Mutation E252C Increases Drastically the $K_m$ Value for Na$^+$ and Causes an Alkaline Shift of the pH Dependence of NhaA Na$^+/H^+$ Antiporter of Escherichia coli*

Received for publication, August 14, 2003, and in revised form, October 28, 2003
Published, JBC Papers in Press, November 5, 2003, DOI 10.1074/jbc.M309021200

Tzvi Tzubery, Abraham Rimon, and Etana Padan‡

From the Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, 91904 Jerusalem, Israel

A single Cys replacement of Glu at position 252 (E252C) in loop VIII–IX of NhaA increases drastically the $K_m$ for Na$^+$ (50-fold) of the Na$^+/H^+$ antiporter activity of NhaA and shifts the pH dependence of NhaA activity, by one pH unit, to the alkaline range. In parallel, E252C causes a similar alkaline pH shift to the pH-induced conformational change of loop VIII–IX. Thus, although both the Na$^+/H^+$ antiporter activity of wild type NhaA and its accessibility to trypsin at position Lys$^{249}$ in loop VIII–IX increase with pH between pH 6.5 and 7.5, the response of E252C occurs above pH 8. Furthermore, probing accessibility of pure E252C protein in dodecyl maltoside solution to 2-(4’-maleimidylanilino)-naphthalene-6-sulfonic acid revealed that E252C itself undergoes a pH-dependent conformational change, similar to position Lys$^{249}$, and the rate of the pH-induced conformational change is increased specifically by the presence of Na$^+$ or Li$^+$, the specific ligands of the antiporter. Chemical modification of E252C by N-ethylmaleimide, 2-(4’-maleimidylanilino)-naphthalene-6-sulfonic acid; [2-(trimethylammonium)ethyl]methane thiosulfonate, or (2-sulfonatoethyl)methanethiosulfonate reversed, to a great extent, the pH shift conferred by E252C but had no effect on the $K_m$ of the mutant antiporter.

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 (reviews in Refs. 1–3).

*Escherichia coli* has two antiporters, NhaA (4) and NhaB (5), which specifically exchange Na$^+$ or Li$^+$ for H$^+$. Only NhaA is indispensable for adaptation to high salinity, for challenging toxicity, and for growth at alkaline pH (in the presence of Na$^+$ (1–3)).

NhaA is an electronegatic antiporter with a stoichiometry of 2H$^+$/Na$^+$ (6–8). Recently two-dimensional crystals of NhaA diffracting at 4 Å were obtained. Cryoelectron microscopy of these crystals showed that NhaA exists as a dimer of monomers composed each of 12 helices as predicted (9–11). In the native membrane NhaA forms oligomers within which the monomers physically and functionally interact (12). Based on the two-dimensional crystals, a three-dimensional map of NhaA was obtained (13), the first insight into the architecture of the protein.

One of the most interesting characteristics of NhaA is its dramatic dependence on pH; both in isolated membrane vesicles and when purified in proteoliposomes its rate of activity changes more than 3 orders of magnitude between pH 7 and 8 (3, 6). Amino acid residues involved in the pH response of NhaA have been identified in both loops and TMSs. For example, His$^{225}$ in loop VII–VIII was found essential for the pH response of the antiporter (14, 15). Gly$^{338}$ of TMS XI also affects the pH response of NhaA; its replacement with serine (G338S) produced a transporter that, in contrast to the wild type protein, lacks pH control (16).

NhaA undergoes a conformational change upon its activation by pH. Monoclonal antibody 1F6 raised against the NhaA antiporter (17) identified that the N terminus of NhaA, its epitope, responds to pH (18). The antibody binds NhaA at pH 8.5 but not at pH 4.5. Furthermore, H3C/H5C, a double mutation in this domain, changes the pH profile of NhaA (18).

Probing with trypsin digestion showed that Loop VIII–IX is another domain that changes its conformation with pH (19). Both in everted membrane vesicles as well as in DM micelles, NhaA is completely resistant to trypsin below pH 6.5 and with increasing pH is progressively cleaved at Lys$^{249}$, reaching a maximum at pH 8.5. Furthermore, two NhaA mutants (H225R (19) and G338S (16)) with a modified pH profile are susceptible to trypsin, in isolated membrane vesicles, at Lys$^{249}$, only at the pH range where they are active and reflecting the level of activity.

Two pieces of evidence suggest that Loop VIII–IX not only responds to pH but is also required for pH regulation: (a) Loop VIII–IX is located in the interface between monomers of NhaA dimer (12). Cross-linking between these loops of the NhaA dimer with a rigid and short cross-linking agent caused a dramatic change in the pH response as opposed to no effect of a long and flexible cross-linking reagent (12). (b) Insertion mutation Lys$^{249}$-IEG-His$^{250}$ and Cys replacement mutations E241C and V254C in loop VIII–IX cause an acidic shift in the pH profile of NhaA (19).

