Probing the Conformation of NhaA, a Na^+/H^+ Antiporter from *Escherichia coli*, with Trypsin†

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ABSTRACT: One of the most striking features of NhaA, an *Escherichia coli* Na^+/H^+ antiporter, is its extreme sensitivity to pH. The activity of NhaA increases 2000-fold between pH 6.5 and 8.5. In this work, we investigated whether the activation of NhaA by pH is accompanied by conformational changes which can be detected using trypsin as a probe. We have found that NhaA is susceptible to proteolytic digestion at the pH range where it is activated, suggesting that these two events may be related; at alkaline pH, the protein becomes active and adopts an "open" conformational state in which more domains are exposed to the enzyme. This idea was further supported by results from two mutants of NhaA in which His-225, a residue involved in pH sensing, has been replaced by either Arg or Asp. The mutant H225R is activated at more acidic pH values, while H225D at more alkaline pH. In accordance with the results described for the wild-type protein, H225R was susceptible to digestion by trypsin at the pH at which it undergoes main activation. NhaA has many potential tryptic cleavage sites. However, analysis of the tryptic digestion fragments suggests that at alkaline pH, the protein is exposed to cleavage mainly at hydrophilic loops 6, 7, and 8. Thus, upon activation, NhaA appears to undergo a change in conformation that is reflected in specific regions of the protein.

NhaA is a Na^+/H^+ antiporter from *E. coli* which plays a central role in adaptation to high salinity and alkaline pH (*1*). Its expression is regulated by the intracellular Na^+ concentration (*2*) while its activity is highly regulated by pH (*3*). NhaA is 388 amino acids long (calculated molecular mass 41 355 Da), and it is predicted to cross the membrane 12 times with 2 unusually short TMS[1] 14 and 16 amino acids long (*4*). NhaA has been purified to homogeneity and reconstituted in proteoliposomes (*3*). With the purified antiporter, it was found that the H^+/Na^+ stoichiometry of NhaA is $2H^+/Na^+$ and that the rate of transport is drastically dependent on pH, changing its V_{max} over 3 orders of magnitude from pH 7 to pH 8 (*3*).

The increase in activity of NhaA with pH is in line with the proposed role of NhaA in regulation of intracellular pH (*1*, *3*). The experimental evidence suggests that the activity of NhaA is regulated by pH in such a manner so that an increased influx of protons can be achieved when necessary, i.e., upon rising of the intracellular pH. Mutagenesis of His-225 modifies this behavior of the protein, suggesting that this residue influences the pH sensor of the protein (*5*, *6*).

An inherent feature of transporters is that they are intrinsically very dynamic. Their overall structure undergoes continuous changes in conformation which are associated with the various transport steps and also with activation when present. Ligand-induced conformational changes have been demonstrated for example in several transporter proteins by means of proteolytic digestion studies (*7*, *8*, *9*) and Cys-scanning mutagenesis with site-directed sulfhydryl modification in situ or site-directed fluorescence spectroscopy (*10*, *11*).

In this paper, we have used proteolysis by trypsin as a means of investigating structural changes in NhaA accompanying changes in pH. We report that, at acidic pH, under conditions at which it is inactive, NhaA is very resistant to proteolysis by trypsin, even after solubilization with detergents. A dramatic increase in the sensitivity to trypsin is observed when the pH is shifted to alkaline values, conditions under which NhaA becomes fully active. These results suggest that, upon activation, NhaA undergoes a change in conformation. This change appears to be reflected in specific regions of the protein.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The bacterial strain used in this study is TA15, an *E. coli* K-12 derivative (*12*). The plasmid carrying wild-type *nhaA* was pGM36 (*13*). Plasmids carrying *nhaA* mutants H225R and H225D were pYG2 (*5*) and pH225D (*6*), respectively. Plasmids used for exclusive labeling of NhaA were pGP1-2 (*14*) and pT7-5 derivatives (*13*, *14*): pDT62 for the wild-type (*13*), pYG62-2 for H225R (*5*), and pYG62-3 for H225D, respectively.

Construction of Plasmid pYG62-3. The *nhaA* mutant H225D was fused to the bacteriophage T7 promoter by digesting plasmid pDT62 with *Bgl*II and *Hin*dIII, and the *Bgl*II−*Hin*dIII (3.6 kb) fragment was ligated with a *Bgl*II−*Hin*dIII (1.46 kb) fragment excised from pH225D to create plasmid pYG62-3.

