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Biochemistry

Histidine-226 is part of the pH sensor of NhaA, a Na^+/H^+ antiporter in *Escherichia coli*

(pH regulation/membrane protein/transport)

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ABSTRACT **The *nhaA* gene of *Escherichia coli*, which encodes a pH-activated Na^+/H^+ antiporter, has been modified; six of its eight histidine codons were mutated to arginine codons by site-directed mutagenesis, yielding the mutations H254R-H257R (a double mutant), H226R, H39R, H244R, and H319R. In addition a deletion (Δ*nhaA*1–14) lacking the remaining two histidines, His-3 and His-5, has been constructed. By comparing the phenotypes conferred by plasmids bearing the various mutations to the phenotype of the wild type upon transformation of strains NM81 (Δ*nhaA*) or EP432 (Δ*nhaA*, Δ*nhaB*) we found that none of the NhaA histidines are essential for the Na^+/H^+ antiporter activity of the NhaA protein. However, the replacement of His-226 by Arg markedly changes the pH dependence of the antiporter. All mutants except H226R confer to NM81 and EP432 Na^+ resistance up to pH 8.5 as well as Li^+ resistance. Cells bearing H226R are resistant to Li^+ and to Na^+ at neutral pH, but they become sensitive to Na^+ above pH 7.5. Analysis of the Na^+/H^+ antiporter activity of membrane vesicles derived from H226R cells shows that the mutated protein is activated by pH to the same extent as the wild type. However, whereas the activation of the wild-type NhaA occurs between pH 7 and pH 8, that of H226R antiporter occurs between pH 6.5 and pH 7.5. Furthermore, while the wild-type antiporter remains almost fully active at least up to pH 8.5, H226R is reversibly inactivated above pH 7.5, reaching 10–20% of the maximal activity at pH 8.5. We suggest that His-226 is part of a pH-sensitive site that regulates the activity of NhaA.**

Sodium/proton antiporters are ubiquitous membrane proteins which are found in the cytoplasmic and organelle membranes of cells of many different origins, including plants, animals, and microorganisms (1–3). This ubiquity fits the important roles ascribed to these antiporters. In addition to being involved in cell energetics, they play primary roles in signal transduction and in regulation of intracellular pH, cell Na^+ content, and cell volume (for review see ref. 4).

Escherichia coli has two antiporters which specifically exchange Na^+ or Li^+ for H^+ (5). The genes *nhaA* and *nhaB* have been cloned (6, 7). Deleting *nhaA*, *nhaB*, or both genes together allowed deduction of the role of these antiporters in cell physiology. *nhaA* is indispensable for adaptation to high salinity, for challenging Li^+ toxicity, and for growth at alkaline pH (in the presence of Na^+) (5). *nhaB* by itself confers a limited sodium tolerance to the cells but it becomes essential when NhaA activity is limiting growth (8).

Expression of *nhaA* is regulated by Na^+ in a pH-dependent manner (9). NhaR, a protein that on the basis of sequence homology belongs to the LysR family of positive regulators, has been shown to be a positive regulator of expression of *nhaA* (10).

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The NhaA protein has been purified to homogeneity and reconstituted in a functional form in proteoliposomes (11). Using the purified system, we showed that NhaA is electrogenic, catalyzing an exchange of $2H^+$ per $1Na^+$ (ref. 11 and D.T., E.P., and S.S., unpublished data). Furthermore, the activity of NhaA was found to be highly dependent on pH, changing more than a 1000-fold over the pH range between 7 and 8 (11). This pH dependence of NhaA is expressed also in isolated membrane vesicles (5, 11, 12) and is unique to NhaA. The activity of NhaB is pH independent (5, 7).

The steep pH dependence of NhaA is expected from the primary role assigned to it in pH homeostasis of the cytoplasm at alkaline pH (13, 14). It has been suggested that when the pH increases the antiporter is activated so that it can acidify the cytoplasm back to the "resting pH_{in}" in a self-regulated mechanism (3). This pattern of a molecular pH meter and titrator in the same molecule exists also in completely different molecules: in the animal Na^+/H^+ antiporter (15), which shows very little similarity with NhaA (4), and in the nonerythroid Cl^-/HCO_3^- exchanger (16). The pH at which half-maximal activity (the set point) of the human protein is observed seems to be regulated by various hormones through phosphorylation of the cytoplasmic domain of the protein (17). In none of these systems are the protein residues involved in pH sensing known. It is also not clear whether the H^+-sensing and the ion-transporting sites are identical, overlapping, or different.