In addition to Glu$^{241}$, loop VIII–IX contains only one other Glu (at position 252). In this paper, we therefore, replaced Glu$^{252}$ with Cys and explored the properties of the E252C

*This work was supported first by a Israel Science Foundation Grant 501/03-16.2 administered by the Israel Academy of Sciences and Humanities and then by funds from the German-Israeli Foundation for Scientific Research and Development (to E. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of the Massimo and Adelina Della Ferra Chair in Life Sciences. To whom correspondence should be addressed. Tel.: 972-2-6585694; Fax: 972-2-6586947; E-mail: etana@vms.huji.ac.il.

‡ The abbreviations used are: TMS, transmembrane segment; NEM, N-ethylmaleimide; MIANS, 2-(4’-maleimidylanilino)-naphthalene-6-sulfonic acid; MTSET, [2-(trimethylammonium)ethyl]methane thiosulfonate; MTSES, (2-sulfonatoethyl)methanethiosulfonate; BTF, 1,3-bis[tris(hydroxymethyl)methyl]amino]propane; NTA, nitritotriacetic acid; MOPS, 3-N-morpholino)propanesulfonic acid; DM, α-dodecyl-β-D-maltoside; CL, Cys-less NhaA.
mutant both in the membrane and after solubilization and purification of the protein in DM. We found that in contrast to E241C and the other two mutations in loop VIII–IX that induce an acidic shift in the pH profile of activity of NhaA, the single amino acid change, E252C, causes a dramatic alkaline shift in the pH dependence of the Na\(^+/\)H\(^+\) antiporter activity of NhaA and increases drastically the \(K_m\) of the antiporter to both Na\(^+\) and Li\(^+\). In parallel, the E252C mutation also caused a similar alkaline shift in the pH dependence of the conformational change in loop VIII–IX. Remarkably, this conformational change at position E252C was found sensitive specifically to both Na\(^+\) and Li\(^+\), the specific ligands of the NhaA antiporter.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—EP432 is an *E. coli* K12 derivative, which is *melBld, Δ nhaAΔ::kan*, Δ nhaBΔ::cat, Δ lacZΔ, thy1 (5). TA16 is NhaA−/−nhaB−/−lacZΔ and otherwise isogenic to EP432 (6). The cells were grown in modified L broth (LBK) in which NaCl was replaced by KCl (67 mM, pH 7.5). When indicated, the media was buffered by 60 mM Tris. The cells were also grown in minimal medium A without sodium citrate (20) with glycerol (0.5%) as a carbon source. Thiamine (2.5 \(\mu\)g/ml) was added to all minimal media.

For EP432, threonine (0.1 mg/ml) was also added (4). For plates, 1.5% agar was used. The antibiotics and their concentrations were 100 \(\mu\)g/ml ampicillin and 50 \(\mu\)g/ml kanamycin. Resistance to Li\(^+\) and Na\(^+\) was tested as described previously (4).

**Plasmids**—The plasmids encoding wild type NhaA used in this study were as follows. pECO2, a derivative of pECO (21), was constructed by purification of the BstXI-BstXI fragment (5188 bp) obtained from pECO (21), teeming with T4 DNA polymerase and blunt end ligation and destroying the two BstXI sites of the plasmids. pBSTX is a derivative of pEC02 that contains a silent mutation introducing a BstXI site and only one of the two BstXI sites of the plasmids. pBSTX is a derivative of pEC02 that contains a silent mutation introducing a BstXI site and only one of the two BstXI sites of the plasmids.

**Site-directed Mutagenesis**—Site-directed mutagenesis was conducted following the polymerase chain reaction-based protocol (23). pBSTX-E252C and pCL-BSTX-E252C were constructed using plasmids pBSTX and pCL-BSTX as a template and the mutagenic primers described in Table I. In each case the entire fragment originated by PCR and cloned in a plasmid was sequenced through the ligation junction to verify the accuracy of mutagenesis. pAXH2-E252C and pCL-XH2-E252C were obtained by replacing the MluI-BglII fragments of plasmids pAXH2 and pCL-XH2 with MluI-BglII fragments excised either from pBSTX-E252C or pCL-BSTX-E252C to obtain the mutation in either wild type or CL genetic background respectively. The plasmids carrying the Cys mutations in loops were previously described (22).

**DNA Sequencing**—Sequencing of DNA was conducted by an automated DNA sequencer (ABI PRISM \(^{TM}\) 377; PerkinElmer Life Sciences).

**Isolation of Membrane Vesicles and Assay of Na\(^+/\)H\(^+\) Antipporter Activity**—Assays of Na\(^+/\)H\(^+\) antipporter activity were conducted on everted membrane vesicles of EP432 cells (24, 25) transformed with the respective plasmids. A fluorescence assay of antipporter activity was performed as described (25) using acridine orange to measure generation of \(\Delta\)pH (the pH difference across the membrane). Energization was achieved with either Tris-\(\alpha\)-lactate or ATP (1.6 mM each).