Preparation of Membranes Containing Labeled NhaA. *E. coli* (TA15) cells carrying plasmid pGP1-2 were transformed with plasmids pDT62, pYG62-2, or pYG62-3 and grown on minimal medium A, containing 0.5% glucose, 50 μg/mL kanamycin, and 100 μg/mL ampicillin at 30 °C. When the culture reached an $A_{600} = 0.5$, it was transferred to 42 °C

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[1] Abbreviations: TMS, transmembrane segments; RSO, right-side-out; ISO, inside-out; DM, *n*-dodecyl β-D-maltoside.

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for 15 min, rifampicin was added to 200 μg/mL, and 10 min later the culture was transferred back to 30 °C for 40 min. Then, [^{35}S]methionine (1350 Ci/mmol) was added to a final concentration of 10 (μCi/mL for 40 min. Cells were centrifuged and washed with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and sonicated twice for 10 s using a probe type sonicator. Undisrupted cells were removed by centrifugation at 10000*g* for 20 min. The supernatant was centrifuged at 180 000*g* for 60 min, and the pellet was washed and resuspended in the same buffer at a concentration of about 10^8 cpm/mL and kept at −70 °C.

Preparation of Inside-Out and Right-Side-Out Membrane Vesicles for Digestion with Trypsin. E. coli (TA15) cells transformed with plasmids pGP1-2 and pDT62 were labeled with [^{35}S]methionine as described above. Inside-out membrane vesicles were prepared by passage through a French pressure cell at 4000 psi (*15*). Right-side-out membrane vesicles were prepared by lysozyme−ethylenediaminetetraacetic acid (EDTA) treatment and osmotic lysis as described (*16*).

Preparation of Solubilized Membranes for Digestion with Trypsin. Before solubilization, nonlabeled inside-out membrane vesicles (1 mg of protein) derived from TA15 cells transformed with plasmid pGP1-2 and plasmids pGM36, pYG2, or pH225D were combined with the desired amount of [^{35}S]methionine-labeled membranes to achieve a concentration of about 9 mg of protein/mL and 2727 cpm/μL. Each volume of membranes was solubilized by the addition of 2 volumes of a mixture containing 1.5% *n*-dodecyl (β-D-maltoside (DM) (Sigma) and 7.5 mg/mL *E. coli* phospholipids, 150 mM MOPS, pH 7, 30% glycerol, and 3 mM DTT. After 20 min at room temperature, the sample was clarified by centrifugation at 180,000 x g for 60 min. The supernatant was used immediately or frozen in liquid nitrogen and kept at −70 °C. The final concentration of the solubilized membranes was about 3 mg of protein/mL.

Proteolysis of Membrane Vesicles and Solubilized Membranes with Trypsin. Inside-out and right-side-out membrane vesicles containing NhaA labeled with [^{35}S]methionine were suspended to a final concentration of 0.140 mg of protein/mL in a medium that contained 100 mM KCl, 0.7 mM K-EDTA, 1 mM $CaCl_2$, and 10 mM Tris−Hepes at the desired pH (32 μL final volume). The reaction was initiated by the addition of 15.6 μg/mL trypsin type II from Sigma, incubated for 1 h at 40 °C, and stopped by the addition of 46.8 μg/mL trypsin inhibitor type II-S from Sigma. Detergent-solubilized membranes containing [^{35}S]methionine-labeled NhaA were brought to a final concentration of 0.147 mg of protein/mL in a medium that contained 100 mM KCl, 0.7 mM K-EDTA, 1 mM $CaCl_2$, 0.1% DM, and 10 mM Tris−Hepes at the desired pH (60 μL final volume). The reaction was initiated by addition of trypsin 2.6 μg/mL, incubated for 2 h at 37 °C, and stopped by addition of 7.8 μg/mL trypsin inhibitor. In control experiments it was shown that addition of the trypsin inhibitor prior to trypsin fully inhibited the digestions observed.

