, for calculation of the percentage of dimers and monomers (100% = dimers + monomers (17)).

![Figure 4](image-url)

**FIGURE 4.** Intermolecular cross-linking of purified single Cys replacement NhaA variants CL-NhaA/S246C (A–C) and CL-NhaA/Δ(Pro45-Asn58)/S246C (D) as a function of β-DDM concentration. Membrane vesicles (2 mg of membrane protein/ml) of the NhaA variants CL-NhaA/S246C and CL-NhaA/Δ(Pro45-Asn58)/S246C were extracted in 1% DDM, and the NhaA variants (10 μg/ml) were affinity-purified in the presence of the indicated (top panel) DDM concentrations: Lane a, 0.03% untreated control; lane b, 0.015%; lane c, 0.03%; lane d, 0.1%; lane e, 0.3%; lane f, 1%. Intermolecular cross-linking with the indicated cross-linking reagents was performed in the presence of the respective DDM concentrations. The proteins were precipitated in trichloroacetic acid (10%), resuspended in sample buffer (10 μl) lacking β-mercaptoethanol, in the case of MTS-2-MTS, and resolved (12 μg of protein) on SDS-PAGE. The gels were Coomassie-stained and
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linking vanished. It could be argued that the high concentration of detergent interferes with the accessibility of the protein to the cross-linking reagent in solution. This possibility was excluded using fluorescein maleimide, a fluorescent SH reagent that titrates all free SH residues (for details see “Experimental Procedures” and Ref. 35). The protein prepared at different DDM concentrations was first exposed to the cross-linking agent and then to fluorescein maleimide and resolved on SDS-PAGE. The fluorescent intensity was determined on the gels. Whereas, the untreated control showed 100% fluorescence, the cross-linking treated protein even at 1% DDM showed less than 5% fluorescence (data not shown).

Taken together, these results suggest that the oligomeric state of NhaA variants in the DDM micelles (0.015–0.03%) is very similar to that in the native membrane. It is noteworthy that in the preliminary experiment conducted with a CL-NhaA variant containing a Cys replacement in the β-hairpin (CL-NhaA/E54C), very similar dependence on DDM concentration of intermolecular cross-linking was observed.5

Remarkably, minor intermolecular cross-linking was obtained with NhaA-CL/Δ(Pro45-Asn58)/S246C with any of the cross-linking reagents at any of the DDM concentrations (Fig. 4D and data not shown). This may reflect a low level of aggregation because it also occurred in the untreated control. Thus, the β-hairpin is required for the dimeric native form of wild-type NhaA in the DDM micelles as in the native membrane.

Co-expressed HA- and (His)6-tagged NhaA/Δ(Pro45-Asn58) Proteins Do Not Co-purify on the Ni2+-NTA Column in Contrast to the Corresponding Versions of the Wild-type NhaA That Do Co-purify—The cross-linking results can be interpreted in two ways. (a) The β-hairpin is essential for the native dimerization of NhaA in the membrane and therefore CL-NhaA/Δ(Pro45-Asn58)/S246C is a monomer in the membrane and cannot perform intermolecular cross-linking. (b) The β-hairpin mutant still forms dimers in the membrane, albeit with physical contacts that differ from those of the native NhaA. To exclude the latter possibility, physical contacts between differentially tagged NhaA variants were sought in the membrane. We have previously shown that, as expected for a dimer, physical contacts exist between the wild-type NhaA monomers co-expressed from a pair of compatible plasmids, NhaA-(His)6 and NhaA-HA-co-purified via the His tag on a Ni2+-NTA affinity column (17). We, therefore, constructed another pair of compatible plasmids encoding NhaA-(His)6/Δ(Pro45-Asn58) and NhaA-HA/Δ(Pro45-Asn58) and tested the capacity of the co-expressed proteins to co-purify from the membrane compared with similarly tagged wild-type NhaA versions. Cells were co-transformed with one pair of the plasmids or with each plasmid separately. Membrane vesicles were isolated from each type of cell. DDM (1%) extracts were prepared, and samples containing identical amounts of membrane proteins were taken to ensure expression of each polypeptide by Western analysis. The antibodies used were mAb 1F6 (32) specific to the N terminus of NhaA (12) and therefore expected to recognize all NhaA variants (Fig. 5A) or a mAb specific to the HA epitope that recognizes only HA-tagged variants (Fig. 5B). Indeed, whether co-expressed (Fig. 5, A and B, lanes a and e) or separately expressed (Fig. 5, A and B, lanes b, c, f, and g), both wild type and Δ(Pro45-Asn58)-hairpin is required for the dimeric native form of wild-type NhaA in the DDM micelles as in the native membrane.

