A pH-dependent Conformational Change of NhaA Na\(^+\)/H\(^+\) Antiporter of Escherichia coli Involves Loop VIII–IX, Plays a Role in the pH Response of the Protein, and Is Maintained by the Pure Protein in Dodecyl Maltoside*

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Digestion with trypsin of purified His-tagged NhaA in a solution of dodecyl maltoside yields two fragments at alkaline pH but only one fragment at acidic pH. Determination of the amino acid sequence of the N terminus of the cleavage products show that the pH-sensitive cleavage site of NhaA, both in isolated everted membrane vesicles as well as in the pure protein in detergent, is Lys-249 in loop VIII–IX, which connects transmembrane segment VIII to IX. Interestingly, the two polypeptide products of the split antiporter remain complexed and co-purify on Ni\(^2+\)-NTA column. Loop VIII–IX has also been found to play a role in the pH regulation of NhaA; three mutations introduced into the loop shift the pH profile of the Na\(^+\)/H\(^+\) antiporter activity as measured in everted membrane vesicles. An insertion mutation introducing Ile–Glu–Gly between residues Lys-249 and Arg-250 (K249-I250) and Cys replacement of either Val-254 (V254C) or Glu-241 (E241C) cause acidic shift of the pH profile of the antiporter by 0.5, 1, and 0.3 pH units, respectively. Interestingly, the double mutant E241C/V254C introduces a basic shift of more than 1 pH unit with respect to the single mutation V254C. Taken together these results imply the involvement of loop VIII–IX in the pH-induced conformational change, which leads to activation of NhaA at alkaline pH.

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).

*Escherichia coli* has two antiporters, NhaA (5) and NhaB (6), which specifically exchange Na\(^+\) or Li\(^+\) for H\(^+\) (3). NhaA is indispensable for adaptation to high salinity, for challenging pH profile of the Na\(^+\)/H\(^+\) antiporter, and is active; G338S, which lost pH control, is active and exposed to dium tolerance to the cells, but becomes essential when the lack of NhaA activity limits growth (11).

Both the NhaA and NhaB are electrogenic antiporters that have been purified to homogeneity and reconstituted in a functional form in proteoliposomes (12–14). The H\(^+\)/Na\(^+\) stoichiometry of NhaA is 2H\(^+\)/Na\(^+\) and that of NhaB 3H\(^+\)/2Na\(^+\); NhaB but not NhaA is sensitive to amiloride derivatives, and the rate of activity of NhaA but not of NhaB is drastically dependent on pH, changing its V\(_{\text{max}}\) over 3 orders of magnitude from pH 7 to pH 8 (12).

Interestingly, a strong pH sensitivity is characteristic of antiporters as well as other transporters that are involved in pH regulation (reviewed in Ref. 4). Identifying the amino acid residues involved in the pH sensitivity of these proteins is important for understanding the mechanism of pH regulation. NhaA contains eight histidines, none of which were found essential for the Na\(^+\)/H\(^+\) antiporter activity of NhaA (15). However, replacement of histidine 225 by Arg (H225R) suggested that His-225 has an important role in the pH sensitivity of the antiporter. Whereas the activation of the wild-type NhaA occurs between pH 7.5 and pH 8, that of H225R antiporter occurs between pH 6.5 and pH 7.5. In addition, while the wild-type antiporter remains almost fully active, at least up to pH 8.5, H225R is reversibly inactivated above pH 7.5, retaining only 10–20% of the maximal activity at pH 8.5 (15). Furthermore, replacement of His-225 with either cysteine (H225C) or serine (H225S) but not alanine (H225A) yielded an antiporter with a wild-type pH-sensitive phenotype, implying that polarity and/or hydrogen bonding, the common properties shared by His, Cys, and Ser, are essential at position 225 for pH regulation of NhaA (16). Glycine 338 affects the pH response of NhaA; its replacement with serine (G338S in TMS1 XI) produced a transporter, which in contrast to the wild-type protein lacks pH control; it is active between pH 6.5 and 8.5 (17).