SDS−PAGE, Western Blots, and Phosphor-autoradiography. Samples containing equal amounts of total protein, as determined (*17*) prior to trypsin digestion, were mixed with half the volume of sample buffer (40% glycerol, 4% β-mercaptoethanol, 8% SDS, and 0.26 M Tris-HCl, pH 6.8) and analyzed on a 16.5% tricine gel prepared according to Shagger and Jagow(*18*). The dry gel was exposed to a Phosphor-imager FUJIX BAS 1000 for several hours, and radioactive bands were quantitated. In the case of membrane vesicles, proteins were transferred to a poly(vinylidene difluoride) membrane (Millipore) prior to reaction with an antibody raised against the C terminus of NhaA (*5*) and detection using alkaline phosphatase coupled to anti-rabbit IgG (*19*). Prestained molecular weight markers were used to estimate the molecular weights of the different digestion products.

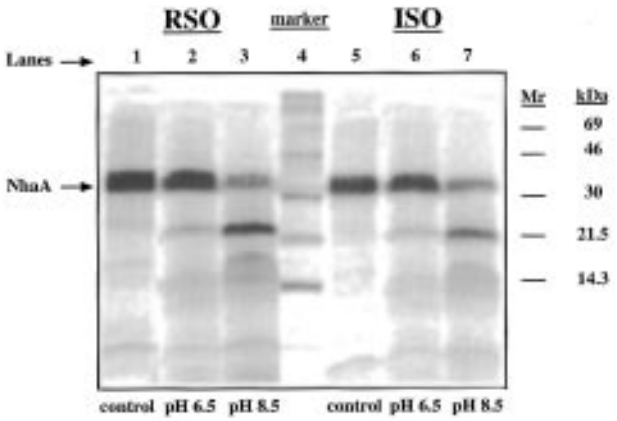

FIGURE 1: Digestion of [^{35}S]methionine-labeled NhaA by trypsin as a function of pH in right-side-out and inside-out membrane vesicles. RSO (lanes 1−3) and ISO (lanes 5−7) membrane vesicles containing NhaA, labeled specifically with [^{35}S]methionine, were suspended to a final concentration of 0.140 mg of protein/mL and incubated with 15.6 μg/mL trypsin as described under Materials and Methods, either at pH 6.5 (lanes 2 and 6) or at pH 8.5 (lanes 1, 3, 5, and 7). In lanes 1 and 5, trypsin inhibitor was added prior to trypsin. The samples were loaded on a 16.5% tricine gel, and the gel was exposed to a phosphor-imager as described under Materials and Methods.

RESULTS AND DISCUSSION

NhaA Is Resistant to Proteolysis at Slightly Acidic pHs. To test the digestion of NhaA in membrane vesicles, the protein was specifically labeled with [^{35}S]methionine. Specific labeling was achieved using the T7-polymerase system as previously described so that NhaA is the major (>90%) protein labeled (Figure 1, lanes 1 and 5) . When right-side-out (RSO) and inside-out (ISO) membrane vesicles are prepared from the cells and exposed to trypsin at slightly acidic pH values (<6.7), less than 10% of the protein is digested even after 1 h incubation of either type of vesicles (Figure 1, lanes 2 and 6). This resistance is intrinsic to NhaA and is not due to low activity of trypsin because under these conditions even an increase of 12-fold in the amount of the protease (up to a trypsin:membrane protein ratio of 1.2:1 μg/μg) does not significantly change the overall resistance pattern (Figure 2A). In addition, under the same experimental conditions, trypsin (3 μg) fully digests bovine serum albumin (30 μg) in 1 h at 37 °C (not shown). The resistance to proteolytic digestion is independent of the sidedness of the membrane even though in ISO membranes there is a much larger number of potential trypsinization sites in the hydrophilic loops (Figure 3). Solubilization of NhaA with the detergent dodecyl maltoside did not dramatically modify this resistance pattern (Figure 4, lanes 1−4) even though the amount of NhaA degraded increases with the trypsin concentration to about 30−35% of the total and remains stable thereafter (Figure 2B). From these results, it would