FIGURE 5. Co-expressed HA-tagged and (His)6-tagged NhaA/Δ(Pro45-Asn58) are not co-purified from the membrane via Ni2+-NTA, as opposed to co-purification of the respective wild-type derivatives. TA16 cells transformed with plasmids expressing the indicated proteins (lanes a–c and e–g) were induced by isopropyl-1-thio-β-D-galactopyranoside for overexpression, and high pressure membranes (2 mg of membrane protein/ml) and DDM (1%) extracts were prepared. In lanes d and h, each cell type was grown and processed separately, and the membranes were mixed in equal amounts prior to the DDM extraction. Samples of the detergent extracts (25 μg of membrane protein each) were separated on SDS-PAGE (A and B) or subjected (50 μg of membrane protein each) to Ni2+-NTA affinity chromatography (0.03% DDM) with acidic elution and separated on SDS-PAGE (C and D). Detection of the proteins in each case was conducted by Western analysis. Blots were probed with either mAb 1F6, recognizing the N terminus of NhaA (1F6), or an anti-hemagglutinin epitope mAb (anti HA). The cells used were as follows: (a) TA16/p184AXH/p100HA, (b) TA16/p184AXH, (c) TA16/p100HA, (d) TA16/p184AXH and TA16/p100HA, (e) TA16/p184AXH/Δ(Pro45-Asn58)/p100HA/Δ(Pro45-Asn58), (f) TA16/p184AXH/Δ(Pro45-Asn58), (g) TA16/p100HA/Δ(Pro45-Asn58), (h) TA16/p184AXH/Δ(Pro45-Asn58) and TA16/p100HA/Δ(Pro45-Asn58).

5 K. Herz and E. Padan, unpublished results.
Asn\textsuperscript{58} variants were detected in the membrane according to their epitope. For unknown reasons, although clearly detected, the HA-tagged derivatives were less reactive with mAb 1F6 compared with the other variants (Fig. 5A, lanes c and g). All HA-tagged variants were detected by the anti-HA specific antibody (Fig. 5B).

To test if the two differentially tagged NhaAs co-expressed in the membrane are in physical contact with each other, the DDM (1%) extracts were loaded on a Ni\textsuperscript{2+}-NTA column, and the His-tagged NhaA variants were affinity-purified (in the presence of 0.03% DDM). With regard to membrane vesicles expressing only one of the tagged proteins, as expected affinity-purified NhaA-(His)\textsubscript{6} and NhaA-(His)\textsubscript{6}/Δ(Pro\textsuperscript{45}-Asn\textsuperscript{58}) were obtained from the DDM extracts and recognized by mAb 1F6 but not by anti-HA (compare Fig. 5C, lanes b and f to Fig. 5D, lanes b and f). No proteins were affinity-purified by Ni\textsuperscript{2+}-NTA column from membranes, singly expressing the HA-tagged respective polypeptides (Fig. 5, C and D, lanes c and g).

With regard to membranes co-expressing the differentially tagged variants, as expected affinity-purified NhaA-(His)\textsubscript{6} and NhaA-(His)\textsubscript{6}/Δ(Pro\textsuperscript{45}-Asn\textsuperscript{58}) were obtained from the DDM extracts and recognized by mAb 1F6 but not by anti-HA (compare Fig. 5C, lanes b and f to Fig. 5D, lanes b and f). No proteins were affinity-purified by Ni\textsuperscript{2+}-NTA column from membranes, implying that wild-type NhaA exists in the membrane as an oligomer. In marked contrast, although expressed in the membrane, NhaA-HA/Δ(Pro\textsuperscript{45}-Asn\textsuperscript{58}) did not co-purify from the membrane with NhaA-(His)\textsubscript{6}/Δ(Pro\textsuperscript{45}-Asn\textsuperscript{58}) (Fig. 5D, lane a). Taken together, these results strongly support our contention that whereas, native NhaA is a dimer in the membrane, NhaA/Δ(Pro\textsuperscript{45}-Asn\textsuperscript{58}) is a monomer, and the β-hairpin is essential for the native dimerization of NhaA.

As shown before (17), when extracts (DDM, 1%) of membranes separately expressing either the wild type (Figs. 5A and 4B, lanes d) or the mutant (Fig. 5, A and B, lanes h) variants were mixed, no co-purification was obtained (Fig. 5D, compare lane a to lanes d and h) with either variant. These results imply that the oligomerization of the wild-type NhaA occurred in the membrane and not during extraction and purification. The deletion mutant did not oligomerize at any of these steps.  