Recently, we have found that NhaA undergoes a conformational change upon its activation by pH which can be probed by trypsin (18). At acidic pH the protein in everted membrane vesicles is completely resistant to trypsin, while at alkaline pH it is digested in a pattern reflecting the pH profile of the antiporter activity. Furthermore, two mutants with a modified pH profile are susceptible to trypsin in isolated membrane vesicles only at the pH range, where they are active and reflecting the level of activity (18). H225R, the mutant with a pH profile shifted toward acidic pH, is digested at the pH where it is active; G338S, which lost pH control, is active and exposed to

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1 The abbreviations used are: TMS, transmembrane domain; DM, n-dodecyl β-maltoside; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Bis-Tris propane, 1,3-bis[(tris(hydroxymethyl)methylamino)propane].

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trypsin throughout the entire pH range of activity. Although NhaA has many trypsin-cleavable sites, only two main fragments were observed following digestion of isolated membrane vesicles at alkaline pH. This observation suggests that only one cleavage site is exposed by pH while all the other sites are masked. It was therefore inferred that the trypsin cleavage site is located in, and therefore serves as a tag of, that part of the protein which undergoes a conformational change in response to pH. Identification and study of this site was therefore undertaken in this study. The results show that loop VIII–IX is important for the pH regulation of NhaA and bears the trypsin cleavage site, which is involved in the pH-dependent conformational change of NhaA. This change is maintained by the pure protein in DM.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions—**EP432 is an E. coli K12 derivative, which is melBldl, ΔnhaA1-cat, ΔnhaB1:cat, ΔlacZ, thr1 (11). TA16 is nhaA::nhaB lacZ and otherwise isogenic to EP432 (12). DH5α (U. S. Biochemical Corp.) and JM109 were used as hosts for construction of plasmids. Cells were grown in modified L broth in which NaCl was replaced by KCl (Ref. 7; 87 mM, pH 7.5). Where indicated, the medium was buffered by 60 mM Bis-Tris propane, and pH was titrated with HCl. Cells were also grown in minimal medium A without sodium citrate (19) with either glyceral (0.5%) or melibiose (10 mM) as a carbon source. Thiamine (2.5 μg/ml) was added to all minimal media. For plates, 1.5% agar was used. Antibiotics were 100 μg/ml ampicillin, and/or 25 μg/ml kanamycin, and/or 12 μg/ml chloramphenicol, and/or 12.5 μg/ml tetracycline. Resistance to Li⁺ and Na⁺ was tested as described previously (15).

**Plasmids—**pGM36 and pGMAR100 are pBR322 derivatives (17, 20); the first bears nhaA and most of the nhaR gene. The latter carries nhaA and C-terminal truncated nhaB. pPAR100 is a pACYC184 derivative, which carries an insertion identical to that of pGMAR100 (17). pPAR4 is a pACYC184 derivative, which carries lacZ′ (kindly provided by E. Bibi, Weizman Institute of Science, Rehovot, Israel).

pPAH is a plasmid carrying Xa-His-tagged NhaA. It was constructed previously (21) and contains NhaA fused at its N terminus to the tac promoter for over expression and at its C terminus to a sequence encoding in tandem two factor Xa protease cleavage sites and 6 His. pPMXH was constructed by digestion of pPAH by MluI and XhoI, followed by end filling with T4 DNA polymerase and self-ligation of the 4960-base pair fragment. The resulting plasmid carries nhaA fusion encoding NhaA, of which RRPSV C terminus is replaced by EHHHHH.

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

In the case of E241C and V254C, the resulting mutated DNA (1295 base pairs) was digested with NheI and MluI, yielding a fragment of 879 base pairs, which was ligated either to the 4436-base pair NheI-MluI fragment of pGMAR100 to yield plasmids pV254C or pE241C or to the 4139-base pair NheI-MluI fragment of pAXH to yield plasmids pV254C-XH or pE241C-XH. In the case of ΔLys-242–His-253, the resulting 1259-base pair polymerase chain reaction fragment was digested as above to yield an 820-base pair fragment and cloned as above to yield pGMAR100 derivative pΔLys-242–His-253. In the case of the IEG insert between Lys-249 and Arg-250 (K249-IEG-R250), the 1304-base pairs polymerase chain reaction fragment was digested as above to yield an 888-base pair fragment and cloned as above into both pGMAR100 and pAXH to yield plasmids pK249-IEG-R250 (5324 base pairs) or pLys-249-IEG-R250A-XH (5027 base pairs, respectively).