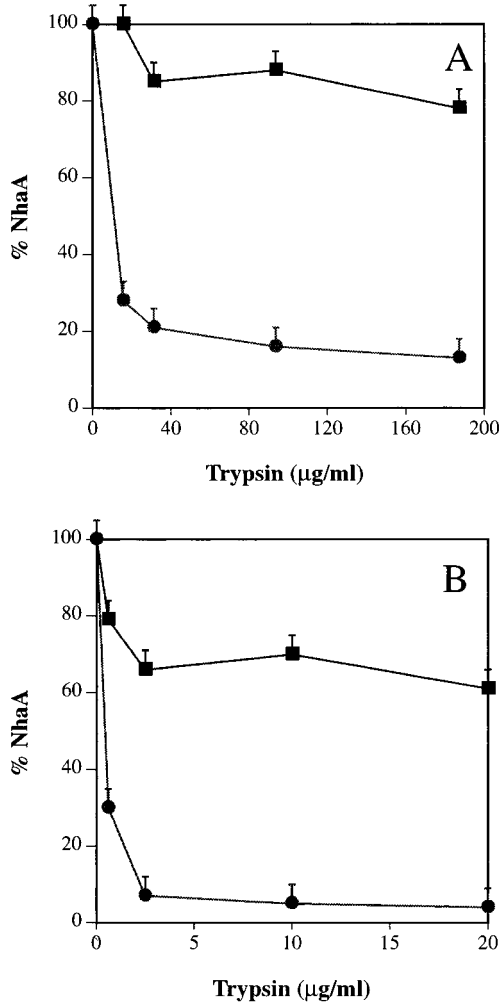

FIGURE 2: Digestion of NhaA as a function of trypsin concentration. (A) ISO membrane vesicles containing NhaA labeled specifically with [^{35}S]methionine were suspended to a final concentration of 0.140 mg of protein/mL and incubated with different concentrations of trypsin at either pH 6.3 (■) or pH 8.6 (●) as described under Materials and Methods. The reactions were terminated by addition of a trypsin inhibitor, and the samples were loaded on a 16.5% tricine gel. The gel was exposed to a phosphor-imager, and the results were quantitated as described under Materials and Methods. The percentage of protein remaining after digestion with increasing concentrations of trypsin is shown. (B) Detergent-solubilized membranes, labeled specifically with [^{35}S]methionine, were suspended to a final concentration of 0.147 mg of protein/mL and incubated with different concentrations of trypsin at pH 6.5 (■) or 8.5 (●) as described under Materials and Methods. Experimental details as in (A).

seem that about one-third of NhaA in the detergent-solubilized preparation may be in a more "open" state but, at this pH, the bulk is resistant to proteolysis even after a 30-fold increase of the enzyme. As previously shown, also carboxypeptidase B digests only the short hydrophilic C-terminus of the detergent-solubilized NhaA (*4*). Taken together, these results suggest a compact, closed conformation of NhaA in which most of the protein is inaccessible to proteolytic digestion by trypsin. In this respect, the conformation of the bulk of the detergent-solubilized protein at low pH is similar to that of the membrane-embedded protein.

Sensitivity to Trypsin Increases Dramatically with Activation of NhaA at Alkaline pH. We next asked whether the activation of NhaA by pH is accompanied by conformational changes which can be detected using trypsin as a probe. A dramatic difference in the sensitivity to trypsin is observed at pH 8.5. More than 70% of NhaA is digested both in RSO and in ISO membranes (Figure 1, lanes 3 and 7, respectively). In detergent-solubilized membranes, also most of the protein is degraded at alkaline pH (Figure 4, lane 7). This dramatic difference is innate to NhaA and not due to lack of activity of trypsin at low pH. As described above, trypsin digests bovine serum albumin at pH 6.5. When trypsin activity was assessed by measuring the rates of hydrolysis of the artificial substrate *p*-toluenesulfonyl-L-arginine methyl ester as measured by an increase in absorbance at 247 mμ (*20*), the rate at pH 8.8 was only 3-fold higher than that at pH 6.3.