**Protein Determination**—The protein was determined according to Ref. 26.

**Detection and Quantitation of NhaA and Its Mutated Proteins in the Membrane**—Detection and quantitation of NhaA and its mutated derivatives in membranes of EP432 were conducted by Western analysis as described previously (14) using the NhaA-specific monoclonal antibody 1P6 (17). The amount of affinity-purified NhaA and its mutants was determined by Coomassie staining of the gel after SDS-PAGE as described previously (12).

**Overexpression and Affinity Purification of His-tagged Antiporters by Ni\(^{2+}\)-NTA Chromatography**—To overexpress the plasmids encoding the His-tagged antiporters, TA16 cells transformed with the respective plasmids were used as described (6), and high pressure membranes were prepared as described (6). His-tagged NhaA was affinity-purified on Ni\(^{2+}\)-NTA-agarose resin (Qiagen) by miniscale purification and eluted either with imidazole (10, 22) for probing digestion with trypsin or acid (8) for labeling with MIANs. For probing digestion with trypsin—For trypsin treatment (19), 15 \(\mu\)g of affinity-purified protein was resuspended in 0.5 ml containing 0.1 M KCl, 0.7 mM EDTA, 1 mM CaCl\(_2\), 0.1% DM, and 50 mM Tris-HEPES at the indicated pH values. After the addition of 0.3 \(\mu\)g of trypsin (Sigma, type III), the suspension was incubated at 37°C for 1 h. The reaction was terminated by adding 0.9 \(\mu\)g of trypsin inhibitor type II (Sigma) dissolved in 1 mM HCl (200 \(\mu\)g/ml). Then the protein was precipitated in 10% trichloroacetic acid for 0.5 h at 4°C, centrifuged (Eppendorf, 14,000 rpm, 30 min, 4°C), resuspended in sampling buffer, titrated to neutrality with Tris base, and loaded on the gel for SDS-PAGE as described (27).

**Treatment with SH Reagents**—Everted membrane vesicles or high pressure membranes were isolated from EP432 cells transformed with the indicated plasmids. The membranes (0.5 mg of membrane protein) were resuspended in a reaction mixture (0.5 ml) containing 5 mM MgSO\(_4\), 100 mM potassium phosphate (pH 7.5), 1 mM NEM (Sigma), and MTSES or MTSET (10 mM each) and incubated for 20 min at 25°C with gentle shaking. The reaction was stopped by the addition of 20 mM dithiothreitol and 3 ml of TSC solution containing 10 mM Tris/Cl (pH 7.5), 250 mM sucrose, and 140 mM choline chloride. The membranes were centrifuged (Beckman, TLA 100.4, 7500 rpm, 20 min, 4°C) and resuspended in the same buffer (5–10 mg of membrane protein/ml). For measurement of Na\(^+/\)H\(^+\) antiporter activity of the treated membranes, ATP was used to energize the membranes because NEM inactivates the lactate-dependent respiration.

To determine accessibility to NEM or other SH reagents, the membranes (0.5 mg of membrane protein) were resuspended in 1.15 ml of TSC supplemented with 14% glycerol, 1% DM, and 0.06 M MOPS (pH 7). The suspension was incubated for 20 min at 4°C and centrifuged (Beckman, TLA 100.2, 75000 rpm, 20 min, 4°C). The supernatant was added to 100 \(\mu\)l of Ni\(^{2+}\)-NTA-agarose (Qiagen) and incubated with agitation for 1 h at 4°C. The beads were then washed twice in binding buffer (10) at pH 7.4 and resuspended in 100 \(\mu\)l of membrane buffer containing 0.2 mM fluorescein 5-maleimide (Molecular Probes) with gentle tilting for 30 min at 25°C and washed in washing buffer (10) at pH 7.4. The protein was eluted in 20 \(\mu\)l of SDS-PAGE sampling buffer supplemented with 300 mM imidazole, agitated for 20 min at 4°C, and collected in the supernatant after centrifugation (Eppendorf, 14,000 rpm, 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 ultraviolet light (260 nm) as described (22).

**Labeling of NhaA Mutants with MIANs and Fluorescence Measurement**—For treatment with MIANs (Molecular Probes) the protein was affinity-purified on Ni\(^{2+}\)-NTA with acid elution. The reaction mixture (2.5 ml) contained 0.03% DM, 50 mM BTP/Cl at the indicated pH values.

