Oligomerization of NhaA, the Na^+/H^+ Antiporter of *Escherichia coli* in the Membrane and Its Functional and Structural Consequences†

Yoram Gerchman,‡ Abraham Rimon,‡ Miro Venturi,§ and Etana Padan*,‡

Division of Microbial and Molecular Ecology, Institute of Life Sciences, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel, and Abteilung Molekulare Membranbiologie, Max-Planck-Institut für Biophysik, Heinrich-Hoffmann Strasse 7, D-60528 Frankfurt/Main, Germany

Received November 20, 2000; Revised Manuscript Received January 3, 2001

ABSTRACT: Recently, a two-dimensional crystal structure of NhaA, the Na^+/H^+ antiporter of *Escherichia coli* has been obtained [Williams, K. A., Kaufer, U. G., Padan, E., Schuldiner, S. and Kühlbrandt, W. (1999) *EMBO J., 18,* 3558−3563]. In these crystals NhaA exists as a dimer. Using biochemical and genetic approaches here we show that NhaA exists in the native membrane as a homooligomer. Functional complementation between the polypeptides of NhaA was demonstrated by coexpression of pairs of conditional lethal (at high pH in the presence of Na^+) mutant alleles of *nha*A in EP432, a strain lacking antiporters. Physical interaction in the membrane was shown between the His-tagged NhaA polypeptide which is readily affinity purified from DM-solubilized membranes with a Ni^{2+}-NTA column and another which is not; only when coexpressed did both copurify on the column. The organization of the oligomer in the membrane was studied in situ by site-directed cross-linking experiments. Cysteine residues were introduced—one per NhaA—into certain loops of Cys-less NhaA, so that only intermolecular cross-linking could take place. Different linker-size cross-linkers were applied to the membranes, and the amount of the cross-linked protein was analyzed by mobility shift on SDS−PAGE. The results are consistent with homooligomeric NhaA and the location of residue 254 in the interface between monomers. Intermolecular cross-linking of V254C caused an acidic shift in the pH profile of NhaA.

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

NhaA is the Na^+/H^+ antiporter that is essential for $H^+$and Na^+ homeostasis in *Escherichia coli*. In logarithmic growth the expression of *nha*A is positively regulated by NhaR, a member of the LysR family, and is induced by Na^+ in a pH-dependent manner (reviewed in refs *5* and *6*).

The NhaA protein is predicted to have a putative secondary structure consisting of 12 TMS[1] connected by hydrophilic loops (*7*). This topology has been substantiated by using *pho*A fusions combined with epitope mapping, exposure to proteolysis, and accessibility of site-directed Cys replacements from each side of the membrane (*7*, *8*).

NhaA is an electrogenic antiporter that has been purified to homogeneity and reconstituted in a functional form in proteoliposomes (*9*). The H^+/Na^+ stoichiometry of NhaA is $2H^+/Na^+$ (*10*). The activity of NhaA is highly dependent on pH, with V_{max} changing by over 3 orders of magnitude from pH 7 to pH 8, suggesting that NhaA is equipped with both a pH sensor and a transducer to increase its activity with pH (*9*, *10*).

The amino acid residues His-225 (*11*, *12*) and Gly-338 (*13*) are involved in the pH sensitivity of NhaA. A pH-induced conformational change is required for the pH regulation of NhaA. The conformational change involves loop VIII−IX (*14*) and the N-terminal loop of NhaA (*15*). It is clear that structural information is needed for a deeper understanding of the mechanism of activity of NhaA and its regulation. However, as yet neither NhaA nor any other secondary transporters have been solved at atomic resolution.

Overexpression of His-tagged NhaA (*14*), providing 4−5 mg of pure active NhaA/L of cell culture opened the way to structural studies. As determined by gel filtration analysis, the pure protein in DM micelles might exist as a dimer (*16*). Recently, a two-dimensional crystal structure of NhaA has been obtained (*17*, *18*). In this crystal, the first known for a transporter, NhaA also exists as a dimer. These studies raise the questions as to whether the dimerization of NhaA occurs in the native membrane and whether it is essential for the

† This work was supported by a grant from the BMBF and the International Bureau of the BMBF at the DLR [German−Israeli Project Cooperation on Future-Oriented Topics (DIP)] (to E.P.) and the Moshe Shilo Minerva Center for Biogeochemistry. E.P. was awarded the Massimo and Adelina Della Pergolla Chair in Life Sciences.

* Corresponding author. Tel: 972-2-6585094. Fax: 972-2-6586947. E-mail: etana@vms.huji.ac.il.

‡ The Hebrew University of Jerusalem.

§ Max-Planck-Institut für Biophysik.

[1] Abbreviations: TMS, transmembrane domain; DM, *n*-dodecyl β-D-maltoside; PAGE, polyacrylamide gel electrophoresis; BMH, 1,6-bis-(maleimido)hexane; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; *p*-PDM, *N*,*N*′-*p*-phenylenedimaleimide; CuPh, copper (1,10-phenanthroline)$_3$; DTT, dithiothreitol; Ni-NTA, Ni^{2+} nitrilotriacetic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate; BTP, 1,3-bis-[[tris(hydroxymethyl)methyl]amino]propane; H225R, NhaA-His225 replaced with Arg. All other *nha*A mutations are accordingly depicted.

10.1021/bi002669o CCC: $20.00
Published on Web 02/17/2001

Na^+/H^+ antiporter activity and/or its regulation. In the present study we show, using both genetic and biochemical approaches, that NhaA is a homooligomer in the membrane.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions. EP432 is an *E. coli* K12 derivative, which is *mel*BLid, Δ*nha*A1::*kan*, Δ*nha*B1::*cat*, Δ*lac*ZY, *thr*1 (*19*). TA16 is *nha*A^+*nha*B^+*lac*I^Q and otherwise isogenic to EP432 (*9*). DH5α (United States Biochemical Corp.) was used as a host for construction of plasmids. BL21[(DE3)Gold] was from Stratagene. Cells were grown either in L broth (LB) or in modified L broth (LBK) in which NaCl was replaced by KCl [(*20*) 87 mM, pH 7.5]. The media were buffered by 60 mM BTP, and pH was titrated with HCl. Cells were also grown in minimal medium A without sodium citrate (*21*) with either glycerol (0.5%) or melibiose (10 mM) as a carbon source. Thiamin (2.5 μg/mL) and threonine (0.1 mg/mL) were added to all minimal media. For plates, 1.5% agar was used. The antibiotics and their concentrations were 100 μg/mL ampicillin, 50 μg/mL kanamycin,12 μg/mL chloramphenicol, 12.5 μg/mL tetracycline, and 20 μg/mL zeocin (Invitrogen, Groningen, The Netherlands). Resistance to Li^+ and Na^+ was tested as described previously (*11*).

Plasmids. The plasmids used in this study are described in Table 1. Since plasmids derived from pBR322 are compatible with the ones derived from pACYC184, pGMAR100 and pAR100 were used for constructing the plasmid pairs used in the complementation experiments.

Site-Directed Mutagenesis. Site-directed mutagenesis was conducted following a polymerase chain reaction-based protocol (*22*). DNA of pGMAR100 or pC-less was used as a DNA template. The end primers and the mutagenic primers are described in Table 2.

Isolation of Membrane Vesicles and Assay of Na^+/H^+ Antiporter Activity. Assays of Na^+/H^+ antiport activity were conducted on everted membrane vesicles (*23*). The assay of antiport activity was based upon the measurement of Na^+-induced changes in the ΔpH as described (*24*, *25*). The reaction mixture contained 50−100 μg of membrane protein, 0.5 μM acridine orange, 140 mM KCl, 50 mM BTP, and 5 mM $MgCl_2$, and the pH was titrated with HCl.