It is conceivable that residues involved in pH sensing or H^+ transport undergo protonation at the physiological pH range of activity. Histidines (pK 6.0 in solution) have been implicated in the mechanism of H^+ transport in the lactose carrier (18, 19), in the photosynthetic reaction center (20), and also in the Na^+/H^+ antiporter activity of *E. coli* (21).

We therefore deleted or mutated by site-directed mutagenesis the histidines of NhaA. We found that none of the eight histidines of NhaA are necessary for activity, while His-226 is required for the response of the protein to pH.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions. Bacterial strains used in this study are *E. coli* K-12 derivatives. TA15 is *melB*Lid, *nhaA*$^+$, Δ*lacZY* (6). NM81 is *melB*Lid, Δ*nhaA1*, *kan*$^+$, Δ*lacZY*, *thr-1* (5). EP432 is *melB*Lid, Δ*nhaA1*, Δ*nhaB1*, *kan*$^+$, *cam*$^+$, Δ*lacZY*, *thr-1* (8). Cells were grown in modified L broth (LBK) in which NaCl was replaced by KCl (87 mM, pH 7.5). Where indicated the medium was buffered with 60 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol, and pH was adjusted with KOH. Cells were also grown in minimal medium MTC (5) or A (22) without sodium citrate. Thiamin (2.5 μg/ml) and threonine (0.1 mg/ml) were added. Carbon sources were 10 mM melibiose or 0.5% glucose.

Plasmids. Plasmids carrying wild-type *nhaA* were pGM36, a pBR322 derivative (23); pEP3T, a pKK223-3 (Pharmacia)

derivative (11); and pDT62, a pT7-5 derivative (23, 24). pGP1-2 was described in ref. 24.