**TABLE I**

| Mutation | DNA sequence of mutagenic oligonucleotide | Codon change observed | New restriction site |
|----------|------------------------------------------|----------------------|---------------------|
| E252C    | GCCACTGGTGTCACGGTGTTCACCC               | GAG → TGT           | Eco21               |
| A248     | GTTCTCCAGCGACGACTGGCATGGTGTG           | GGG → GCC           | BstXI               |

**end primer a**

CTGATCGAGGATCGCTAGCCAGC

**end primer b**

GAAGGCTGCGACGCTCCGGCCGCTAC

GCTCATTTCTCCTCCGTAGAAC

**end primer c**

GTTCTCCAGCCAAGCGACTGGAGCATGTG

**end primer d**

A248

CTGATCGAGGATCGCTAGCCAGC

**end primer e**

GAAGGCTGCGACGCTCCGGCCGCTAC

GCTCATTTCTCCTCCGTAGAAC

**end primer f**

GTTCTCCAGCCAAGCGACTGGAGCATGTG

**end primer g**

A248

CTGATCGAGGATCGCTAGCCAGC

**end primer h**

GAAGGCTGCGACGCTCCGGCCGCTAC

GCTCATTTCTCCTCCGTAGAAC

**end primer i**

GTTCTCCAGCCAAGCGACTGGAGCATGTG

**end primer j**

A248

CTGATCGAGGATCGCTAGCCAGC

**end primer k**

GAAGGCTGCGACGCTCCGGCCGCTAC

GCTCATTTCTCCTCCGTAGAAC
monitored with acridine orange at the indicated pH in a reaction mix-
orange, 150 mM KCl, 50 mM BTP, and 5 mM MgCl2. At the onset of the
affinity-purified protein (20
H9262
WT
EP432 cells transformed with plasmids
E252C at various pH values.
fluorescence quenching was recorded until a steady state level of
had been reached (100%). NaCl (10 mM) was then added (°
and MIANS (4 μm). The reaction was initiated by the addition of the
affinity-purified protein (20 μg of protein). Fluorescence was monitored
continuously at 22 °C with a spectrofluorometer (PerkinElmer Life
Sciences) using an excitation wavelength of 330 nm (8-nm slit) and an
emission wavelength of 415 nm (4-nm slit).

RESULTS

Cys Replacements Mutants of Loop VIII–IX—The Cys-less-
NhaA (CL-NhaA) is expressed similar to the wild type protein
and is as active (Fig. 1D and Refs. 10 and 22). We constructed
the Cys replacement mutation E252C in loop VIII–IX of both
wild type and CL-NhaA. The mutated NhaA proteins were
designated E252C and CL-E252C, respectively. To characterize
the phenotypic properties conferred by the NhaA mutations,
the mutant plasmids were transformed into EP432, a

nhaB strain. This strain can grow in high Na⁺-selective media
(0.6 mM NaCl at pH 7 or 8.3) only when it expresses a functional
NhaA. Hence, when transformed with plasmids bearing NhaA
mutations, this host allows characterization of the effect of the
mutations on cell and membrane phenotype.

Fig. 1D shows that both mutants of NhaA, E252C and CL-
E252C, were expressed in EP432 cells, even better than the
wild type and the CL-NhaA, their respective controls. As
compared with E252C mutant, CL-E252C mutant showed some-
what lower expression (Fig. 1D).

When Na⁺ was not added to the growth medium, the growth
at both pH 7 and 8.3 of EP432 expressing either E252C or
CL-E252C was similar to that expressing the wild type anti-
porter or its CL derivative (data not shown). In the selective
medium, the growth of EP432 expressing E252C was very
similar to that expressing the wild type protein with respect to
both number and size of the colonies (Table II). The colony size
of both strains was slightly smaller at alkaline pH than at
neutral pH (Table II). On the other hand, the mutant CL-
E252C conferred preferential growth at alkaline pH, although
similar at both pH 7 and pH 8.3, the number of colonies of all
strains was similar, the colony size of cells expressing CL-E252C
was very small at pH 7, but at alkaline pH, it was even larger than
that of wild type, CL-E252C, and E252C (Table II). It is not
clear why CL-E252C but not E252C exhibits a pH-shifted
growth phenotype.

E252C Shifts the pH Dependence of the Na⁺/H⁺ Antiporter Activity of NhaA to the Alkaline Range—To determine the
Na⁺/H⁺ antiporter activity, everted membrane vesicles were
isolated from EP432 expressing either E252C or CL-E252C.
The Na⁺/H⁺ antiporter activity was measured at various pH
values. The assay was based on the measurement of the ΔpH
maintained across the membrane by respiration or H⁺/ATPase
as determined from fluorescence quenching of acridine orange.
The Na⁺/H⁺ antiporter activity was assessed from the de-
quenching caused by the addition of Na⁺. Membranes derived
from EP432 cells transformed with the vector plasmid have no
Na⁺/H⁺ antiporter activity (data not shown and (5)). Transfor-
mation with a plasmid expressing wild type NhaA restores
Na⁺/H⁺ antiporter activity (5) that as shown previously
changes dramatically as a function of pH (Refs. 3 and 6 and
Figs. 1, A–C, and 2). The results show that both NhaA mutants,
E252C and CL-E252C, behave differently; at pH 7 where wild
type NhaA shows between 15 and 20% of its maximal activity,
both E252C mutants do not show any activity (Figs. 1A and 2).
Furthermore, at pH 8 (in the presence of 10 mM Na⁺) when the
wild type exhibits 100% activity (Figs. 1B and 2), E252C and
CL-E252C mutants show about 30% of their maximal activity