**Growth Phenotype, Na\textsuperscript{+}/H\textsuperscript{+} Antipporter Activity and pH Dependence of NhaA/Δ(Pro\textsuperscript{45}-Asn\textsuperscript{58}) Mutant**—To characterize NhaA/Δ(Pro\textsuperscript{45}-Asn\textsuperscript{58}) with respect to growth and antipporter activity, the mutated plasmid was transformed into EP432, an *E. coli* strain that lacks the two Na\textsuperscript{+}-specific antiporters (NhaA and NhaB). This strain, neither grows at the routinely used selective media (Fig. 7, A and B). Therefore, we increased the stress conditions in two ways: (a) decreased expression by using the native nhaA promoter (pGMAR100 plasmid derivative in EP432 cells), (b) increased both the concentration of Na\textsuperscript{+} and pH. Practically, very similar results were obtained whether the extreme stress conditions were applied on growth on agar or in liquid culture. At up to 0.7 M NaCl, at pH 7, both the wild-type, and the deletion strain grew similarly (Fig. 7A and data not shown). Whereas, increasing the pH to 8.5 in the presence of 0.6 M NaCl slowed down only slightly the growth of the mutant NhaA/Δ(Pro\textsuperscript{45}-Asn\textsuperscript{58}) (Fig. 7A), a drastic effect was observed in the presence of 0.7 M at pH 8.5 compared with the wild-type strain (Fig. 7, A and B). The doubling time of the mutant was reduced by, at least, 3-fold. Further increase of the Na\textsuperscript{+} concentration to 0.8 M reduced the growth rate of the wild type but stopped completely the growth of the mutant (Fig. 7B). Because the growth effect on the mutant was pH-dependent, it could not be ascribed to an effect of increased osmosality. To further
Functional/Structural Implications of NhaA Monomers and Dimers

Everted membrane vesicles were prepared from EP432 cells transformed with pAXH3 or pAXH3/(Pro45-Asn58) grown in LBK (pH 7) and expressing NhaA or NhaA/Δ(Pro45-Asn58) from EP432 bearing the vector plasmid pBR322, a negative control. The 2-fold lower expression of the mutant compared with the wild type (Fig. 7C) cannot account for the much larger effect of the extreme stress conditions on growth of the mutant compared with the wild type (Fig. 7, A and B). In line with this conclusion, the difference in expression was constant and not dependent on the growth conditions. It should also be noted that both variants were expressed from multicopy plasmids, so that their proteins were readily detected by Western analysis (Fig. 7C). These expression levels are above that obtained from a single chromosomal gene that confers a wild-type phenotype (42).

Hence, it is concluded that the native dimerization state of NhaA is beneficial over the monomeric NhaA/Δ(Pro45-Asn58) mutant under the extreme stress condition, a combination of alkaline pH (8.5) in the presence of 0.7 M NaCl or 0.1 M LiCl.

**DISCUSSION**

By constructing a NhaA mutant deleted of the β-hairpin at the periplasmic face of the membrane and studying the effect of the deletion mutation on the tertiary structure, Na$^+$/H$^+$ antiporter activity, pH regulation of NhaA, and the conferred growth phenotype, we obtained direct evidence that the β-hairpin is essential for the formation and/or maintenance of NhaA dimers. The deletion yielded exclusively NhaA monomers both in the native membrane and when purified in DDM solution. Furthermore, the study revealed that the NhaA monomers are fully functional and stable in cells (under the routinely used stress conditions), isolated membranes, and reconstituted proteoliposomes. Remarkably, the dimeric state of NhaA was found to be beneficial under extreme stress conditions.

We have previously obtained, experimental evidence both in vivo (17) and in membrane (17, 18), implying that NhaA exists in the native membrane exclusively as dimers. Only NhaA dimers were observed in two-dimensional crystals of NhaA by cryo-electron microscopy (20, 21).

The recently determined three-dimensional crystal structure of NhaA, while providing essential insights into the mechanism of activity and pH regulation of NhaA (15) has also provided a basis for predicting the structure of the native NhaA dimer (18, 22) and modeling it (19). In support of previous biochemical data (17); the helix packing of the monomers of the native dimer is identical to that of the monomers in the crystal structure, and the dimer interface is very limited in size. At the periplasmic side, two β-hairpins of the two NhaA monomers form an anti-
parallel β-sheet, supposed to hold the two monomers together in the dimer; at the cytoplasmic side, only very few contacts between the two monomers exist involving the N terminus of TMS IX (17, 19, 22).

These studies have not provided an answer as to which of the elements lining the interface are essential for the tertiary structure and/or functionality of NhaA. However, it has implied that an NhaA deletion mutation, lacking the β-hairpin, should yield monomeric NhaA if the two β-hairpins of the monomers are essential in forming and/or maintaining the NhaA native dimers. Furthermore, these studies guided us in the construction of the β-hairpin deletion mutation.