**Isolation of Membrane Vesicles, Assay of Na⁺/H⁺ Antiporter Activity, and Quantitation of NhaA in the Membranes—**Assays of Na⁺/H⁺ antiporter activity were conducted on everted membrane vesicles (23). The assay of antiporter activity was based upon the measurement of Na⁺ (or Li⁺)-induced changes in the A447 as described (5, 24).

High pressure membrane vesicles were prepared essentially as everted membrane vesicles but the pressure used was 20,000 p.s.i. (French pressure cell press; SLM Aminco).

Quantitation of the NhaA and its mutated derivative in membranes was determined by Western analysis as described previously (16).

**Overexpression and Affinity Purification of His-tagged Antiporters—**To overexpress the wild-type and the mutated antiporters, plasmids overexpressing the His-tagged proteins in TA16 cells were used. The transformed cells were grown in minimal medium to A600 0.6, induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside, grown for 2 h to A600 1.2, harvested (12), and used for preparation of high pressure membranes either after storage overnight at 4°C or after freezing at −20°C. Xa-His-tagged NhaA was affinity-purified on Ni⁺⁻NTA-agarose column (Qiagen, Hilden, Germany) as described (17).

**Digestion by Trypsin—**Purified antiporter was subjected to trypsin in a 30-μl reaction mixture containing 10 μg of antiporter protein, 30 ng of trypsin (type III from bovine pancreas, Sigma T-8253), 0.1% DM, 8.3 mM potassium acetate, 200 mM KCl, 6.5% glycerol, 0.7 mM NaEDTA, 20 mMK$_2$HPO$_4$ (pH 8 if not indicated otherwise), 1 mM CaCl$_2$. Incubation was for 30 min at 37°C. The reaction was terminated by the addition of 100 ng of trypsin inhibitor (type II-S from soybean, Sigma T-9128). Samples of 5 μg of protein were run on SDS-PAGE (17).

Digestion of membrane vesicles (200 μg of protein, 4 μg of trypsin) was carried out in 100 μl of reaction mixture containing 140 mM KCl, 10 mM Tricine (pH 8), and 0.5 mM MgCl$_2$ and incubation conducted for 1 h at 37°C. This reaction mixture, which is also used for the Na⁺/H⁺ antiporter activity assay, gave similar digestion pattern and was occasionally used also with pure proteins as indicated.

**Separation and Isolation of NhaA Tryptic Fragments and Determination of the Amino Acid Sequence of Their N Termi—**The protein sample (30 μg) was resuspended in SDS-PAGE sampling buffer and loaded on 12.5% bisacrylamide gel. After separation the polypeptides were transferred (400 mA for 60 min) to polyvinylidene difluoride type (Millipore Immobilon™-P) transfer membranes in transfer buffer containing 25 mM Tris, 192 mM glycine, 10% methanol, 0.025% SDS (pH 8.4). The filters were then washed in distilled water, stained for 5 min in 0.1% Coomassie (R-250) in 50% methanol, destained for 5–10 min in 10% acetic acid in 50% methanol, and washed for 5–10 min in distilled water. The stained bands were cut and subjected to N-terminal se