FIG.1 .

| Encoded NhaA variant | Plasmid          | Growth (0.6 M NaCl) | K m (pH 8) |
|----------------------|------------------|--------------------|------------|
|                      |                  | pH 7               | pH 8.3     | NaCl | LiCl |
| NhaA                 | pBR322           | + + +              | +         | 0.2  | 0.02 |
| CL-NhaA              | pBSTX            | + + +              | +         | 0.2  | ND   |
| E252C                | pCL-BSTX         | + + +              | +         | 9.9  | ND   |
| CL-E252C             | pCL-BSTX-E252C   | + + +              | +         | 11.3 | 4    |

\[\text{TABLE II}\]

Cell and membrane phenotype of E252C and CL-E252C mutations
EP432 cells transformed with the indicated plasmids were grown on
selective agar plates containing 0.6 mM NaCl at either pH 7 or pH 8.3. —, no growth on selective agar. + + +, wild type growth with respect to
both number and size of colonies; + +, number of colonies identical
but colonies size progressively smaller. The Na⁺/H⁺ antiporter activity was determined in everted membrane vesicles, and the K m values for Na⁺ and Li⁺ were determined. All of the experiments were repeated at least three times, and the results were essentially identical. ND, not determined.
as observed at pH 9 (Figs. 1, B and C, and 2). The results suggest that whether in a wild type or CL genetic background, the E252C mutation causes a drastic alkaline shift, of 1 pH unit, in the pH dependence of NhaA.

The Effect of E252C on the $K_m$ of NhaA—The $K_m$ values for Na$^+$ of the Na$^+$/H$^+$ antiporter activity of E252C and CL-E252C mutants as compared with those of the wild type were measured at both pH 8 (Table II) and pH 8.5 (data not shown). At both pH values, the apparent $K_m$ for the Na$^+$ ion (10 mM) of both mutants was drastically increased as compared with the wild type (0.2 mM). The $K_m$ for Li$^+$ was also increased by the mutations, but the effect was less drastic than that for Na$^+$ (Table II). The results summarized in Fig. 2 show that the drastic shift in the pH profile of the Na$^+$/H$^+$ antiporter activity of both E252C and CL-E252C mutants is maintained even at saturating Na$^+$ concentrations. Hence, the mutation E252C has an effect on the pH dependence of NhaA that is independent of its effect on the $K_m$ of the antiporter.

E252C Causes an Alkaline Shift to the pH-dependent Conformational Change of NhaA as Probed by Trypsin—Although NhaA has many trypsin-cleavable sites, trypsin digests NhaA, either in the native membrane or when purified in DM micelles, only at Lys$^+$ in loop VIII, A118C of loop III, IV (22), or H225C of loop VII (21). The accessibility of E252C (data not shown) as compared with that of NhaA was also increased by the mutations. We therefore tested accessibility of E252C in isolated membrane vesicles and in the pure protein (in DM micelles) to various SH reagents. For control we used other single Cys replacements that also reside in NhaA loops but, in contrast to E252C, do not affect the pH response of NhaA.

Several SH reagents have been used to modify Cys replacement mutations to determine accessibility, including maleimides (10, 29–31) and MTS reagents (32, 33). Modification of Cys by these reagents depends upon the ionization of the Cys sulfhydryl group (38, 39) but becomes fluorescent only when its maleimide group preferentially in an aqueous environment and to depend on pH, given that the $pK_a$ of ionization of the sulfhydryl residue falls given by these reagents depends upon the ionization of the Cys sulfhydryl group (38, 39) but becomes fluorescent only when its maleimide group preferentially in an aqueous environment and to depend on pH, given that the $pK_a$ of ionization of the sulfhydryl residue falls.

To further probe the accessibility of E252C, we used MIANS, a larger and negatively charged maleimide reagent that like NEM reacts specifically and covalently with thiol groups (38, 39) but becomes fluorescent only when its maleim-
ide group reacts with Cys (40). We first tested MIANS labeling of Cys replacements in loops that have no effect on either the pH dependence of activity or conformation of NhaA as probed by trypsin. The reaction of MIANS with purified CL-NhaA containing single Cys residues H225C in loop VII–VIII (Fig. 4) can readily be followed fluorimetrically. Above pH 6.5 the rate of the reaction with MIANS increased with pH reaching a maximal rate at pH 8.5 and maximal fluorescence within 200–300 s (Fig. 4A). The results obtained at pH 9 were very similar to those obtained at pH 8.5, and increasing the concentration of MIANS had no effect on maximal fluorescence level, suggesting that all Cys residues had been modified. Very similar pattern of reaction with MIANS was obtained at position K221C of loop VII–VIII and N177C of loop V–VI (data not shown). Furthermore, the pH dependence of the reaction at these positions was very similar to that of the reaction with NEM (data not shown).