Construction of Mutations in Histidines of NhaA. The NhaA protein has eight histidines (Fig. 1). The mutant Δ*nhaA*1–14, in which His-3 and His-5 are deleted, was constructed by digestion of pEP3T with *Ava* I and ligation of the *Ava* I–*Ava* I fragment (5 kb) with the *Ava* I–*Ava* I fragment (1.45 kb) derived by digestion of pKK223-3 (Pharmacia). The plasmid obtained, pMJ, contains the *nhaA* gene with 36 nucleotides deleted from the N-terminal-encoding region, fused to the *tac* promoter at the *Ava* I site of the cloning cartridge of pKK223-3. The DNA sequence of the resulting fusion site was determined and is shown below; the sequence derived from pKK233-2 is in normal type and that from *nhaA* is in boldface type.

```
                    Ptrc
ITGACAIATTAATCATCGGCTCGITATAATI
GTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAA
M  W  N  C  E  R  I  T  I  S  H  R  K
          Ava I
CAGAATTCCCIGAGGCATTATTCTTATCATTGCCGCTAT
G  N  S  R  G  I  I  L  I  I  A  A  I
CCTGGCGATGATT...
 L  A  M  I  ...
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The resulting mutated gene shows that 14 amino acids of the N terminus of NhaA were replaced with 17 different amino acids. Note that the frame of the remaining sequence of *nhaA* was not altered.

All other histidine mutants were obtained by site-directed mutagenesis of specific histidines to arginines. Mutations H244R and H319R were obtained by using the *in vitro* mutagenesis kit of Amersham (version 2.0). For this purpose recombinant M13 mp8 and M13 mp9 bearing *nhaA* were constructed by inserting the *Hin*dIII–*Sal* I (3.1-kb) fragment of pGM36 (23) into the *Hin*dIII–*Sal* I (6.9-kb) fragment of the M13 replicative form DNA. Both mutations were verified by sequencing. Then the *Sal* I–*Hin*dIII fragments bearing the mutations were excised from the respective mutagenized replicating forms and inserted into the *Sal* I–*Hin*dIII (3.7-kb) fragment of pBR322 to yield plasmids, pY01 and pY02, respectively.

The rest of the mutants were obtained by following a PCR-based protocol (25). DNA of pGM36 was utilized as a DNA template to obtain mutants H254R-H257R (a double mutant), H226R, and H39R. The outside primers in the case of the first two mutations were homologous to bp 310–329 of *nhaA* and 466–432 downstream of *nhaA*. In both cases, the resulting mutagenized DNA obtained (1450 bp) was digested with *Bgl* II and *Mlu* I and the *Bgl* II–*Mlu* I fragments (680 bp) were inserted into the *Bgl* II–*Mlu* I (6.2-kb) fragment of pGM36 to yield plasmids pYG1 and pYG2, respectively. The outside primers for the third mutation were homologous to bp 796–772 of *nhaA* and 423–405 upstream of *nhaA*, and the resulting mutagenized DNA (1.1 kb) was digested with *Ssp* I and *Bgl* II. The *Ssp* I–*Bgl* II fragment (731 bp) was inserted into the *Ssp* I–*Bgl* II fragment (6.1 kb) of pGM36 (obtained by partial digestion) to yield plasmid pYG3. In each case the entire fragment originated by PCR and placed in the recombinant plasmid was sequenced to verify the mutation. In the case of pYG3, a new restriction site, *Nsu* I, created by the mutation was also verified. Sequencing of single- and double-stranded DNA was conducted with the Sequenase kit (version 2.0, United States Biochemical).

Isolation of Membrane Vesicles and Assay of Na^+/H^+ Antiporter Activity. Assays of Na^+/H^+ antiport activity were conducted on everted membrane vesicles prepared from cells grown in LBK at pH 7.5 (26). The assay of antiport activity was based upon the establishment of a ΔpH (transmembrane pH gradient) by addition of D-lactate and then the decrease of that ΔpH upon the subsequent addition of NaCl, LiCl, or KCl. The ΔpH was monitored with acridine orange as a probe (14, 27) at an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