High-pressure membranes were prepared as described (*14*).

Overexpression and Affinity Purification of His-Tagged Antiporters by Ni^{2+}-NTA Chromatography. To overexpress the plasmids encoding the His-tagged antiporters, TA16 transformed with the respective plasmids was used as described (*9*).

His-tagged NhaA was affinity purified on a Ni-NTA-agarose column (Qiagen, Hilden, Germany) and, if not otherwise stated, eluted with imidazole, dialyzed, and stored as described (*14*). For copurification of coexpressed polypeptides and for the cross-linking experiments miniscale purification was performed basically as described (*8*), but the elution was acidic; the resin-bound and washed His-tagged NhaA was washed again with a solution containing 500 mM NaCl, 20 mM Tris (HCl), pH 7.9, 0.1% DM, and 10% glycerol to remove imidazole and then eluted in a solution containing 25 mM potassium acetate, pH 4, 300 mM KCl, 0.1% DM, and 10% glycerol. The protein was precipitated in 10% TCA for 0.5 h at 4 °C, resuspended in sampling buffer, titrated to neutrality with Tris, and loaded on the gel.

Reconstitution of Proteoliposomes and Assay of ΔpH-Driven Sodium Uptake. Reconstitution of NhaA in proteoliposomes and assay of ΔpH-driven ^{22}Na uptake by proteoliposomes were as described (*9*).

Protein Determination. Protein was determined according to ref *26*.

DNA Sequence. Sequencing of DNA was conducted by an automated DNA sequencer (ABI PRISM 377, Perkin-Elmer).

Detection and Quantitation of NhaA and Its Mutated Proteins in the Membrane. Detection and quantitation of NhaA and its mutated derivatives in membranes were by Western analysis as described previously (*12*). The antibodies used were mAb 1F6 (*16*) or anti-hemagglutinin mAb (HA.11, BabCO).

Site-Directed Cross-Linking. Membranes were prepared from TA16 cells transformed with one of the plasmids: pC-less-H3C-H5C-XH, pC-less-S52C-XH2, pC-less-S87C-XH2, pC-less-H225C-XH2, pC-less-E241C-XH, pC-less-V254-XH, pC-less-L316C-XH, and pC-less-S352C-XH2. Membranes (1 mg of protein) were resuspended (1 mL) in a buffer containing 100 mM KP_i and 10 mM $MgSO_4$ (pH 7.3), and one of the cross-linkers was added [BMH (Pierce) or CuPh (Sigma), 5 mM each, or *o*-PDM (Sigma) or *p*-PDM (Sigma) (1 mM each)]. The suspension was sonicated for 10 s and incubated at room temperature for 30 min. The reaction, except the one with CuPh, was terminated by the addition of 10 mM DTT. The membranes were washed in the buffer, and the pellet was resuspended in a solution containing 150 mM choline chloride, 250 mM sucrose, and 10 mM Tris-HCl (pH 7) solubilized by the addition of DM (1%) and the NhaA-$(His)_6$ protein affinity purified on a Ni-NTA column (miniscale) and eluted by acid elution. The samples were processed as described in Figure 3.

Labeling with [^{35}S]Methionine and Trypsinization of NhaA $(His)_6$ To Remove the $(His)_6$ Tag. BL21/pAXHΔ*tac* cells were grown in minimal medium A (glycerol) to A_{600} 0.6, induced by 0.4 mM IPTG, and grown for 1 h, and 200 μg/mL rifampicin was added. After 40 min at 37 °C [^{35}S]-methionine (20 nCi/mL) was added and incubation continued for an additional 45 min. The cells were harvested and membranes prepared by sonication (*12*). NhaA $(His)_6$ was purified (miniscale procedure) and subjected to trypsin at pH 6.5 as described (*14*). Under these conditions the $(His)_6$ tail is removed from NhaA (*14*).

RESULTS

Functional Complementation of Growth by Coexpression of Two Lethal Alleles. Coexpression of mutant alleles within the same cell and study of the resulting phenotype are powerful methods to study the potential interaction between polypeptides.

For example, restoration of viability by coexpression of two lethal mutants (functional complementation) provides evidence for oligomeric organization (reviewed in ref *27*). It is assumed that in the oligomer the functional complementation results when mutant polypeptides combine to form an active hybrid protein such that each mutant chain provides

Table 1: Plasmids Used in This Study

plasmid	plasmid parent (origin of replication)	NhaA derivatives[a]	reference or procedure
pBR322			
pACYC184			
pGM36	PBR322	wild type	*42*
pDT62	PBR322	wild type	*42*
pAR100	PACYC184	wild type	*13*
pGMAR100	PGM36	wild type	*13*
pG338S	PGMAR100	G338S	*11*
pH225R (*Sty*I)	PGMAR100	H225R	this study; in addition codon 227 → *Sty*I
pAR-H225R (*Sty*I)	PAR100	H225R	this study; *Bgl*II−*Mlu*I (682 bp) replaced with that of pH225R (*Sty*I)
pAR-G338S (pNR1)	PAR100	G338S	*13*
pG338S	PGMAR100	G338S	this study; *Bgl*II−*Mlu*I replaced with that of pAR-G338S
pD164C	PDT62	D164C	Rothman, Padan, and Schuldiner, unpublished results
pD163C	PDT62	D163C	Rothman, Padan, and Schuldiner, unpublished results
pAR-D163C	PAR100	D163C	this study; *Bgl*II−*Mlu*I replaced with that of pD163C
pD163C-G338S	pG338S	D163C-G338S	this study; *Bgl*II−*Bam*HI^{537bp} replaced with that of pD163C
pD164C-G338S	pG338S	D164C-G338S	this study; *Bgl*II−*Bam*HI replaced with that of pD164C
pH225R-G338S	pG338S	H225R-G338S	this study; *Bgl*II−*Bam*HI replaced with that of pH225R (*Sty*I)
pBR322Δ*tet*	PBR322		this study; excision of *Hin*dIII−*Eco*130I, end filling, self-ligation
pVgRXR (Zeo^+)	PBR322		invitrogen
p184-Zeo (Zeo^+Tet^-)	PACYC184		this study; *Bam*HI−*Bam*HI (1.4 kb) of pVGRXR inserted into *Bam*HI
pAXH	PBR322	wild-type fused to two factor Xa sites and $(His)_6$ tag [NhaA-$(His)_6$]	*8*
pA-HA-H	PAXH	factor Xa codons replaced with hemagglutinin epitope (HA)[b]	this study
pC-less	PGMAR100	all Cys codons replaced with Ser	*8*
pC-less-XH	PAXH	all Cys codons replaced with Ser	*8*
pAXH2	PAXH	wild type	this study; excision of *BSa*B1−*BSa*B1 (252 bp) and self-ligation
pC-less-XH2	PAXH2	wild type	this study; *Bgl*II−*Mlu*I (682 bp) replaced with that of pC-less
p100-HA	PGMAR100	wild type fused to HA epitope (NhaA-HA)	this study; an insert produced by digestion of pA-HA-H with *Xho*I, end filling, and *Nhe*I digestion (944 bp) ligated with a fragment (3.6 kb) obtained from pGMAR100 by *Hin*dIII digestion, end filling, and *Nhe*I digestion
p184-AXH	PACYC184	wild-type $(His)_6$	this study; *Kpn*2I−*Kpn*2I (1.62 kb) of pAXH inserted into *Kpn*2I
pC-less-H3C-H5C-XH	pC-less	C-less L221C-$(His)_6$	this study[c]
pC-less-S52C-XH2	pC-less	C-less V254C-$(His)_6$	this study[d]
pC-less-S87C-XH2	pC-less	C-less G241C-$(His)_6$	this study[d]
pC-less-H225C-XH2	pC-less	C-less L316C-$(His)_6$	this study[d]
pC-less-E241C-XH	pC-less	C-less S52C-$(His)_6$	this study[c]
pC-less-V254C-XH	pC-less	C-less S87C-$(His)_6$	this study[c]
pC-less-L316C-XH	pC-less	C-less S352C-$(His)_6$	this study[c]
pC-less-S352C-XH2	pC-less	C-less H225C-$(His)_6$	this study[d]
pAXHΔtac	PAXH2	wild-type $(His)_6$	excision of *Xba*I−*Eco*RI (4.51 kb), end filling, and self-ligation
pC-less-V254C	pC-less	C-less V254C	this study on pC-less