However, a striking difference was observed in the pH de-
Mutation E252C Increases $K_m$ and Shifts the pH Response of NhaA

The Rate of Labeling of E252C by MIANS Is Increased Significantly and Specifically by Na$^+$ and Li$^+$—Because E252C increases the $K_m$ of the antiporter to its specific ligands, Na$^+$ and Li$^+$, we tested the effect of Na$^+$ on the rate of labeling of the NhaA protein with MIANS. In these experiments the ion was added at the indicated concentrations to the reaction mixture used for MIANS labeling (Fig. 5). For controls we used Cys replacements in loops that affect neither the pH response nor the specific labeling of NhaA. An example, CL-K221C of loop VII–VIII, is shown in Fig. 5A. It is evident that both Na$^+$ and Li$^+$ could be interpreted as an increase in quantum yield of E252C caused by the E252C Mutation—Most likely because of the Cys replacement at position E252, a negative charge was removed from loop VIII–IX of NhaA. Therefore, we modified E252C membrane vesicles expressing the NEM-treated CL-E252C protein with each type of added ion. Whereas K$^+$ (20 mM) had no effect, Na$^+$ (10–20 mM) (Fig. 5B) or Li$^+$ (10 mM; data not shown) significantly enhanced the rate of labeling of E252C.

The increase in MIANS fluorescence caused by Na$^+$ and Li$^+$ could be interpreted as an increase in quantum yield of E252C bound MIANS or as an increase in accessibility of the protein to MIANS. We prefer the latter alternative because we did not find any change in the absorption or emission spectrum of the fluorophore. Chemical Modifications of E252C Alleviate the pH Shift Caused by the E252C Mutation—Most likely because of the Cys replacement at position E252, a negative charge was removed from loop VIII–IX of NhaA. Therefore, we modified E252C membranes by various SH reagents that chemically modify Cys while introducing either a negative charge (MTSET) or positive charge (MTSET) or that cause neutral modification (NEM) and measured the effect of the chemical modification both on the Na$^+/\mathrm{H}^+$ antiporter activity of the protein and its sensitivity to trypsin as a function of pH. The results summarized in Fig. 6A show the Na$^+/\mathrm{H}^+$ antiporter activity at pH 8 of NEM-treated CL-E252C membranes as compared with the untreated mutant or wild type membranes. It is evident that alkylation by NEM suppressed significantly the phenotype conferred by the mutation. Thus, at pH 8 when the activity of the wild type is maximal and untreated CL-E252C is very low, NEM treatment increases the activity of CL-E252C by about 3-fold. Fig. 6B shows that the effect of NEM on CL-E252C was saturated at 1 mM NEM.

We then explored the pH dependence of the Na$^+/\mathrm{H}^+$ antiporter activity of the NEM-treated CL-E252C membranes (Fig. 7). It is evident that the alkaline shift conferred by E252C mutation on the pH dependence of activity was, to a great extent, alleviated in the NEM-treated membranes. However, NEM treatment did not change the $K_m$ of E252C.

Most importantly, similar to the effect on the antiporter activity, NEM treatment alleviated the alkaline pH shift conferred by the mutation on the pH-dependent conformational change as probed by trypsin (Fig. 3C). The pH dependence of the digestion by trypsin of the NEM-treated CL-E252C protein was very similar to that of the wild type (Fig. 3A). We conclude...
that NEM alkylation of E252C reversed to a great extent the effect of the mutation on the pH response of the antipporter as reflected in the pH dependence of activity and the conformational change of the protein.

Treatment by MTSET and MTSES reagents had the same effect on E252C as that of NEM treatment (data not shown). Therefore, changing the native structure by the mutation E252C had a dramatic effect on the pH response of NhaA that could be suppressed by a chemical modification without restoring a negative charge at that position.

**DISCUSSION**

The pH regulation of NhaA (41), as of other both eukaryotic (42–44) and prokaryotic Na\(^+/\)H\(^–\) antiporters (2, 45–47), involves pH sensors and conformational changes in different parts of the protein. Accordingly, many amino acids in various domains of the protein participate in this regulation. Hence, to understand the mechanism underlying the pH regulation of NhaA, it is essential to identify the amino acid residues and domains involved and to elucidate the pH-induced conformational changes. In the present work, we have identified a novel mutation, E252C in loop VIII, that has a very drastic effect on the pH response of NhaA. Whereas other mutations in this loop (E241C and V254C (19)) and other loops (15, 18) cause acidic shift in the pH dependence of the activity of NhaA, Cys replacement mutation E252C causes an alkaline shift of one pH unit. As yet, such an alkaline shift of the pH response of NhaA has previously been found only in mutations at position 127 in the middle of TMS IV (21).