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**TABLE I**

*Primers used for construction of NhaA mutants*

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Mutagenic primera</th>
<th>Locationb</th>
<th>Codon change</th>
<th>New restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>E241C</td>
<td>TTCTTTGAAAtgtAAGCATGGGCG</td>
<td>710-735</td>
<td>GAG → TGT</td>
<td>None</td>
</tr>
<tr>
<td>V254C</td>
<td>TTCTTTGAAAtgtAAGCATGGGCG</td>
<td>751-778</td>
<td>GTG → TGC</td>
<td>StyI</td>
</tr>
<tr>
<td>Δ(K242-H253)</td>
<td>ACCTCGAGACACGGCGGTTCGG</td>
<td>707-723</td>
<td>Codons to amino acids</td>
<td>SaaI, SgrAI</td>
</tr>
<tr>
<td>K249-IEG-R250</td>
<td>TCTCGAGACACGGCGGTTCGG</td>
<td>737-747</td>
<td>Insert codons for amino acids</td>
<td>As above</td>
</tr>
<tr>
<td>End primer</td>
<td>TCTCTCTCTGGAGATATAGTCGATGATTCGGGCG</td>
<td>748-760</td>
<td>242-253 deleted</td>
<td>SaII, XhoI</td>
</tr>
</tbody>
</table>

a All primers start at 5′. Mutated codons are shown in lowercase. The mutated codons introducing new restriction site are underlined and indicated consecutively from the 5′ end.

b Locations are relative to the initiation codon. The nhaA sequence appears in the GenBank™ data base (accession no. J03879).
obtained the trypsin inhibitor at time 0. 

Trypsin digest at pH 6.5, but the former purified in DM (0.1%), subjected to trypsin inhibitor added at time 0. H.F., heavy fragment; L.F., light fragment. B, lane a, molecular size markers; lanes b and c contained samples of the trypsin digest at pH 6.5, but the former obtained the trypsin inhibitor at time 0. The SDS-PAGE was run for 90 min.

**RESULTS**

The pH Profile of Trypsin Digestion of Pure Xa-His-tagged NhaA in DM—For affinity purification of NhaA, we have previously engineered NhaA fused at its C terminus to two factor Xa cleavage sites and 6 His residues (designated henceforth Xa-His-tagged NhaA instead of pYG10; Ref. 21). Trypsin digestion of native NhaA (18) and Xa-His-tagged NhaA (17) in everted membrane vesicles showed identical pH profile, which is similar to that of the activity of the antiporter, and thus has been suggested to reflect a native active conformational change of the antiporter. Both activities are shut off at acidic pH and increase above pH 7 to reach the maximum at pH 8.5. The digestion products of each protein are two main fragments similar in size. Given that the trypsin digestion pattern probes a native conformation of the protein, it was interesting to use trypsin digestion to test whether this conformation of NhaA is maintained by the pure protein in DM.

Xa-His-tagged NhaA was affinity-purified in DM, subjected to digestion by trypsin at various pH values, and the products separated on SDS-PAGE (Fig. 1A). Upon alkalization, two protein fragments appear in a pH-dependent fashion, similar in size to that previously obtained by trypsin digestion of isolated membrane vesicles (17). In the acidic pH digest, a single fragment was observed, suggesting that the cleavage site, which splits the pure protein at alkaline pH, is masked. Hence, the main cleavage site of trypsin that is exposed by pH and splits NhaA, each of the two fragments (heavy and light) obtained from the trypsin digest of Xa-His-NhaA at alkaline pH (Fig. 1) were isolated from the gels and subjected to N-terminal sequencing (Fig. 2). The N terminus of the heavy fragment was found (with less than 1% contaminations) identical to that of the native protein. The light fragment fraction contained mainly (about 90%) a fragment with a N-terminal sequence that overlaps a sequence introduced by the Xa tag. Indeed, as shown below, deleting the factor Xa sequences yielded His-tagged NhaA, which is not clipped by trypsin at the C terminus.

The pH-induced Conformational Change in NhaA

Since the His tag was fused at the C terminus of NhaA, binding to Ni²⁺-NTA column was used to test whether the C terminus is intact. Hence, Xa-His-tagged NhaA was treated with trypsin either at acidic or alkaline pH and the capacity of the products to affinity-purify on Ni²⁺-NTA tested. Neither the acidic nor the basic pH digestion products bound to the column, implying that at both pH values the C terminus together with the His tag was trimmed off. Similar results were obtained when the trypsin treatment was conducted on everted membrane vesicles overexpressing Xa-His-tagged NhaA (data not shown).