[a] Mutations expressed by the letter code of amino acids: first the wild-type, followed by the codon number, and then the replacement. [b] *Xba*I−*Not*IX fragment (4986 bp) ligated to the indicated linker encoding the HA epitope and introducing the *Nde*I site:

NdeI
GGCCAGCTACCCATATGACGTGCCCGATTATGCTC
TCGATGGGTATACTGCACGGGCTAATACCGAGTCGA
Y P Y D V P D Y A

[c] The respective mutations produced on pC-less were introduced into pC-less-XH by replacing restriction fragments (XH was added to the plasmid name). [d] The respective mutations were produced on pC-less and introduced into pC-less-XH2 (XH2 was added to the plasmid name).

or corrects the structural elements required for the function of the oligomer.

The *E. coli* strain EP432 is devoid of its two Na^+-specific Na^+/H^+ antiporters, *nha*A and *nha*B, and therefore does not grow at pH 8.3 in the presence of 0.6 M NaCl [high pH, high Na^+, nonpermissive growth conditions (*19*)]. Cells of EP432 transformed with a plasmid carrying wild-type *nha*A are rescued and grow under the nonpermissive growth conditions (*19*; see Table 3). We have isolated several *nha*A mutants that are expressed normally in EP432 but do not support growth of EP432 under the nonpermissive growth conditions. We therefore assumed that if NhaA functions as an oligomer, pairs of these mutants coexpressed on compatible plasmids within EP432 should show functional comple-

Table 2: Primers Used for Construction of NhaA Mutants

mutation	mutagenic primer[a]	location[b]	codon change	new restriction site
start primer	TTTAACGATGATTCGTGGCGG	−67 to −47		
H3C H5C	GTGAAAtgTCTctgcAGATTCTTTAGC	1 to 27	CAT → GTG and CAT → TGC	*Pst*I
S52C	CGGGTTGGTTgtCTaGAAATCAAC	145 to 168	TCA → TGT	*Xba*I
S87C	CTGATGCAAGGGTgcCTAGCCAGC	247 to 270	TCG → TGC	*Ban*I added and *Nhe*I removed
H225C	ATCGGGGGTTtgCGCAACTCTGGCG	663 to 687	CAC → TGC	*Fsp*I
H225R (*Sty*I)	GGGGGTTCggGCAACctTGGCG	666 to 687	CAC → CGG	*Sty*I
E241C	TTCCTTTGAAAtgtAAGCATGGGCG	710 to 735	GAG → TGT	
V254C	CTGGAGCATtgcTTGCACCCaTGGGTGG	751 to 778	GTG → TGC	*Nco*I
L316C	CGTTTGAAAtgcGCaCATCTGCCTGAG	937 to 963	CTG → TGC	*Fsp*I
S352C	GCCTTTGGTtGtGTtGAcCCAGAACTG	1045 to 1071	AGC → TGT	*Hin*cII
end primer	GCTCATTTCTCTCCCTGATAAC	1233 to 1211		

[a] All primers start at 5′. Mutated codons are shown in lower case. The mutated codons introducing new restriction sites are underlined. [b] Locations are relative to the initiation codon. The *nha*A sequence appears in the GenBank database (accession no. J03879).

Table 3: Functional Complementation between Pairs of *nha*A Mutants

plasmid 1 (pBR322 ori)	plasmid 2 (pACYC184 ori)	mutations	growth on 0.6 M NaCl, pH 8.3[a]
pGMAR100	pACYC184	wild type	+++
pBR322Δ*tet*	pAR100	wild type	+++
pD164C	pAR-D163C	D163C + D164C	−
pD163C	pAR-H225R (*Sty*I)	D163C + H225R	++
pD164C	pAR-H225R (*Sty*I)	D164C + H225R	++
pD163C	pAR-G338S	D163C + G338S	++
pD164C	pAR-G338S	D164C + G338S	++
pH225R (*Sty*I)	pAR-G338S	H225R + G338S	++
pD163C	pACYC184	D163C	−
pD164C	pACYC184	D164C	−
pH225R (*Sty*I)	pACYC184	H225R	−
pBR322Δ*tet*	pAR-G338S	G338S	−
pBR322Δ*tet*	pACYC184		−
Double Mutants (Both on the Same Gene)			
pD163C-G338S		D163C G338S	−
pD164C-G338S		D164C G338S	−
pH225R-G338S		H225R G338S	−

[a] EP432 cells transformed with the indicated plasmids were grown in LBK with the respective antibiotics and replica plated on LB containing 0.6 M NaCl at pH 8.3. Key: +++, wild-type growth; ++, number of colonies identical but size slightly smaller than that of the wild type; −, no growth.

mentation. A summary of complementation experiments of this type is shown in Table 3.

When EP432 was transformed with either of the plasmidic *nha*A mutants encoding H225R (*11*; see Table 3) or G338S (*13*; see Table 3), neither of the transformed cells grew under the nonpermissive growth conditions due to a modification of the pH regulation of NhaA; in the former mutant the antiporter activity was too low to support growth (*11*); in the latter mutant pH control was lost, and the uncontrolled activity of NhaA killed the cells by overacidification of the cytoplasm (*13*). However, when EP432 was cotransformed with both plasmids, the cotransformed cells grew under the nonpermissive conditions (Table 3). The growth both on plates (Table 3) or in liquid medium (data not shown) was stable, reproducible, and very similar to that of the wild type. Only the lag period was slightly prolonged and the colony size slightly reduced with the cotransformed cells. Both D163 and D164 were found to be essential for growth and could not be replaced (*28*; A. Rothman, E. Padan, and S. Schuldiner, unpublished results; Table 3). Coexpression of the pair D163C with D164C was also not functional (Table 3). However, coexpression of D163C or D164C with either H225R or G338S restored growth (Table 3). When the pairs of the complementing mutations were constructed within one *nha*A gene (a double mutant) and transformed on a single plasmid into EP432, none of the transformants EP432/pH225R-H338S or EP432/pD163C-G338S or EP432/pD164C-G338S could grow under the nonpermissive growth conditions (Table 3). Hence, in order to functionally complement, both mutated polypeptides must be coexpressed within EP432 cells.

Nevertheless, these results could be explained in other ways: (1) suppression of the mutations by a chromosomal suppression mutation; (2) genetic recombination between the DNA of the two plasmids. To exclude the latter possibility, it was essential to show that the two compatible plasmids remained intact, separable from each other, and therefore the cotransformed cells were curable from each of the plasmids.

Restriction enzyme analysis and DNA sequencing of the various plasmids showed that apart from the respective mutation the DNA was unchanged. However, with these approaches small amounts of recombinant products could have escaped detection.