Construction and Expression of pYG62-2 and pYG62-36. The wild-type and mutated *nhaA* were 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 *Bgl* II–*Hin*dIII (1.46-kb) fragments excised from either pGM36 or pYG2 to create plasmids pYG62-36 and pYG62-2, respectively. For labeling with [^{35}S]methionine each plasmid was used to transform TA15 carrying pGP1-2 (24). Strains TA15/pGP1-2/pYG62-2 and TA15/pGP1-2/pYG62-36 were grown to OD_{600} of 0.5 at 30°C in MTC (supplemented with kanamycin, ampicillin, and glucose) at pH 7.5 or at pH 8.5 and labeled with [^{35}S]methionine (11, 24).

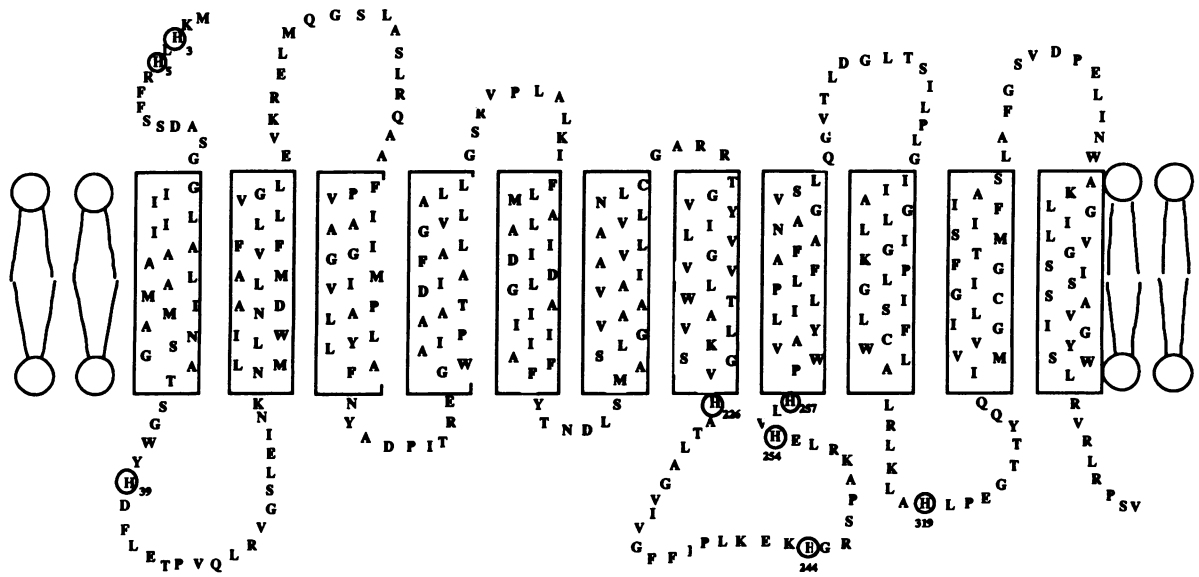

FIG. 1. Secondary structure model of NhaA showing the positions of histidine residues. The model is based on the hydropathy of the deduced amino acid sequence (11, 23) (*nhaA* nucleotide sequence is in GenBank, accession no. J03879). The single-letter amino acid code is used and histidine residues are circled and numbered.

To follow the turnover of the protein 20 mM methionine was added after 5 min of labeling and incubation was continued at 30°C. Samples were withdrawn and treated as described (23) except that the protease inhibitors ϵ-amino-*n*-caproic acid, benzamidine, and phenylmethylsulfonyl fluoride, 1 mM each, were added prior to sonication.

Preparation of Antibodies Against the C Terminus of NhaA and Quantitation of NhaA in the Membranes. Rabbits were immunized with the peptide GYSWLRVRLRPSV, corresponding to the C terminus of NhaA (23), coupled to thyroglobulin and boosted with the same peptide coupled to bovine serum albumin. Antibodies were purified from total serum by an affinity column prepared by coupling the peptide to Affi-Gel (Bio-Rad). For Western blots, membranes (1 mg) were resuspended in 200 μl containing 6 M urea, 10 mM Tris·HCl at pH 7.5, and 75 mM NaCl, incubated for 30 min at 4°C, centrifuged, and resuspended in 50 μl of sample buffer (28). Proteins were resolved by Tricine/sodium dodecyl sulfate/polyacrylamide gel electrophoresis (28), in gels which contained 6 M urea, 6.75% acrylamide, and 0.21% bisacrylamide, and transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore) prior to reaction with the antibody and detection using alkaline phosphatase coupled to anti-rabbit IgG (29).

Protein was determined according to ref. 30.

RESULTS

Growth Phenotype of Histidine Mutants of NhaA. NhaA, the Na^+/H^+ antiporter of *E. coli*, has eight histidine residues (Fig. 1). To study the role of these residues in the activity and/or pH sensitivity of the antiporter, we constructed various mutants of *nhaA*. The mutation Δ*nha*1–14 is the result of a deletion of 36 bp of the N-terminus-encoding region of the gene and a fusion to the DNA of the plasmid. The resulting putative protein is devoid of two histidines, His-3 and His-5, and its N-terminal sequence is described in *Experimental Procedures*. The other mutations were obtained by site-directed mutagenesis of the histidines to arginines. One of these carried a double mutation, H254R-H257R, and the four others carried a single arginine substitution for the respective histidine, H39R, H244R, H226R, and H319R.