In contrast to Xa-His-tagged NhaA, native NhaA in isolated membrane vesicles did not seem to be cleaved by trypsin at its C terminus (18); the size of the protein following acidic digestion did not change; Western analysis with a site-directed polyclonal antibody against the C terminus recognized the apparently uncut protein obtained at acidic pH and the light tryptic fragment obtained at alkaline pH. This antibody did not recognize the intact Xa-His-tagged protein, most probably due to steric hindrance of the tags. We thus assumed that the trypsin cleavage site at the C terminus of Xa-His-tagged NhaA was introduced by the Xa tag. Indeed, as shown below, deleting the factor Xa sequences yielded His-tagged NhaA, which is not clipped by trypsin at the C terminus.

The N terminus remained intact following trypsin digestion of pure Xa-His-tagged protein; as shown below, the large fragment obtained at alkaline pH starts with the original N-terminal sequence of the native protein (Fig. 2).

**The Unique Trypsin Cleavage Site Exposed by pH in Pure Xa-His-tagged NhaA Is in Loop VIII–IX**—To identify the trypsin cleavage site that is exposed by pH and splits Xa-His-tagged NhaA, each of the two fragments (heavy and light) obtained from the trypsin digest of Xa-His-NhaA at alkaline pH (Fig. 1) were isolated from the gels and subjected to N-terminal sequencing (Fig. 2). The N terminus of the heavy fragment was found (with less than 1% contaminations) identical to that of the native protein. The light fragment fraction contained mainly (about 90%) a fragment with a N-terminal sequence that overlaps a sequence between Arg-250 and Leu-264 (Arg-250–Leu-264) of loop VIII–IX. In addition, it contained small amounts (about 5% each) of two additional peptides with sequences overlapping Ser-246–Ala-250 in loop VIII–IX and Val-50–Asn-64 in loop I–II, respectively. Hence, the main cleavage site of trypsin that is exposed at alkaline pH is in Lys-249 of loop VIII–IX.

**Lys-249 in Loop VIII–IX Is Also the Site Exposed to Trypsin in Situ at Alkaline pH in Isolated Membrane Vesicles**—The similarity in the pH profile of the trypsin digestion and the size of the products have suggested that the cleavage site in situ in everted membrane vesicles is identical to that of the pure protein in DM. However, proving this suggestion was difficult since it is a very laborious task to purify the products that lost the His tag during the in situ digestion by trypsin, from the membranous fraction. We therefore constructed a plasmid (pMXH) from pAXH, which encodes His-tagged NhaA with no factor Xa cleavage sites. Everted membrane vesicles isolated from cells overexpressing this protein exhibit a Na/’H’ antiporter activity and a pH profile identical to that of the wild-type protein (data not shown).

These everted membrane vesicles were exposed to trypsin both at acidic (data not shown) and alkaline pH (Fig. 3), the treated membranes solubilized in DM, the solubilized fraction affinity-purified on Ni²⁺-NTA column, and the eluted polypeptides separated on SDS-PAGE (Fig. 3). Following treatment at
acidic pH, the protein purified on the column behaved in SDS-PAGE in a fashion similar to that of the undigested control: one band, of a size identical to the untreated control. These results suggest that following trypsinolysis the His tag remains intact in this recombinant protein and allows the affinity purification of the protein. The results also show that indeed the trypsin cleavage site at the C terminus of the Xa-His-tagged NhaA resides in the factor Xa cleavage site. When alkaline pH digest of His-tagged NhaA was applied to the column, two fragments were affinity-purified on the Ni$^{2+}$-NTA (Fig. 3, lanes b and c): a heavy fragment similar in size to that observed following treatment of Xa-His-tagged NhaA, and a slightly shorter light fragment as expected on the basis of the difference between the two C termini of Xa-His-tagged and His-tagged NhaA. In contrast, none of the tryptic polypeptides derived from Xa-His-tagged NhaA subjected to the same treatment were purified by the column (Fig. 3, lane e). It is remarkable that despite the trypsin split in His-tagged NhaA two fragments co-purify via the His tag: the C-terminal fragment with His tag and the N-terminal fragment without it. This result implies that both C-terminal and N-terminal fragments formed by the trypsin split are bound to each other and do not separate in DM.