To show that the plasmids are separable and maintain their individual properties, the cells (Amp^+Tet^+) containing plasmid pairs were cured from one of the plasmids by many transfers in the presence of only one selective antibiotic (Table 4). About 98% of these cells lost either the Amp^+ or the Tet^+ resistance and accordingly the capacity of growth at high Na^+/high pH conditions (Table 4). When the cells containing the single plasmid were retransformed with a plasmid identical to the lost one, all Amp^+ Tet^+ cotransformants grew at high Na^+/high pH conditions. Table 4 also shows that despite the separation attempts in several experiments very few cotransformants remained with unseparable plasmids. The separation procedure was repeated with these cells, and again over 98% of the cells contained separable plasmids. Hence, although the single antibiotic added selected only one plasmid, a certain percentage of the cell population could maintain both plasmids. It is suggested that the contaminating Na^+ in the medium [which can reach 10 mM in LBK (*2*)] confers upon the cells a selective pressure, even without the antibiotics, to maintain both plasmids and therefore a functional antiporter.

We therefore conducted a more drastic plasmid curing experiment. EP432 coexpressing plasmid pairs was trans-

Table 4: Functional Complementation Is Lost by Curing from One Plasmid[a]

original plasmid pair	selection by growth on	selected colonies, % growth on Amp	Tet	0.6 M NaCl, pH 8.3
pD163C + pAR-H225R (*Sty*I)	Amp	100	0	0
pD163C + pAR-H225R (*Sty*I)	Tet	1	100	1
pD164C + pAR-H225R (*Sty*I)	Amp	100	2	2
pD164C + pAR-H225R (*Sty*I)	Tet	1	100	1
pD163C + pAR-G338S	Amp	100	0	0
pD163C + pAR-G338S	Tet	0	100	0
pD164C + pAR-G338S	Amp	100	2	2
pD164C + pAR-G338S	Tet	3	100	3
pH225R (*Sty*I) + pAR-G338S	Amp	100	0	0
pH225R (*Sty*I) + pAR-G338S	Tet	3	100	3

[a] EP432 cells transformed with the indicated pairs of plasmids grown on LB and 0.6 M NaCl, pH 8.3, were transferred on agar plates (4−5 times) and then in liquid medium (10 generations) in the presence of only one of the selective antibiotics (either Amp or Tet) as indicated. The plasmids were then isolated, diluted 100-fold in water, and/or digested with the restriction enzyme unique to the unselected plasmid and retransformed into EP432. The transformed cells were plated on either LBK-Amp or LBK-Tet, respectively, and then each colony was scored for growth on the indicated selective plates: Amp, LBK-Amp; Tet, LBK-Tet; 0.6 M NaCl, pH 8.3 (LB). In each case about 1000 colonies were examined.

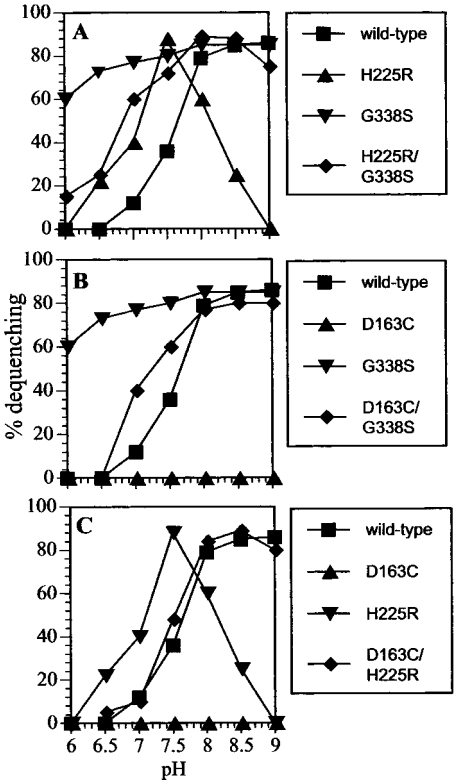

FIGURE 1: Na^+/H^+ antiporter activity of coexpressed pairs of functionally complementing NhaA mutants. EP432 cells transformed with single plasmids (■, ▲, ▼) or plasmid pairs (◆) encoding the indicated proteins were grown on LBK with the respective antibiotics. Then everted membrane vesicles were isolated, and the Na^+/H^+ antiporter activity was tested at various pH values as indicated. At the onset of the experiment, lactate (2 mM) was added, and the fluorescence quenching was recorded until a steady-state level of ΔpH had been reached (100%). NaCl (10 mM) was then added, and the new steady state of fluorescence obtained (dequenching) was monitored. All experiments were repeated at least twice, and the results were essentially identical. Plasmids used: pGMAR100, encoding wild-type NhaA; pH225R-(*Sty*I), encoding H225R; pAR-G338S, encoding G338S; pD163C, encoding D163C. The respective compatible vectors, pACYC184 or pBR322Δ*tet*, were cotransformed into cells expressing a single NhaA variant.

formed with either plasmid p184-Zeo or pVgRXR, both carrying a gene conferring zeocin resistance (Zeo^+) but differing in the origin of replication (Table 1). The first is incompatible with plasmids derived from pACYC184 and the second with plasmids derived from pBR322. The transformed cells were grown in the presence of zeocin with either tetracycline or ampicillin. After several transfers (*8*−*10*) both the capacity to grow at high pH and high Na^+ and the antibiotic resistance of the cells were tested. In all cases one antibiotic resistance was lost according to the curing plasmid used. For example, transformation of EP432/pD163C/pAR-H225R with pVgRXR yielded Tet^+ Zeo^+ cells (EP432/pAR-H225R/pVgRXR), while transformation with p184-Zeo produced Amp^+ Zeo^+ cells (containing pD163C and p184-Zeo). Furthermore, cells cured of one plasmid lost, at the same time, the growth capacity at high pH and high Na^+. Taken together, these experiments show that the pairs of plasmids cotransformed into EP432 are separable and the cells curable. The curing experiment also showed that the growth phenotype was determined by the plasmids and not by a chromosomal mutation. Therefore, a functional complementation was established between the coexpressed mutated NhaA polypeptides.

The NhaA Na^+/H^+ Antiporter Activity of Everted Membrane Vesicles Isolated from the Functionally Complemented Cells. Figure 1A shows the Na^+/H^+antiporter activity of everted membrane vesicles isolated from the cotransformed cells, EP432/pH225R(*Sty*I)/pAR-G338S, and from cells transformed with each plasmid separately or the wild-type plasmid. As shown before, the pH dependence of both mutants differs markedly from each other and from that of the wild type. The wild-type NhaA is activated between pH 7 and pH 8.5 (*9*) and is still at the maximum activity at pH 9; G338S protein is not regulated by pH and is active even at pH 6 (*13*); H225R is regulated, but its activation is shifted by half a pH unit toward acidic pH (between pH 6 and pH 7.5), and beyond pH 7.5 it is progressively inactivated to zero activity at pH 9 (*11*). Remarkably, Na^+/H^+ antiport activity of the membranes isolated from the cotransformed cells exhibits a pH dependence which is different from that of either mutants and from that of the wild-type NhaA. Similar to H225R it shows an acidic pH shift in the pH profile by half a pH unit as compared to the wild type. In contrast to H225R and similar to G338S and the wild type, it is not inactivated beyond its maximum at pH 8 and remains at the maximum up to pH 9. These results highly suggest that the complementation occurs in the membrane between the polypeptides of the mutated NhaA, creating a novel phenotype.