To determine the growth phenotype conferred by the various mutations of *nhaA*, the mutated genes were introduced into pBR322 or pKK233-3. The respective plasmids were used to transform NM81, a strain lacking the *nhaA* antiporter gene (Δ*nhaA*) (5), or EP432, a strain lacking both *nhaA* and *nhaB* antiporter genes (Δ*nhaA*, Δ*nhaB*) (8). As previously described (5), NM81 bearing either of the vector plasmids, pBR322 or pKK233-3, does not grow on minimal medium agar plates in the presence of 100 mM LiCl. However, upon transformation with a high copy number plasmid bearing wild type *nhaA* (pGM36 or pEP3T) it acquires resistance to Li^+. The various plasmids carrying the *nhaA* histidine mutants also confer resistance to Li^+ on NM81 (data not shown).

The Na^+ tolerance of *E. coli* is dependent on the *nhaA* gene and this dependence increases with pH; the Δ*nhaA* strain, NM81/pBR322, is sensitive to NaCl (>400 mM, pH 7.5), and this sensitivity is augmented by pH [100 mM, pH 8.5 (5)]. To determine the Na^+ tolerance conferred by the various histidine mutants, NM81 cells carrying the respective plasmids were grown on LB agar plates in the presence of different concentrations of NaCl at various pH values. At both pH 7.5 and pH 8.5 all plasmids bearing arginines instead of histidines, except pYG2, conferred to NM81 tolerance to sodium (0.7 M and 0.5 M, respectively) similar to the wild-type plasmid, pGM36 (or pEP3T). These results indicate that none of the histidines (His-3, His-5, His-39, His-244, His-254, His-257, or His-319) are essential for the activity of NhaA either at pH 7.5 or at pH 8.5.

Similar results were obtained when EP432 rather than NM81 was used as a host to test the halotolerance conferred by the various plasmids. EP432 is more sensitive to Na^+ (50 mM at pH 7.5 and pH 8.5) than NM81. Nevertheless all plasmids bearing mutated *nhaA*, except pYG2, conferred upon EP432 the wild-type level of Na^+ tolerance at both pH 7.5 and 8.5, as did pGM36 (data not shown).

At pH 7.5 the H226R-bearing strains (NM81/pYG2 and EP432/pYG2) grew similarly to the strains carrying a wild-type *nhaA* plasmid (NM81/pGM36 or EP432/pGM36) in liquid media containing up to 600 mM NaCl (Fig. 2 and data not shown). However, at pH 8.5 the growth of strains carrying H226R was impaired. NM81/pYG2 behaved very similarly to the strain bearing only the vector plasmid; its doubling time (110 min) in 100 mM NaCl was twice as long as that of NM81/pGM36, and at 400 mM its growth was totally arrested (Fig. 2). In EP432 pYG2 conferred very limited Na^+ tolerance at pH 8.5 (doubling time 140 min in 200 mM NaCl) as compared to none conferred by pBR322. However, in comparison to the pGM36-bearing strain the salt tolerance of EP432/pYG2 was drastically reduced, as was that of NM81/pYG2 (Fig. 2). It is concluded that whereas at pH 7.5 the H226R NhaA confers Na^+ resistance to NM81 and EP432 to the same extent as the wild-type protein, it fails to do so at pH 8.5. This defect of H226R is specific to Na^+ at alkaline pH, since the mutation had no effect on growth of EP432/pYG2 at pH 8.5 in the presence of 400 mM KCl instead of NaCl (Fig. 2).

Properties of the H226R Protein. To show that the H226R protein is inserted into the membrane normally and is stable at alkaline pH we cloned H226R downstream of the T7 promoter (plasmid pYG62-2). A similar plasmid, pYG62-36, carrying the wild-type *nhaA* served as a control. Using these plasmids, we followed the synthesis of wild-type NhaA and the H226R mutant at both pH 7.5 (not shown) and pH 8.5 (Fig. 3*A*) and found that within 5 min the mutated protein was labeled with [^{35}S]methionine to an extent similar to the wild type. After a chase of unlabeled methionine both proteins turned over very slowly and with similar kinetics. After 2 hr of chase all of the radioactivity was still found in both proteins (data not shown). Thus, the phenotype observed at pH 8.5 is not due to changes in the turnover rate of the mutated protein.