To verify that the trypsin cleavage site of His-tagged NhaA in situ (in the membrane) is identical to that of the pure protein, the light C-terminal fragments obtained from the trypsin digest of His-tagged NhaA membranes were isolated by SDS-PAGE and subjected to N-terminal sequencing. The results show that indeed the trypsin cleavage site that is exposed in situ at alkaline pH is identical to that identified in the pure protein in DM, namely Lys-249 of loop VIII–IX. Also similar to the pure protein is a minor split occurring in situ at Arg-245.

Mutations in Loop VIII–IX Affect the pH Regulation but Not the Activity of NhaA—Since loop VIII–IX changes its conformation with pH, the question arises as to whether loop IV–IX plays any role in the activity of NhaA or its regulation by pH. To answer this question, three types of mutations have been introduced to loop VIII–IX.

The first type is a deletion mutation lacking 12 amino acids from Lys-242 to His-253 ($\Delta$Lys-242–His-253). This mutant was expressed to a very low level (2% of the expression of the wild type; Table II) and did not grow in the presence of 0.6 M NaCl...
either at pH 7 or at pH 8.3. As measured in isolated membrane vesicles, the mutant did not show any Na\(^{+}/H^+\) antiporter activity at pH 7 but at pH 8.5 a very low but reproducible activity was monitored (Fig. 4). Whereas, with the wild-type membranes, 100% of dequenching of the fluorescence was obtained within 30 s, 30% of this activity was obtained by the mutant only after 10 min. This low activity was ascribed to the mutant-NhaA since the control membranes derived from cells transformed with the vector (pBR322) did not show any activity.

A spontaneous suppressor mutation was obtained by growing ΔLys-242–His-253 at pH 7 in the presence of 0.6 M NaCl. The mutation was cloned and identified as P257S in ΔLys-242–His-253 (ΔLys-242–His-253) P257S. The suppression was only partial since the second-site mutation restored growth in the presence of Na\(^+\) only at neutral pH but not at pH 8.3. The expression of the ΔLys-242–His-253 P257S nhaA was 2-fold higher as compared with the original mutant (Table II). Although similar to mutant ΔLys-242–His-253, mutant ΔLys-242–His-253 P257S did not show any antiporter activity in everted membrane vesicles at pH 7; its activity at pH 8.5 was substantially higher in rate but not in extent as compared with the original mutant (Fig. 4). This phenotype most probably accounts for the improved growth of the double mutant at pH 7 in the presence of Na\(^+\) as compared with the original mutant, which did not grow. Interestingly, despite their low activity, both mutants show pH sensitivity being inactive at pH 7 and active at pH 8.5 (Fig. 4).

The second type of mutation was an insertion mutation; amino acids IEG were inserted between Lys-249 and Arg-250 of loop VIII–IX, creating factor Xa protease cleavage site (IEGR). This insertion mutation, designated K249-IEG-R250, was expressed as good as the wild-type (Table II) and allowed us to create very specifically a single split in NhaA in loop VIII–IX with factor Xa protease at the same site as trypsin. The size of the fragments obtained following factor Xa digestion were indeed as expected for a unique split in loop VIII–IX (data not shown).

The Na\(^{+}/H^+\) antiporter activity in everted membrane vesicles containing K249-IEG-R250 NhaA was similar in its maximal activity to the wild-type control (Fig. 5A). However, the pH profile of the activity of the mutant was shifted by about half a pH unit toward acidic pH (Fig. 5A). Hence, the insertion mutation into loop VIII–IX affects the pH sensitivity of NhaA.

The third mutation was three point mutations, each in a separate plasmid; these were introduced to loop VIII–IX of NhaA by site-directed mutagenesis: E241C, V254C, and...
E241C/V254C. All mutants were equally and fully expressed (Table II). EP432 transformants of the three plasmids showed growth phenotype identical to that of the wild-type strain.