The crystal structure shows that amino acid residues from Pro45 to Asn58 comprise the β-hairpin and that their deletion (Δ(Pro45-Asn58)) would leave a long loop from Ser31 at the C-terminal end of helix I to Thr44 at the N-terminal end of helix II (Fig. 1), assuming that the helix packing is not impaired by the deletion mutation. Thus, the shortened loop was expected to be long enough to span the distance (19.5 Å between the α-carbons of Asn50 and Met59) between helices I and II at the periplasmic side of the molecule (15) and not to impose constraints on the NhaA monomer that may lead to misfolding and or impaired insertion of NhaA into the membrane. Indeed, compared with the wild-type protein, the mutant protein was significantly expressed in the membrane in an active form (see below) and efficiently affinity-purified on Ni²⁺-NTA column in a functional form, evidenced by its reconstitution into active proteoliposomes.

Unfortunately, determination of the quaternary structure of a membrane protein in detergent solution is not trivial. A wide variety of techniques, chemical cross-linking, analytical ultracentrifugation, size exclusion chromatography, and electron microscopy have been used (16). No single technology is entirely satisfactory. Biochemical or hydrodynamic means lead to overestimation of the number of subunits in an oligomeric membrane protein because of unknown amounts of lipids and detergents in mixed micelles. Denaturing SDS gel results in underestimation because of partial unfolding of the hydrophobic protein by harsh detergents.

Blue native gel electrophoresis has been successfully used to study the oligomeric state of purified membrane proteins,
including LacS (43), SecYEG complex (44), and recently BetP (45) and CaiT (46). Here, we found conditions for the determination of the oligomeric state of native NhaA by blue native gel electrophoresis and revealed that the concentration of the detergent was critical. To obtain NhaA dimers in the blue native gels the DDM concentration (1%) in which the protein was solubilized from the membrane had to be reduced to 0.015–0.03% during affinity purification on the Ni²⁺-NTA column and separation on the gel (Fig. 2C). The wild-type protein could be stored at 0.03% DDM, for at least a month at 4 °C (data not shown) and retained its dimer mobility in the blue native gel.

Furthermore, intermolecular cross-linking data ascertained that the dimers in the DDM micelles performed intermolecular cross-linking very similar to the dimers in the native membrane. Thus, CL-NhaA/S246C, a Cys-less NhaA variant bearing a Cys replacement in the native dimer interface, at the N terminus of helix IX, performed very similar extensive intermolecular cross-linking in the native membrane, reconstituted proteoliposomes, and when prepared in DDM solution at 0.015–0.03%. Importantly, the wild-type dimers (Fig. 2C) converted into monomers and CL-NhaA/S246C (Fig. 4, A–C) lost the capacity to cross-link in a similar detergent-dependent fashion.

In marked contrast to the wild-type protein, the NhaA/Δ(Pro⁴⁵-Asn⁵⁸) protein exists as a monomer both in the native membrane and detergent micelles: (a) The mobility of affinity-purified NhaA/Δ(Pro⁴⁵-Asn⁵⁸) in blue native gels was that of a monomer (Fig. 1B) irrespective of DDM concentrations (0.015–4%), and no aggregation or dimeric forms appeared for at least one month incubation in 0.03% DDM at 4 °C (data not shown). (b) Whether free in solution or in the native membrane or reconstituted proteoliposomes, CL-NhaA/Δ(Pro⁴⁵-Asn⁵⁸)/S246C did not perform intermolecular cross-linking as opposed to CL-NhaA/S246C which cross-linked very efficiently with its twin in the native membrane, proteoliposomes, or when in solution containing 0.015–0.03% DDM. (c) Hemagglutinin-tagged and His-tagged variants of wild-type NhaA when co-expressed in the membrane co-purified via the His tag by Ni²⁺-NTA affinity purification. In marked contrast, hemagglutinin-tagged and His-tagged NhaA/Δ(Pro⁴⁵-Asn⁵⁸) were co-expressed but did not co-purify. Taken together, these results strongly support our contention that mutant NhaA/Δ(Pro⁴⁵-Asn⁵⁸) is a monomer in the membrane and in β-DDM micelles, implying that the β-hairpin of NhaA is essential for its dimerization.

To our knowledge, our results present the first example of a polytopic membrane protein in which a β-hairpin of two monomers is essential for dimerization. β-sheets formed by joint β-hairpins are a classical motif of oligomer formation in soluble proteins (47–51).