The results show that digestion of NhaA by trypsin in both intact membranes and detergent-solubilized membranes occurs mainly at alkaline pH, conditions under which NhaA becomes fully active (*3*). The sensitivity to trypsin of detergent solubilized NhaA is about 20-fold higher since already 0.004 μg of trypsin/μg of protein yielded 70% digestion at pH 8.5. However, the general pattern observed with membrane-embedded NhaA is also detected in the detergent-solubilized NhaA since incubation even with 0.13 μg of trypsin/μg of protein at pH 6.5 resulted in digestion of not more than 39% of NhaA (Figure 2B). The sensitivity to trypsin has been tested at various pH values, and the results are shown in Figure 5. The percentage of wild-type NhaA degraded increases dramatically above pH 7.0. While at pH 7.0 no degradation is observed, at pH 7.2 35% is degraded, at pH 7.9 almost 80% is degraded, and at pH 8.5 practically full digestion is observed. The increase in susceptibility to proteolytic digestion occurs at a similar pH range as the activation of NhaA (*3*, *5*). This finding suggests that these two phenomena may be related; at alkaline pH, the protein becomes active and adopts an "open" conformational state in which more domains are exposed to the enzyme. This idea was further tested by proteolytic studies on detergent-solubilized membranes from two mutants of NhaA in which His-225 has been replaced by either arginine or aspartate: H225R is activated at a more acidic pH range, while H225D is activated at more alkaline pH values (*5*, *6*). The pH dependence of trypsin digestion of H225R shows a different pattern: at pH 6.8 already 30% of H225R is digested, at pH 7.0 more than 60% is digested, and at pH 7.2 most of the H225R protein (85%) is digested (Figure 5), while only 35% of NhaA is digested by trypsin in the wild-type NhaA. In contrast, the pattern of digestion of H225D is practically identical to that of the wild-type protein (Figure 5).

The finding that the increase in susceptibility to proteolytic digestion occurs at a similar pH as the activation of NhaA, in both the wild type and the H225R mutant, supports the idea that these two phenomena are related and that upon activation the protein undergoes a change in conformation in which certain regions of the protein become exposed to trypsin. The fact that the sensitivity of H225D does not show a shift to alkaline pH indicates that the change in the conformation as measured by sensitivity to trypsin is only one step in the chain of events leading to activation of NhaA.

Hydrophilic Loops 6, 7, and 8 of NhaA Are Exposed to Proteolysis at Alkaline pH. The results presented in this paper suggest that at alkaline pH, NhaA is digested by trypsin mainly at cleavage sites located in hydrophilic loops 6, 7, and 8 (Figure 3). The pattern of digestion is clearly different for right-side-out and inside-out membrane vesicles. In right-