Everted membrane vesicles isolated from the three mutants showed maximal $\text{Na}^+ / \text{H}^+$ activity similar in magnitude to that of the wild type (Fig. 5, B and C). However, in marked contrast with the wild-type, the pH profile of the $\text{Na}^+ / \text{H}^+$ activity of V254C was shifted by 1 pH unit toward acidic pH; whereas the activity of the wild-type protein reduces drastically with acidification showing very low activity (6%) at pH 7 and completely shuts off at pH 6.5. The mutant showed 81% and 30% activity at the respective pH values and was shut off only at pH 6. It is concluded that the mutation V254C in loop VIII–IX has a marked affect on the pH profile of NhaA.

Although less pronounced, mutant E241C also showed acidic shift in its pH profile (Fig. 5C). Most interestingly, the double mutant E241C/V254C caused a shift in the pH profile toward basic pH (Fig. 5C).

Both (V254C)-XH and (Lys-249-IEG-His-250)-XH proteins were affinity-purified on Ni$^{2+}$-NTA column and the purified proteins subjected to trypsin digestion at various pH values. The results summarized in Fig. 6 show that the acid shift in the pH profiles of the activity of the proteins (Fig. 5) is reflected in a similar acidic pH shift in the pH profiles of the digestion by trypsin of the purified proteins in DM solution.

**DISCUSSION**

Using site-directed and random mutagenesis, we have previously shown that the drastic regulation by pH of NhaA, the main $\text{Na}^+ / \text{H}^+$ antiporter of *E. coli*, is essential for the capacity of the cells to grow at alkaline pH in the presence of $\text{Na}^+$ (15, 17). We have also shown that activation of NhaA by pH is accompanied by a conformational change that can be detected using trypsin digestion as a probe. Thus, both wild-type NhaA (17, 18) and its mutants with altered pH profiles are susceptible to proteolytic digestion, each only at the pH range where it is activated: the wild type at pH 7 to pH 8.5 (18), the mutant H225R at pH 6.5–8 (18), and the mutant G338S at the entire pH range between 6 and 8.5 (17). Similar correlation was found in the present study with the mutants V254C and K249-IEG-R250; the mutations caused an acidic shift in the pH profile of both the $\text{Na}^+ / \text{H}^+$ activity and the cleavage by trypsin. Most interestingly, despite the many trypsin cleavage sites of NhaA (18), in all these cases only two main tryptic products are...
obtained, suggesting the existence of one main trypsin cleavage site, which is exposed by pH. This is innate property of the NhaA protein, since trypsin digests completely bovine serum albumin under all pH tested.

In the present work, we identified the trypsin digestion sites that are exposed by pH and found that loop VIII–IX bears a pH responsive domain. Both in the purified Xa-His-tagged protein in dodecyl maltoside as well as in situ in membrane vesicles containing His-tagged NhaA, Lys-249 of loop VIII–IX was found to be the site that is exposed to trypsin in a pH-dependent fashion. In both cases, the digestion products are two fragments of NhaA: a large fragment with the native N terminus of NhaA and a short fragment with the N terminus starting at Arg-250 (Fig. 2). Based on the identical site of the split and the protein sequence, in both proteins the size expected for the heavy fragment is 26.4 kDa while 17.1 and 15.2 kDa are expected for the short fragments obtained from the two proteins, respectively. Note that the apparent sizes of the intact protein as well as that of the trypptic fragments as appeared in SDS-PAGE are somewhat shorter than expected. Aberrant mobility in SDS-PAGE is a common known artifact of many membrane proteins (12). In line with these results, two similarly sized NhaA fragments were obtained by trypsinolysis of overtred membrane vesicles containing the native protein (18). However, in this case, it was practically impossible to determine the amino acid sequence of the N termini of the fragments, which were not tagged and thus could not be affinity-purified.