Our finding that high DDM concentration abrogates the native dimeric state of NhaA also provides an insight into the crystallization pattern of NhaA. In the asymmetric unit of the NhaA three-dimensional crystals, NhaA comprises a non-physiological dimer, where the monomers are organized in an upside-down orientation (15). During processing of the protein for crystallization, NhaA is exposed to high detergent concentration at the solubilization step (1% β-DDM), and when it is concentrated together with α-DDM, the anomer yielded the best x-ray diffracting crystals (22). Although further experiments are needed, our preliminary results suggest that at certain steps during crystallization (detergent above 0.03%), the native dimers split, the monomers flip, and acquire the non-physiological orientation.

Many membrane proteins have a strong tendency to associate into oligomers. 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 (52). Secondary transporters are diverse membrane proteins, which have been found in a variety of oligomeric states that tend to be conserved in a given family or sub-family (16). Sugar symporters and phosphate antiporters of the major facilitator family are monomers (53–55), whereas, the ion antiporters such as NhaA are dimers (20, 56–58). A trimeric architecture has been observed in the case of AcrB (59, 60), glutamate transporters (61), BetP (45, 62), and CaiT (46).

Multimerization of membrane proteins provides possibilities for scaffolding, interfaces, and allostery (63). Yet, the role of oligomers in the function of secondary transporters is still an open question in most cases. It has been suggested (64) or shown (65) that monomers cooperate with one another during transport, implying that the oligomeric state is essential for function.

In NhaA cross-linking experiments (17) and ESR, data (18) have suggested that there is functional cross-talk between monomers in the dimeric assembly expected to involve conformational changes at the dimer interface, involving the N terminus of helix IX. Positive complementation was shown between mutants affecting the pH response of NhaA (17). Furthermore, intermolecular cross-linking of V254C by p-PDM, a rigid cross-linker, changed dramatically the pH profile of CL-NhaA/S246C while, BMH, a long and flexible cross-linker had no effect. Nevertheless, it should be emphasized that whereas these results are consistent with a role of the dimers in the pH regulation of NhaA, they do not necessarily mean that the dimeric state is essential for activity and/or pH regulation of the antiporter.

Here, we tested the functional role of NhaA dimers in a most direct approach by producing the NhaA/Δ(Pro⁴⁵-Asn⁵⁸) mutant that exists as a monomer. Comparison of the monomeric mutant to the native dimer, unequivocally, reveals that the functional unit of NhaA is a monomer. Isolated membrane vesicles from cells expressing NhaA/Δ(Pro⁴⁵-Asn⁵⁸) and proteoliposomes reconstituted from pure NhaA/Δ(Pro⁴⁵-Asn⁵⁸) protein show wild-type Na⁺/H⁺ antiporter activity with respect to bioenergetics, kinetic parameters, and pH regulation.

Our results are in full agreement with the NhaA crystal structure predicting that the monomer is the functional unit of NhaA (15). It contains the cytoplasmic and periplasmic funnels and the unique TMS assembly in which TMS IV and XI unwind and cross each other in the middle of the membrane where the putative cation binding site is located and where the ion translocation occurs.

What is the role of the NhaA dimeric state? Most interestingly, here, we have established that the dimeric native form of NhaA is much better than the mutant NhaA/Δ(Pro⁴⁵-Asn⁵⁸) in
confering growth resistance under extreme stress conditions, a combination of alkaline pH (8.5) and either LiCl (0.1 M) or NaCl (0.7 M). These stress conditions affected slightly the wild-type growth but reduced by two orders of magnitude the number of colonies formed by the mutant on agar and drastically decreased the growth rate of the mutant in liquid medium (Fig. 7). The reason for the advantage of the NhaA dimers over the monomers under the extreme stress conditions can be a higher stability of the dimers compared with the monomers, a possibility that is currently under study.

REFERENCES
1. Padan, E., Tzubery, T., Herz, K., Kozachkov, L., Rimon, A., and Galili, L. (2004) Biochim. Biophys. Acta 1658, 2–13
2. Padan, E., Venturi, M., Gerchman, Y., and Dover, N. (2001) Biochim. Biophys. Acta 1505, 144–157
3. Yamaguchi, T., and Blumwald, E. (2005) Trends Plant Sci. 10, 615–620
4. Orlowski, J. I., and Grinstein, S. (2004) Pflugers Arch. 447, 549–565
5. Putney, L. K., Denker, S. P., and Barber, D. L. (2005) Biochim. Biophys. Acta 1729–1734
6. Wakabayashi, S., Hisamitsu, T., Pang, T., and Shigekawa, M. (2003) J. Biol. Chem. 278, 3056–3062