The Na^+/H^+ antiport activity in membranes obtained from EP432/pD163C/pAR-G338S also exhibited a pH dependence which is different from the wild type and intermediate between that of each mutant (Figure 1B). Note that in this pair one of the complementing mutants is completely inactive (D163C).

The Na^+/H^+ antiport activity exhibited by membranes obtained from EP432/pD163C/pAR-H225R(*Sty*I) shows a pH

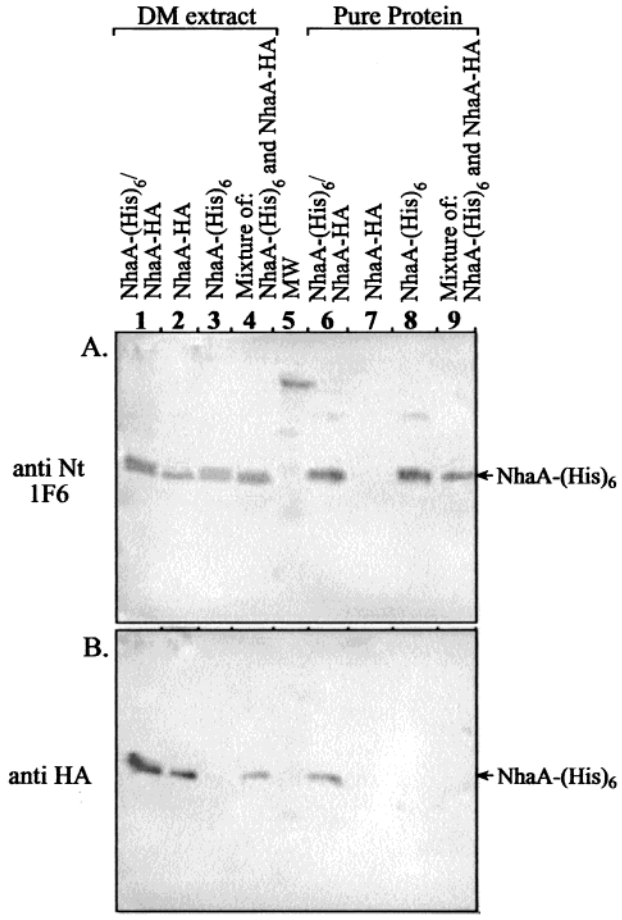

FIGURE 2: Copurification of NhaA-HA and NhaA-$(His)_6$. TA16 cells transformed with plasmids encoding the indicated proteins were induced by IPTG for overexpression, and then high-pressure membranes and their DM extracts were prepared. Samples of the DM extracts (lanes 1−4) were subjected to Ni-NTA chromatography with acidic elution (lanes 6−9) as described in Experimental Procedures. Detection of the proteins [NhaA-HA and NhaA-$(His)_6$] before or after Ni-NTA chromatography was conducted by Western analysis after separation of the proteins of the respective samples (40 μg each) on SDS−PAGE as described in Experimental Procedures. Blots were probed with either mAb 1F6 recognizing the N-terminus of NhaA (anti Nt, panel A) or an anti-hemagglutinin epitope mAb (anti HA, panel B). The cells used were as follows: (lanes 1 and 6) TA16/p184-AXH/p100-HA; (lanes 2 and 7) TA16/pACYC184/p100-HA; (lanes 3 and 8) TA16/p184-AXH/pBR322Δ*tet*; (lanes 4 and 9) TA16/p184-AXH/pBR322Δ*tet* and TA16/pACYC184/p100-HA. Each cell type was grown and processed separately, and the membranes were mixed in equal amounts prior to the DM extraction. Lane 5 shows MW markers. The experiment was repeated three times, and the results were essentially identical.

dependence very similar to that of the wild type (Figure 1C) but very different from that of membranes obtained from EP432 transformed with either plasmid alone (Figure 1C).

Physical Interaction between the Coexpressed NhaA Polypeptides. To test physical interaction between the coexpressed polypeptides within the membrane, we used two compatible plasmids (p100-HA and p184-AXH), each encoding a differently tagged NhaA at the C-terminus (Figure 2); p100-HA encodes NhaA fused to the hemagglutinin epitope (NhaA-HA), and p184-AXH encodes NhaA fused to polyhistidine [NhaA-$(His)_6$]. Cells were cotransformed with this pair of plasmids or with each plasmid separately. Membrane vesicles were isolated from each type of cell, DM extract was prepared, and samples were taken to ensure expression of each polypeptide by Western analysis. The antibodies used were mAb 1F6 (*16*), specific to the N-terminus of NhaA (*15*) and therefore expected to recognize both the NhaA-HA as well as the NhaA-$(His)_6$ (Figure 2A), or a mAb specific to the HA epitope that recognizes only NhaA-HA (Figure 2B). When cells were transformed with the plasmid encoding either NhaA-HA or NhaA-$(His)_6$, each of the proteins was present in the membranes as evidenced by their immunoreactivity with mAb 1F6 (Figure 2A, lanes 2 and 3). As expected, only NhaA-HA was detected by the anti HA specific antibody (Figure 2B, lane 2). When cells were cotransformed by both plasmids, the proteins were coexpressed together [NhaA-$(His)_6$/NhaA-HA] within the membranes and both could be detected in the DM extract (lane 1 in panels A and B of Figure 2). Note that as many other polytopic membrane proteins NhaA runs with aberrant mobility on SDS−PAGE and appears as a fuzzy band (*9*). It occasionally even appears as a double band (lane 1 in panels A and B of Figure 2, lanes 3 and 4 in Figure 2A). Hence, these lanes do not represent the two variants, NhaA-$(His)_6$ and NhaA-HA. Indeed, the two variants differ in length by only one amino acid, and the double band appears also when only one form of NhaA exists in the gel (Figure 2A, lane 3).

To test whether the two differently tagged NhaAs coexpressed in the membrane are in physical contact with each other, the DM extracts were loaded on a Ni-NTA column and the His-tagged NhaA was affinity purified. With regard to membrane vesicles expressing only one of the tagged proteins, as expected affinity-purified NhaA-$(His)_6$ was obtained from the DM extract derived from membrane vesicles containing NhaA-$(His)_6$ (Figure 2A, lane 8) but not from membranes containing NhaA-HA (Figure 2A, lane 7). Remarkably, although lacking a His tag, NhaA-HA copurified with the NhaA-$(His)_6$ (lane 6 in panels A and B of Figure 2).

It could be argued that the two NhaA polypeptides do not interact in the membrane but rather bind to each other during the DM extracting stage, a prerequisite for the solubilization of these hydrophobic polypeptides. To exclude this possibility, the membranes overexpressing each one of the polypeptides separately were mixed prior to the DM extraction (Figure 2A, lane 4), and then the solubilized proteins were resolved on a Ni-NTA column (Figure 2A, lane 9). Under this condition there was no copurification of NhaA-HA with NhaA-$(His)_6$ (compare lane 9 in panels A and B of Figure 2). This lack of interaction between NhaA polypeptides in the DM extract of the membranes could be explained in two ways: (i) The large dilution of the membrane proteins during extraction (10-fold) reduces the concentration of NhaA in the extract to a level below that needed for oligomerization. (ii) The oligomers had already existed in the membrane prior to extraction, and the oligomers are not in equilibrium with their monomers in the DM extract.