These results raise the question as to whether loop VIII–IX of NhaA is involved in the pH response of NhaA or rather its pH-dependent conformational change is a circumstantial episode accompanying the important conformational change. Extensive mutagenesis of loop VIII–IX showed that loop VIII–IX is involved in the pH response of NhaA. Two types of mutations in loop VIII–IX shifted the pH profile of the Na+/H+ antiporter activity of the protein toward acidic pH without affecting the maximal activity or expression of the antiporter: insertion mutation K249-IEG-R250 shifted the pH profile by half a pH unit, point mutations V265C by one pH unit, and E241C by 0.8 pH unit. Interestingly, in the double mutant E241C/V254C, the effect was reversed and the pH profile was shifted back toward basic pH to values slightly even more basic than those of the pH profile of the wild-type protein.

It is clear that resolution of the atomic structure of NhaA is required for a mechanistic interpretation of these results, as to how loop VIII–IX is involved in the pH response of NhaA. Nevertheless, it is possible to envisage at least to ways of involvement of a protein domain in the pH response of NhaA: a pH “sensor” domain that “senses” the pH change and a “transducer domain” that transduces the pH signal to a change in activity of the protein. These two domains can overlap. The phenotype obtained with a mutant deleted of most of loop VIII–IX suggests that the loop VIII–IX is involved at least in the signal transduction event; although the Na+/H+ activity of both mutants, ΔLys-242–His-253 and ΔLys-242–His-253 P257S, was very low, the pH response (increasing activity at alkaline pH) was still maintained in the mutants. It is suggested that, while the “pH sensor” exists in these proteins, the activation step is impaired. The other mutants in loop VIII–IX, which shift the pH profile with no other conspicuous effects, suggest an involvement in the pH sensor. It should be noted that loop VIII–IX is cytoplasmic and links a short putative TMS VIII, which is bordered at its periplasmic face by His-225, a residue previously found to affect dramatically the pH response of NhaA (Refs. 15, 16, 21, 26, and 28; Fig. 2). Two other TMS, XI and IV, also contain residues affecting the pH response of NhaA (Ref. 17; Fig. 2).

Recently a NhaA homologue (named HNhaA) was cloned from Helicobacter pylori (27). Transformation of an E. coli mutant deleted of its antiporter genes with HNhaA complemented the salt-sensitive phenotype of the mutant and evoked membrane vesicles isolated from these transformants exhibited Na+/H+ antiporter activity differing in the pH profile from that of E. coli NhaA (ENhaA): it was active at acidic and neutral pH, where ENhaA is inactive. Remarkably, the primary sequences and the hydropathy profiles of ENhaA and HNhaA were homologous, except for one additional region found in HNhaA. This sequence has about 40 hydrophilic amino acid residues inserted at position next to residue 235 of ENhaA (245 of HNhaA) and includes the loop VIII–IX. Taken together, these results corroborate our results, suggesting that loop VIII–IX has an important role in the pH response of HNhaA.

While the physiological role of HNhaA has not yet been studied, in E. coli we have demonstrated that the pH regulation of the antiporter is essential for growth at alkaline pH in the presence of Na+ with two properties of the regulation being critical: the activation of the antiporter at alkaline pH (15) and its capacity to shut off at acidic pH (17). When each of these properties are absent, the cells die at alkaline pH in the presence of Na+. Furthermore, as long as these properties are maintained, even a shift in the pH profile toward basic pH caused by H225D mutation had a normal growth phenotype (16). In the present work, we show that drastic acidic shift in the pH profile caused by the loop VIII–IX mutants, E241C and V254C, did not affect the growth phenotype of the cells.

It is remarkable that the pH-dependent conformational change as probed by trypsin is maintained by the purified NhaA in a solution of DM. Furthermore, even the two tryptic NhaA polypeptide products remained complexed in DM and copurified on Ni2+-NTA column. Similarly, the lac permease in DM maintains a native conformation (29); its ligand-induced conformational changes were denatured reversibly in vitro. These results are in line with previous results showing that DM is most suitable detergent for purification and subsequent reconstitution in proteoliposomes of many transporters, including NhaA (12).

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A pH-dependent Conformational Change of NhaA Na⁺/H⁺ Antiporter of Escherichia coli Involves Loop VIII–IX, Plays a Role in the pH Response of the Protein, and Is Maintained by the Pure Protein in Dodecyl Maltoside

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