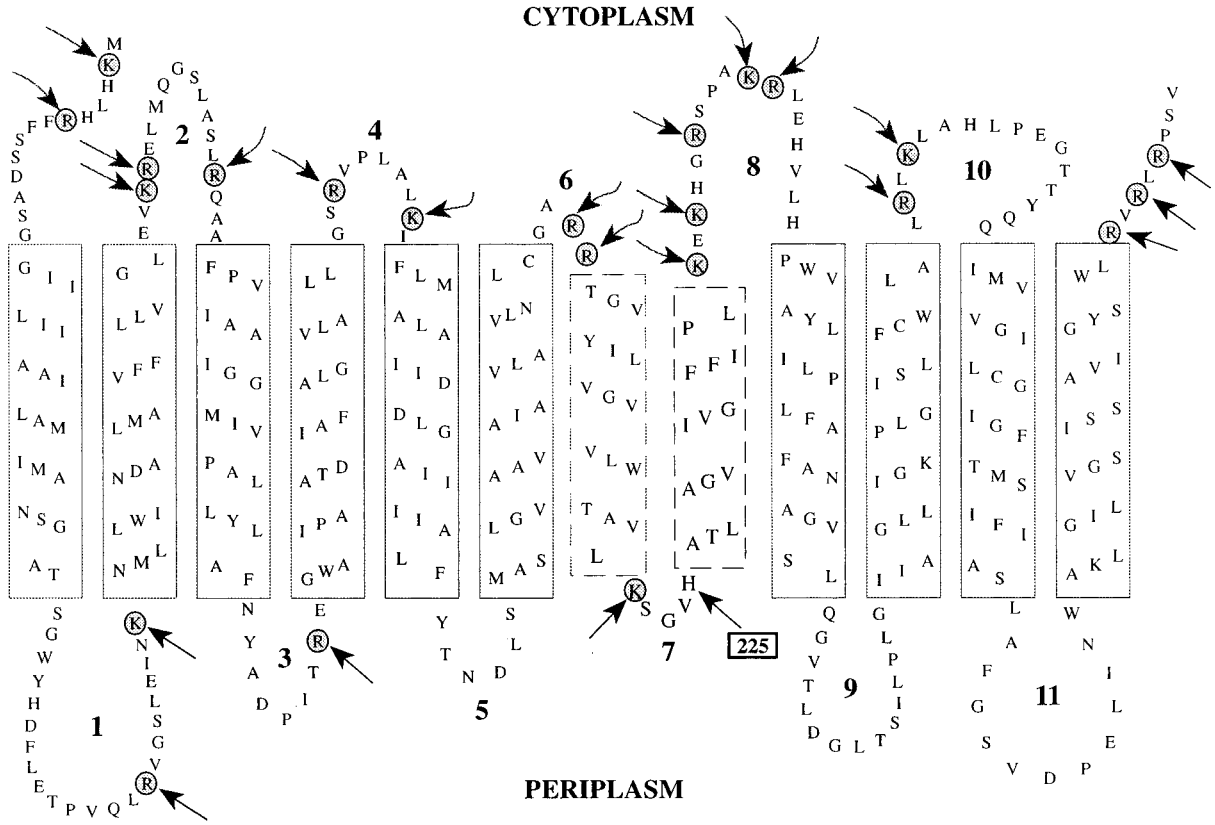

FIGURE 3: Model for the secondary structure of NhaA. The 12 transmembrane segment model proposed (*4*) for the topology of NhaA is shown. The arrows indicate potential trypsin cleavage sites. For reference, the position of His-225 is also indicated.

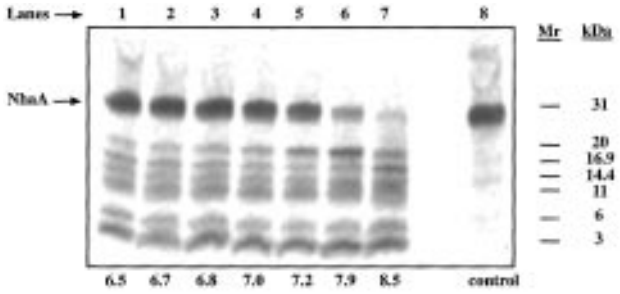

FIGURE 4: pH dependence of digestion by trypsin of detergent-solubilized NhaA. Solubilized membranes containing NhaA labeled specifically with [^{35}S]methionine were suspended to a final concentration of 0.147 mg of protein/mL and incubated with 2.6 μg/mL trypsin as described under Materials and Methods, at the pH values indicated. Reactions were terminated by addition of trypsin inhibitor and were analyzed on a 16.5% tricine gel. The radioactive gel was exposed to a phosphor-imager as described under Materials and Methods. In lane 8, the inhibitor was added prior to the trypsin (pH 8.5).

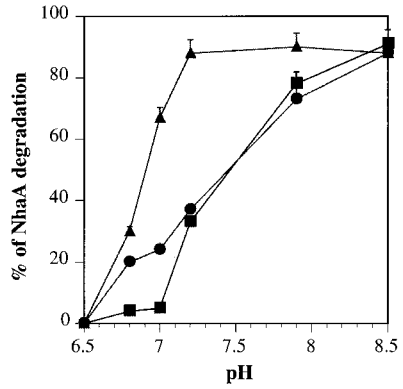

FIGURE 5: pH dependence of trypsin digestion of H225R, H225D, and wild-type NhaA in detergent-solubilized membranes. Solubilized membranes (0.147 mg of protein/mL) containing specifically labeled H225R (▲), H225D (●), and wild-type NhaA (■), were incubated with trypsin (2.5 μg/mL) as described under Materials and Methods, at the pH values indicated. The samples were analyzed on a 16.5% tricine gel; the radioactive gel was exposed to a phosphor-imager as described under Materials and Methods. The degree of digestion observed at pH 6.5 is defined as zero.