To test these alternatives, radioactively labeled [^{35}S]-NhaA-$(His)_6$ was prepared and treated to remove the $(His)_6$ tag (*14*; see Experimental Procedures). Separation on a Ni-NTA column showed that indeed most of the NhaA polypeptides lost their tag and therefore eluted in the void volume. This preparation (1 mg/mL) was mixed with equal concentrations of nonlabeled untreated NhaA-$(His)_6$, incubated for 1 h at 4 °C, and affinity purified on a Ni-NTA column. While full

recovery of the nonlabeled NhaA-$(His)_6$ was obtained, there was no copurification of the radioactively labeled non-His-tagged NhaA (data not shown). These results are in line with alternative ii; once formed, the oligomers of NhaA do not dissociate and do not form hybrids in DM.

Proximity of Loops in the Oligomer. As revealed by electron cryomicroscopy of two-dimensional crystals, NhaA forms dimers with a very short interface between the monomers (*17*, *18*). In this interface only two sites of contacts were identified: one which is formed by identical parts of the two monomers and another by different parts (see Figure 3C in ref *17*). We therefore assumed that the common part of the interface can be identified by screening for cross-linking under conditions that allow only intermolecular cross-linking to take place, i.e., using for the cross-linking reaction single Cys replacements, one per NhaA monomer; only in the common contact site can a single Cys residue cross-link its twin residue. For this purpose we used plasmids (pC-less-XH or pC-less-XH2) that encode a functional cysteine-less derivative of NhaA-$(His)_6$ (*8*) to produce a series of plasmids encoding an antiporter protein bearing a single Cys replacement in one loop of NhaA (Table 1). The mutations were H3C-H5C (loops I), S52C (loops I−II), S87C (loops II−III), or H225C (loops VII−VIII), E241C or V254C (loops VIII−IX), L316C (loops X−XI), and S352C (loops XI−XII). All mutants supported growth of EP432. They were transformed into TA16, and everted membrane vesicles were isolated. All mutants were expressed well in the membranes exhibiting protein bands at MW 32.5 kDa in SDS−PAGE (*8*; for example, see Figure 3, lane 1). Homobifunctional, thiol-specific, cross-linking agents of different chain length between the reactive groups (*29*) were applied to the membranes, the protein was affinity purified on a Ni^{2+}-NTA column, and ran on SDS−PAGE. An experiment conducted with V254C is shown in Figure 3. It is apparent that a prominent 60 kDA band representing a dimer of NhaA was observed only after cross-linking with the cross-linking agents BMH (Figure 3, lane 2) or *p*-PDM (Figure 3, lane 4) or *o*-PDM (Figure 3, lane 3). The amount of NhaA cross-linked was 94%, 90%, and 40%, respectively (Figure 3). The level of spontaneous cross-linking due to oxidation was very low since the difference in the pattern of the SDS−PAGE under reducing conditions (Figure 3, lane 1) or nonreducing, without DTT (data not shown), did not exceed 5%. Furthermore, catalyzed oxidation in the presence of CuPh did not increase the cross-linking of NhaA with the mutation V254C (Figure 3, lane 5).

A summary of similar cross-linking experiments conducted with the other single Cys replacements of NhaA is presented in Figure 4. As noted above, all mutants were well expressed, and a small variation did not affect the interpretation because the quantitative method used to compare one mutant with another was independent of expression level; the amount of dimers was calculated as a percentage of total NhaA for each mutant separately. It is evident that as compared to NhaA-V254C all of the other NhaA mutants showed at least 3-fold less cross-linking capacity. We suggest that in the oligomer two V254C residues exist near or at the interface between monomers so that each of them faces its twin residue in trans and cross-links efficiently two NhaA monomers.

Intermolecular Cross-Linking at V254C Shifts the pH Profile of NhaA. Since both BMH and *p*-PDM cross-linked

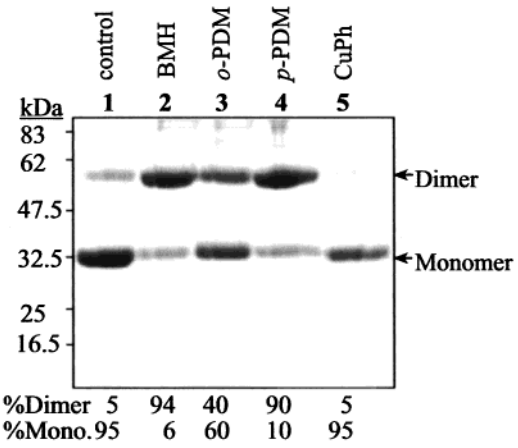

FIGURE 3: Intermolecular cross-linking between NhaA monomers with a single Cys replacement. Everted membrane vesicles were prepared from TA16/pC-less-V254C-XH cells, resuspended (1 mg of protein) in a buffer (1 mL) containing 100 mM KP_i and 10 mM $MgSO_4$, pH 7.3. The membranes were treated with the indicated cross-linkers [BMH or CuPh (5 mM each) or *p*-PDM or *o*-PDM (1 mM each)], and the NhaA-$(His)_6$ was affinity purified on a Ni-NTA column as described in Experimental Procedures. The purification products were TCA (10%) precipitated, resuspended (10 μL) in sample buffer lacking β-mercaptoethanol, and loaded (12 μg of protein each) on the gels for SDS−PAGE and Coomassie staining. For quantitation the gels were scanned (UMAX Astra 122OU), and the percentage of dimers and monomers (100% = dimers + monomers) was calculated (MacBUS, Fuji).

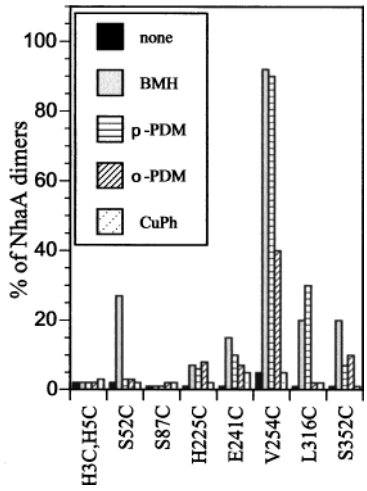

FIGURE 4: Proximity between various loops in the NhaA oligomer. Everted membrane vesicles were isolated from TA16 transformed with one of the plasmids, pC-less derivative, encoding a single Cys replacement of C-less NhaA (Table 1): H3C-H5C, S52C, S87C, H225C, E241C, V254C, L316C, or S352C. The membranes were treated and the results analyzed as described in Figure 3. The data were plotted as a histogram with the amount of disulfide-linked dimers expressed in percent (100% = disulfide-linked dimers plus nonlinked monomers). The histogram represents the mean of results obtained from three independent experiments.

very efficiently V254C (94% and 90%, respectively, Figure 3), it was of interest to determine whether intermolecular cross-linking would change the activity and/or the pH profile of NhaA. Everted membrane vesicles were prepared from C-less-NhaA-V254C, treated with or without either BMH or *p*-PDM, washed, and tested for Na^+/H^+ antiporter activity at various pH values (Figure 5). The results show that cross-linking by BMH had no effect on either the maximal activity or the pH profile of the antiporter. Cross-linking with *p*-PDM

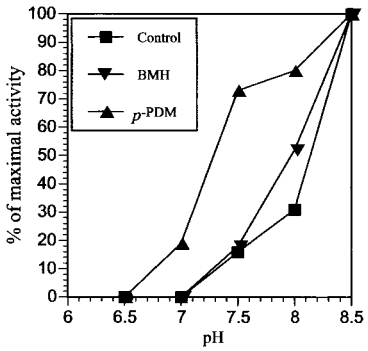

FIGURE 5: Intermolecular cross-linking via V254C shifts the pH profile of NhaA. Everted membrane vesicles were prepared from EP432 cells transformed with pC-less-V254C. The membranes were treated with BMH or *p*-PDM, as described in Figure 3, and washed, and the Na^+/H^+ antiporter activity was determined in the indicated pHs as described in Figure 1. Maximal activity was 80−85% dequenching. Results are expressed as percent of maximal activity. The experiment was repeated three times, and the results were essentially identical.

had also no effect on the maximal activity of the Na^+/H^+ antiport observed at pH 8.5. However, in marked contrast to BMH, *p*-PDM cross-linking changed the pH profile of the protein; while the activity of both BMH treated and nontreated protein reduced with decreasing pH, reaching 18% of the maximal activity at pH 7.5, the activity of the *p*-PDM cross-linked protein reduced much less with decreasing pH and was still 75% active at this pH (Figure 5).