Furthermore, we show here that position E252C undergoes a pH-induced conformational change together with position Lys\(^{249}\), revealing that the pH-induced conformational change of the NhaA antipporter extends along a segment of loop VIII–IX. Using accessibility to digestion by trypsin to probe pH-induced conformational changes in NhaA, we have previously found that position Lys\(^{249}\) in loop VIII–IX changes its conformation with pH, in a pattern that reflects the pH profile of the antipporter activity (19, 28). Using the same approach, we show here that mutation E252C shifts drastically, by one pH unit (to the alkaline range), the pH dependence of the conformational change probed by trypsin at position Lys\(^{249}\) (Fig. 3). Because probing by trypsin is limited to the trypsin-cleavable sites that exist along the protein molecule, this result can be interpreted in two ways: (a) positions Lys\(^{249}\) and E252 in loop VIII–IX undergo a similar pH-induced conformational change and (b) E252C affects indirectly the conformation at position Lys\(^{249}\) rather than participating in the conformational change. It thus has become crucial for the study of the pH-induced conformational changes in NhaA to introduce a probe that monitors conformational changes in a site-directed fashion.

In the present work, we show that accessibility to MIANS of nested Cys replacement mutations is a very good tool to probe, in a site-directed manner, pH-induced conformational changes in loops of NhaA. MIANS, a sulfhydryl reagent that reacts relatively specifically and covalently with thiol groups, is fluorescent only when the maleimide group reacts (38–40). It has previously been used to probe accessibility and conformational changes in various membrane proteins (see for examples Refs. 39 and 48–51). In Fig. 4C, the labeling rate by MIANS of Cys replacement E252C is compared with that of Cys replacement H225C. In contrast to E252C, H225C is a Cys replacement mutation in loop VII–VIII that does not affect the pH response of NhaA (22). The pH profile of the reactivity of the two proteins with MIANS was found to be very different; the pH dependence of reactivity to MIANS of E252C was shifted to the alkaline range by one pH unit as compared with that of H225C. Two other Cys replacement mutations, K221C in loop VII–VIII and N177C in loop V–VI, that, similar to H225C, do not affect the pH regulation of NhaA, behaved similarly to H225C (data not shown). Most importantly, up to pH 9, CL-NhaA did not react with MIANS (Fig. 5B). Hence, the reaction with MIANS is specific to the Cys replacement mutations.

Because the chemical reaction with MIANS depends on the ionized form of the sulfhydryl residue, it is possible that the pK of the Cys in the E252C mutant is different from that of the other Cys replacements tested in the other loops, because of a difference in the immediate environment of E252C in the protein. However, at all pH values, we did not find any difference in either the absorption or emission spectrum (properties that are sensitive to the immediate environment of the fluorophore (39, 52, 53)) of the E252C-bound MIANS (data not shown). Although we cannot exclude the possibility of a change in pK or a difference in the stereospecificity of the reaction with MIANS at position E252C, we prefer an alternative explanation: E252C changes its conformation with increasing pH and moves from a MIANS occluded to a MIANS accessible site. Hence, the pH dependence of the accessibility to MIANS at position E252C reflects a pH-induced change in the conformation at position E252C.

The revealed pH response of mutant E252C strongly supports our contention that loop VIII–IX is involved in the pH regulation of the activity of NhaA by changing its conformation with pH (19). It also shows that the pH-induced conformational change extends along the loop. Thus, two sites (Lys\(^{249}\) and E252C) in loop VIII–IX of mutant E252C are shown to change conformation with pH (Figs. 3B and 4B). Furthermore, the pH dependence of the pH-induced conformational change of E252C reflects the pH dependence of the Na\(^+/\)H\(^–\) exchange activity of mutant E252C (Figs. 1 and 2).