side-out membranes, NhaA cleavage yields two digestion products: an N-terminal fragment of apparent molecular mass 23.8 kDa and a C-terminal fragment of apparent molecular mass 16.9 kDa (Figure 1). That the small peptide is C-terminal is supported by the finding that this peptide, but not the larger one, reacts with an antibody directed against a C-terminal peptide of NhaA (see RSO, pH 8.5, in Figure 6). In inside-out membranes, NhaA cleavage yields two products: an N-terminal fragment of apparent molecular mass 22.1 kDa and a C-terminal fragment of apparent molecular mass 14.4 kDa (Figure 1). Again the assignment is confirmed by probing with an antibody directed against a C-terminal peptide of NhaA (see ISO, pH 8.5, in Figure 6). In these experiments, digestion was performed as in Figure 1 except that part of the protein was Western-blotted and challenged with the antibody raised against a C-terminal peptide. In the results described in Figure 6, the antibody recognizes the intact protein in both RSO and ISO membrane vesicles in the controls at pH 6.5, and in a decreased amount at pH 8.5. Among the tryptic products, only one polypeptide is recognized in both cases: a 16.9 kDa product in the case of RSO membrane vesicles and a 14.4 kDa product in the case of ISO membrane vesicles. In detergent-solubilized membranes, the 14.4 and 16.9 kDa digestion products are apparent. However, the 22.1 and 23.8 kDa digestion products are not resolved as two separate fragments and are

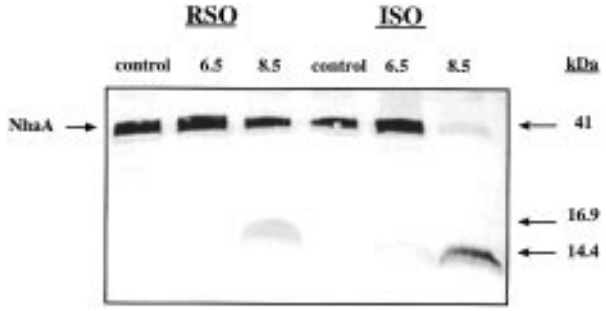

FIGURE 6: Digestion of NhaA with trypsin as detected with an antibody against C-terminal sequences. RSO and ISO membrane vesicles (0.140 mg of protein/mL) containing NhaA labeled specifically with [^{35}S]methionine were incubated with trypsin (15.6 μg/mL) as described under Materials and Methods, at the pH values indicated. The samples were analyzed on a 16.5% tricine gel and were then Western-blotted and detected with an antibody directed against a C-terminal peptide of NhaA.

observed as one band of apparent molecular mass 20 kDa (Figure 4). The smaller digestion products, mainly fragments of apparent molecular mass 11, 6, and 3 kDa, must derive from the N-terminal region because only fragments of 14.4 and 16.9 kDa were detected with the C-terminal antibody (not shown).

Taken as a whole, we suggest that trypsin digests NhaA in RSO membrane vesicles at loop 7 which yields theoretical peptides of about 221 amino acids in the N-terminal region and 167 amino acids in the C-terminal region (Figure 3), very similar to the ones observed according to their electrophoretic behavior. In ISO membrane vesicles, trypsin digests both at loop 6 and at loop 8, yielding 3 predicted peptides (Figure 3): a small peptide of about 39 amino acids which comprises the 2 small transmembrane segment region. This region of the protein contains no methionines and therefore cannot be visualized by labeling with [^{35}S]methionine. The 2 other digestion products are an N-terminal fragment of about 203 amino acids and a C-terminal fragment of about 146 amino acids, very similar to the ones observed according to their electrophoretic behavior. We conclude that at acidic pH when the protein is inactive, NhaA is in a "closed conformation" in which the protein is not exposed to proteolysis. Upon activation at alkaline pH, the protein undergoes a change in conformation upon which loops 6, 7, and 8 become exposed and are accessible to proteolysis.

One of the most interesting aspects of the activity of NhaA is its activation by pH. This type of regulation is shared by several other transporters which are involved in pH regulation, i.e., the mammalian Na^+/H^+ antiporter (*21*) and the nonerythroid anion exchanger (*22*). In all these cases, very little is known about the detailed mechanism of activation by pH even though information regarding residues potentially involved in this process has been recently gathered. In Nhe1, there are several possible pathways of activation of the antiporter. In some of these modes, phosphorylation of residues in the cytoplasmic C-terminus of the transporter has been involved, but more recent data reverse the above hypothesis (*21*). In the case of the nonerythroid anion exchanger, a cluster of His residues seems to play a role in the activation (*22*). In NhaA, His-225 in loop 7, has a clear effect on the pH sensor: replacement with Arg shifts the pH profile of the antiporter toward acidic pH, while replacement with Asp results in a shift toward alkaline pH (*5*, *6*).

In this paper, we show a conformational change that suggests that loops 6, 7, and 8 become more exposed to trypsin at alkaline pH. This conformational change may represent one of the first steps in activation of NhaA.

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