DISCUSSION

The results obtained in this work show that NhaA exists in the native membrane as an homooligomer. These results were obtained by different approaches: (i) Functional interaction between the polypeptides of NhaA was observed when pairs of conditional lethal mutants of *nha*A that cannot grow in the presence of high Na^+ at high pH were coexpressed in the membrane of EP432; the coexpression restored the activity and/or the pH regulation of NhaA and accordingly the growth at high pH in the presence of high Na^+. Thus the mutant polypeptides combine to form an active hybrid protein. (ii) Physical interaction between the coexpressed NhaA polypeptides in the membrane was documented by showing that, when coexpressed, NhaA polypeptide that lacked a His tag copurified with a His-tagged NhaA. The physical interaction between NhaA polypeptides was specific since other cell membrane proteins did not associate to any notable extent with NhaA-$(His)_6$. This was evident by the purity of NhaA-$(His)_6$ following Ni-NTA affinity chromatography (*14*). (iii) Site-directed intermolecular cross-linking experiments showed that residue V254 in loop VIII−IX of NhaA monomers is close to its twin residue in the oligomer, allowing chemical cross-linking to take place.

Most interestingly, NhaA oligomers, when solubilized from the membrane in DM, did not dissociate but maintained their original organization. Neither NhaA monomers were identified in the DM solution (*16*) nor hybrids of differently tagged NhaAs could be formed by their mixing in DM. Therefore, we suggest that NhaA oligomers are formed either in the membrane or during insertion into the membrane.

It was previously proposed (*30*) that the relationship between the initial rate of transport into reconstituted proteoliposomes and the protein/phospholipid ratio should be indicative of the functional oligomeric state formed by the reconstitution; a linear relationship indicates a monomeric form while an exponential relationship a dimeric form. On this basis and other experiments it was concluded that the Lac permease is a monomer (*30*, *31*). A linear relationship was also found between the initial rate of ΔpH-driven Na^+ transport in NhaA proteoliposomes and the NhaA protein/phospholipid ratio (data not shown). Nevertheless, these results are not inconsistent with the suggestion that NhaA is a functional oligomer since a stable undissociated oligomer in the detergent solution that does not change its oligomeric state upon reconstitution would also show such a linear relationship. Indeed, NhaA is a stable oligomer in solution (*16*, *17*; this work).

The functional complementation presented here was observed between conditional lethal mutants under nonpermissive conditions but not with lethal mutants. In each complementing pair, there may have been at least one mechanistically "competent" subunit which is either inactive (H225R) or overactive (G338S) under the nonpermissive conditions due to a change in pH regulation of the mutant protein (*11* and *13*, respectively). In contrast, the dead mutants D163C and D164C did not complement. Therefore, the complementation results can be interpreted in two ways. The first is that NhaA is a functional oligomer and for productive interaction between the mutated polypeptides a certain distance between the mutant sites is required. Alternatively, it is possible that the interaction between subunits is important with respect to the pH regulation of NhaA, but the interaction may well not be required for the function of NhaA monomers. This possibility is also supported by the experiment in which intersubunit cross-linking of the V254C mutant with *p*-PDM alters the pH profile (Figure 5).

Chemical cross-linking between two site-directed Cys replacements in a single protein has been extensively used to assess proximity of pairs of Cys residues and thus helix packing of transport proteins (*32*) and even probe ligand-induced conformational changes within the protein (*29*). Here we have used cross-linking of single Cys replacement per Cys-less protein to test whether NhaA is an oligomer in the membrane and to identify amino acid residues forming the interface between monomers in the NhaA oligomer. Single Cys replacement V254C cross-linked very efficiently (Figure 4). This cross-linking was not observed spontaneously or with seven other mutations in different periplasmic loops of NhaA (Figure 4). At least two of the latter mutated residues were shown to be accessible to SH reagents [H225C (*8*) and H3C-H5C (*15*)]. The results suggest that loop VIII−IX, on the cytoplasmic side of helix VIII, is at the interface between NhaA monomers forming the oligomer.

These results are inconsistent with the notion that oligomerization is a stochastic process resulting from random collisions between monomers. Thus the cross-linking was specific and very efficient and the oligomer was stable; even without cross-linking it did not dissociate when solubilized in DM.

BMH is a homobifunctional thiol cross-linking reagent with a flexible hexyl chain connecting two maleimide groups (ca. 16 Å fully extended). This reagent was very efficient in cross-linking V254C. *o*-PDM and *p*-PDM are rigid homo-

bifunctional reagents in which the maleimido groups are coupled to benzene rings in the ortho or para positions at fixed distances of about 6 or 10 Å, respectively. Interestingly, both cross-linkers cross-linked V254C efficiently. Based on the premise that Cys cross-links can measure distances between the reactive Cys residues, it is possible to suggest that V254C is very close to its respective twin residue in the oligomer (within 10 Å). However, it should be emphasized that cross-link formation is dependent on dynamic collisions, chemical reactivity, and stereochemistry of the residues, not simply their proximity. Hence more direct structural information is needed to assign residue V254C of NhaA a role or a location in the oligomerization domain of NhaA.

Remarkably, the intermolecular cross-linking of V254C by *p*-PDM changed the pH profile of C-less-NhaA-V254C. This effect cannot be ascribed to the chemical modification of V254C since another cross-linker, BMH, had no effect although it bound and cross-linked the protein as efficiently as *p*-PDM (Figures 3 and 4). We have previously shown that loop VIII−IX undergoes a conformational change that is important in the response of NhaA to pH (*14*). We therefore suggest that the rigidity and the short distance between the maleimido groups in the case of *p*-PDM cross-link restrain this conformational change. On the other hand, BMH which provides a flexible long chain between the cross-linked sites had no effect.

It is very likely that the stoichiometry of NhaA polypeptides in the oligomer is 2. This is based on the observation that purified NhaA in DM micelles might exist as a dimer (*16*) and the 2D crystals of NhaA are formed of dimers (*17*, *18*). However, it should be stressed that the stoichiometry within the native membrane has not as yet been determined. The cross-linking experiment described here is based on the usage of NhaA with a single Cys replacement/monomer and a SDS−PAGE step for detection of the products. Therefore, it can detect only covalently linked dimers and is independent of the number of polypeptides in the oligomer. Nevertheless, analysis of the results presented in Figure 2 allows us to get a rough estimate of the coexpressed NhaA monomers. Probing with mAb 1F6 that recognizes the N-terminus of NhaA shows that the differently tagged NhaAs at the C-terminus were both expressed and to a similar level, in the membrane (compare lanes 2 and 3 in Figure 2A). Comparing lane 2 in panels A and B of Figure 2 shows that mAb anti NhaA-HA is about 1.3-fold more reactive with NhaA than mAb 1F6. This ratio allows us to estimate that the purified fraction of the coexpressed polypeptides (lane 6 in panels A and B of Figure 2) contains about one-fourth to one-third NhaA-HA and three-fourths to two-thirds His-tagged NhaA. Given the equal expression and assuming a random association of the polypeptides, this ratio is consistent with a dimer.