Remarkably, in addition to its effect on the pH-induced conformational change, the mutation E252C increases dramatically, by 10-fold, the \(K_m\) of NhaA to Na\(^+\) (Table II). A somewhat lower but significant increase in \(K_m\) was also noted for Li\(^+\). Hence, E252C is the first known mutation in a loop of NhaA that affects the \(K_m\) of the antipporter. Most interestingly, addition of Na\(^+\) (or Li\(^+\)) increases significantly the rate of accessibility to MIANS at position E252C (Fig. 5B). Four lines of evidence lead us to suggest that it is the binding of Na\(^+\), the specific ligand, that affects the conformational change at position E252C: (a) the effect is specific to Na\(^+\), the specific substrate of NhaA and is not a general effect of ionic strength, because K\(^+\) has no effect (Fig. 5B); (b) the concentration of the ion that gives half-maximum increase in the rate of MIANS labeling (Fig. 5C) is within the range of the respective \(K_m\) value of the antipporter activity of E252C mutant (Table II); (c) the effect of Na\(^+\) is observed at the pH range (pH 8.5–9) in which E252C is active (Figs. 1 and 2), and hardly any effect of the ion was discerned at pH 8; and (d) accessibility to MIANS of Cys replacements in other loops (that do not affect either the \(K_m\) or pH response of NhaA) is not sensitive to the presence of Na\(^+\) (Fig. 5A). Our results show that E252C affects both the \(K_m\) and the pH-dependent conformational change of NhaA, and in turn, binding of Na\(^+\) changes the rate of the pH-induced conformational change at position 252. Hence, the study of E252C has provided the first direct evidence that binding of the ligands of the antipporter and the pH-induced conformational change of the antipporter are related.

Although tested previously, we could not find any effect of Na\(^+\) on the pH-induced conformational changes of wild type NhaA. This was most likely due to Na\(^+\) contaminants that persisted in all media to a level similar to that of the \(K_m\) (around 0.2 mM; Table II) of NhaA. In addition the MIANS accessibility assay is much more sensitive than the other meth-
Mutation E252C Increases $K_m$ and Shifts the pH Response of NhaA

E252C is the first mutation in a loop that affects both the pH response of NhaA and its $K_m$ to Na$^+$.

There are many examples including that of NhaA (21) of residues located in TMS that affect the apparent $K_m$ for substrates of transporters. These have been implicated to reside in the translocation passage of the respective substrates (see for example Ref. 30). However, recently more and more examples (36, 54) of residues in loops were found that, similar to E252C in NhaA, affect the kinetics and other parameters of the transporters. It is clear that atomic resolution of NhaA as of the other transporters is needed to get a glimpse as to how and, whether directly or indirectly, loops participate in the translocation mechanism.

The effect of E252C on the pH profile of NhaA cannot be ascribed to a missing single negative charge that changes the overall charge of loop VIII–IX because E241C, a mutation in another Glu in the same loop, causes an acidic rather than alkaline shift in the pH profile of NhaA (19). It can be argued that, specifically at position 252, the negative charge is required to obtain the wild type pH profile. This is also unlikely because chemical modifications of E252C by MTS reagents that add either a negative (MTSES) or a positive charge (MTSET) at position 252 or NEM that does not add any charge upon modification all partially shift the pH profile back to the wild type pH profile (Fig. 7). Hence, it is the presence of the Cys replacement at position 252 that causes the alkaline shift, whereas its modification by various agents reverses the pH profile. We therefore suggest that the size and/or stereospecificity of the residue at position 252 is crucial for the pH response of NhaA.

Acknowledgment—The Moshe Shilo Minerva Center for Biochemistry is acknowledged.

REFERENCES
1. Padan, E., and Schuldiner, S. (1996) in The Handbook of Biological Physics (Konings, W. N., Kaback, H. R., and Leikina, J., eds) Vol. II, pp. 501–531, Elsevier Science, The Netherlands.
2. Padan, E., and Kruithof, T. A. (2000) in Bacterial Stress Responses (Storz, G., and Karmazyn, M., Avkiran, M., and Fliegel, L., eds) pp. 35–47, Academic Publishers, Amsterdam, The Netherlands.
3. Padan, E., Venturi, M., Gerchman, Y., and Dover, N. (2001) Biochem. Biophys. Res. Commun. 284, 1729–1734.
4. Pinnin, E., Kotler, Y., Padan, E., and Schuldiner, S. (1989) J. Biol. Chem. 264, 2619–2623.
5. Taglicht, D., Padan, E., and Schuldiner, S. (1991) J. Biol. Chem. 266, 11289–11294.
6. Taglicht, D., Padan, E., and Schuldiner, S. (1993) J. Biol. Chem. 268, 5382–5387.
7. Venturi, M., and Padan, E. (2002) in A Practical Guide to Membrane Protein Purification (Hunte, C., Van Jagow, G., and Schagger, H., eds) 2nd Ed., pp. 179–190, Academic Press, Amsterdam, The Netherlands.
8. Rothman, A., Padan, E., and Schuldiner, S. (1997) J. Biol. Chem. 272, 1761–1768.
Mutation E252C Increases Drastically the $K_m$ Value for Na$^+$ and Causes an Alkaline Shift of the pH Dependence of NhaA Na$^+$/H$^+$ Antiporter of *Escherichia coli*

Tzvi Tzubery, Abraham Rimon and Etana Padan

*J. Biol. Chem.* 2004, 279:3265-3272.

doi: 10.1074/jbc.M309021200 originally published online November 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M309021200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 25 of which can be accessed free at http://www.jbc.org/content/279/5/3265.full.html#ref-list-1