Similar to many other transport proteins NhaA is a polytopic membrane protein predicted to contain 12 TM domains (*7*). In the case of NhaA this model was strongly supported recently by analysis of the 2D crystals (*17*, *18*). The atomic structure of none of these proteins has yet been elucidated, and for most cases the question whether they are constructed and function as oligomers or monomers is still open. Judging by the size of particles in freeze-fracture images, both the Na-glucose (*33*) and the H-lactose symporters of *E. coli* (*34*) were estimated to be monomers in the membrane. In the Lac permease, functional interaction between monomers was not observed, even when the monomers were covalently linked (*31*).

Nevertheless, studies with an increasing number of other transporters have recently suggested that oligomerization may be an important feature for the structure and possibly also the function of this group of membrane proteins. Functional complementation and structural interaction between monomers have been documented for the TetA transporter (*35*−*37*). Recently, a study of structural and functional interaction between monomers of the serotonin transporter highly suggests that this transporter exists as a dimer or possibly a tetramer with functional interaction between the subunits (*38*). The erythrocyte anion exchanger (*39*), the renal Na^+/H^+ exchanger (NHE1) (*40*), and several neurotransmitter transporters (*41*) were proposed to exist as oligomers. In the case of NHE1 each monomer was shown to function independently within the oligomer (*40*).

Multimerization provides possibilities for scaffolding, interfaces, and allostery. Interestingly, the majority of the transporters which are constructed and/or function as oligomers are antiporters or energy-coupled to two ions with fluxes of opposite directions. Whether this correlation implies a functional significance is an intriguing possibility that remains to be studied.

ACKNOWLEDGMENT

We acknowledge Dr. Helmut Reiländer for recommendation of the Zeo cassette.

REFERENCES

1. Padan, E., and Schuldiner, S. (1992) in *Alkali Cation Transport Systems in Procaryotes* (Bakker, E., Ed.) pp 3−24, CRC Press, Boca Raton, FL.
2. Padan, E., and Schuldiner, S. (1994) *Biochim. Biophys. Acta 1185*, 129−151.
3. Schuldiner, S., and Padan, E. (1992) in *Alkali Cation Transport Systems in Procaryotes* (Bakker, E., Ed.) pp 25−51, CRC Press, Boca Raton, FL.
4. Padan, E. (1998) in *Microbiology and Biochemistry of Hypersaline Environments* (Oren, A., Ed.) pp 163−175, CRC Press, Boca Raton, FL.
5. Carmel, O., Rahav-Manor, O., Dover, N., Shaanan, B., and Padan, E. (1997) *EMBO J. 16*, 5922−5929.
6. Padan, E., and Krulwich, T. (2000) in *Bacterial Stress Responses* (Storz, G., and Hengge-Aronis, R., Eds.) pp 117−130, ASM Press, Washington, DC.
7. Rothman, A., Padan, E., and Schuldiner, S. (1996) *J. Biol. Chem. 271*, 32288−32292.
8. Olami, Y., Rimon, A., Gerchman, Y., Rothman, A., and Padan, E. (1997) *J. Biol. Chem. 272*, 1761−1768.
9. Taglicht, D., Padan, E., and Schuldiner, S. (1991) *J. Biol. Chem. 266*, 11289−11294.
10. Taglicht, D., Padan, E., and Schuldiner, S. (1993) *J. Biol. Chem. 268*, 5382−5387.
11. Gerchman, Y., Olami, Y., Rimon, A., Taglicht, D., Schuldiner, S., and Padan, E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1212−1216.
12. Rimon, A., Gerchman, Y., Olami, Y., Schuldiner, S., and Padan, E. (1995) *J. Biol. Chem. 270*, 26813−26817.
13. Rimon, A., Gerchman, Y., Kariv, Z., and Padan, E. (1998) *J. Biol. Chem. 273*, 26470−26476.
14. Gerchman, Y., Rimon, A., and Padan, E. (1999) *J. Biol. Chem. 274*, 24617−24624.
15. Venturi, M., Rimon, A., Gerchman, Y., Hunte, C., Padan, E., and Michel, H. (2000) *J. Biol. Chem. 275*, 4734−4742.

16. Padan, E., Venturi, M., Michel, H., and Hunte, C. (1998) *FEBS Lett. 441*, 53−58.
17. Williams, K. A., Kaufer, U. G., Padan, E., Schuldiner, S., and Kühlbrandt, W. (1999) *EMBO J. 18*, 3558−3563.
18. Williams, K. A. (2000) *Nature 403*, 112−115.
19. Pinner, E., Kotler, Y., Padan, E., and Schuldiner, S. (1993) *J. Biol. Chem. 268*, 1729−1734.
20. Padan, E., Maisler, N., Taglicht, D., Karpel, R., and Schuldiner, S. (1989) *J. Biol. Chem. 264*, 20297−20302.
21. Davies, B. D., and Mingioli, E. S. (1950) *J. Bacteriol. 60*, 17−28.
22. Ho, S. F., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) *Gene (Amsterdam) 77*, 51−59.
23. Rosen, B. P. (1986) *Methods Enzymol. 125*, 328−336.
24. Schuldiner, S., and Fishkes, H. (1978) *Biochemistry 17*, 706−711.
25. Goldberg, B. G., Arbel, T., Chen, J., Karpel, R., Mackie, G. A., Schuldiner, S., and Padan, E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2615−2619.
26. Zor, T., and Selinger, Z. (1996) *Anal. Biochem. 236*, 302−308.
27. Zabin, I., and Villarejo, M. R. (1975) *Annu. Rev. Biochem. 44*, 295−313.
28. Inoue, H., Noumi, T., Tsuchiya, T., and Kanazawa, H. (1995) *FEBS Lett. 363*, 264−268.
29. Wu, J., and Kaback, H. R. (1997) *J. Mol. Biol. 270*, 285−293.
30. Kaback, H. R. (1989) *Harvey Lect. Ser. 83*, 77−105.
31. Sahin-Toth, M., Lawrence, M. C., and Kaback, H. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5421−5425.
32. Sun, J., and Kaback, H. R. (1997) *Biochemistry 36*, 11959−11965.
33. Eskandari, S., Wright, E. M., Kreman, M., Starace, D. M., and Zampighi, G. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 11235−11240.
34. Costello, M. J., Viitanen, P., Carrasco, N., Foster, D. L., and Kaback, H. R. (1984) *J. Biol. Chem. 259*, 15579−15586.
35. Rubin, R. A., and Levy, S. B. (1990 *J. Bacteriol. 172*, 2303−2312.
36. Rubin, R. A., and Levy, S. B. (1991) *J. Bacteriol. 173*, 4503−4509.
37. McMurry, L. M., and Levy, S. B. (1995) *J. Biol. Chem. 270*, 22752−22757.
38. Kolic, F., and Rudnick, G. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 3106−3111.
39. Casey, J. R., and Reithmeier, R. A. (1991) *J. Biol. Chem. 266*, 15726−15737.
40. Fafournoux, P., Noel, J., and Pouyssegur, J. (1994) *269*, 2589−2596.
41. Haugeto, O., Ullensvang, K., Levy, L. M., Chaudhry, F. A., Honore, T., Nielsen, M., Lehre, K. P., and Danbolt, N. C. (1996) *J. Biol. Chem. 271*, 27715−27722.
42. Karpel, R., Olami, Y., Taglicht, D., Schuldiner, S., and Padan, E. (1988) *J. Biol. Chem. 263*, 10408−10414.

BI002669O