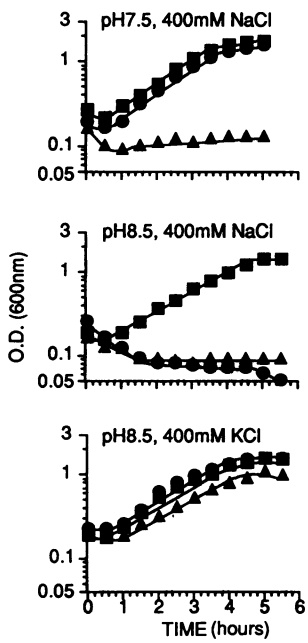

FIG. 2. Growth of the H226R mutant is sensitive to Na^+ at alkaline pH. Cells grown on LBK were diluted 10-fold (OD_{600} = 0.05) into LB medium containing 400 mM NaCl or 400 mM KCl, buffered by 2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol to pH 7.5 or 8.5 as indicated. Growth at 37°C was monitored. ■, EP432/pGM36; ●, EP432/pYG2; ▲, EP432/pBR322.

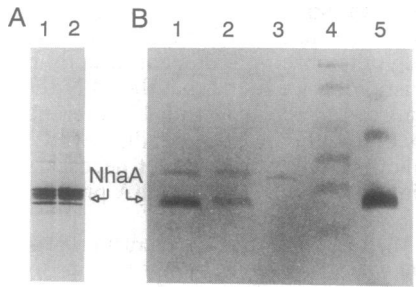

FIG. 3. Synthesis and amount of NhaA and its H226R mutant. (*A*) Cells carrying *nhaA* (TA15/pGP1-2/pYG62-36, lane 1) or H226R gene (TA15/pGP1-2/pYG62-2, lane 2) fused to the T7 promoter were grown at pH 8.5, induced by heat, exposed to rifampicin, and allowed to synthesize proteins in the presence of [^{35}S]methionine (0.2 μM methionine, 37.5 μCi/nmol) for 5 min. The radioactively labeled product in the membrane fraction was separated by electrophoresis on a sodium dodecyl sulfate/polyacrylamide gel and visualized by autoradiography. (*B*) The amount of NhaA was determined in membrane vesicles isolated from EP432/pGM36 (lane 1), EP432/pYG2 (lane 2), and EP432/pBR322 (lane 3), grown at pH 8.5, by using the polyclonal antibody produced against the C terminus of NhaA (each lane contained 50 μg of protein). Prestained molecular weight markers (low range Bio-Rad, lane 4) and pure NhaA protein and its aggregates (11) (lane 5) are also shown.

The absolute level of NhaA protein was also determined in membrane vesicles isolated from EP432/pGM36 and EP432/pYG2 grown at pH 7.5 (not shown) and pH 8.5, using the polyclonal antibody produced against the C terminus of NhaA. The pH of growth did not affect the levels of the wild-type or the mutated protein, which at both pH values amounted to at least 50–60% of the wild type (Fig. 3*B*). Since once the proteins are inserted into the membrane their turnovers are similar, we suggest that slower synthesis or less efficient insertion accounts for the lower level of the H226R protein. It is also possible that the mutant form has an altered immunoreactivity in the Western assay.

To determine whether the mutation H226R affects the activation of NhaA by alkaline pH, we isolated everted membrane vesicles from EP432/pYG2 grown at pH 7.5 and assayed the nonspecific K^+/H^+ antiporter activity and the specific Na^+/H^+ antiporter activity of these membranes as a function of pH (Figs. 4 and 5). The two antiporter activities were monitored by the ability of K^+ (measured first) and Na^+ to decrease the transmembrane pH gradient as measured by acridine orange fluorescence in the presence of choline chloride (140 mM) (Fig. 4). The Na^+/H^+ antiporter activity was also measured in the presence of 140 mM KCl, which saturates the K^+/H^+ antiporter and yields membranes exhibiting only the specific Na^+/H^+ antiporter activity (Fig. 5).

Membrane vesicles isolated from EP432/pBR322 exhibit the nonspecific K^+/H^+ antiporter (31) at both pH 7.5 and 8.5 but do not exhibit either of the two specific Na^+/H^+ antiporters (NhaA and NhaB) at either pH value (Fig. 4 and ref. 8). On the other hand, membrane vesicles isolated from EP432/pGM36 have in addition to the K^+/H^+ activity only the specific Na^+/H^+ antiporter activity of NhaA, which is activated at alkaline pH as previously described (Figs. 4 and 5 and refs. 5, 11, and 12).

At all pH values K^+/H^+ antiporter activity was observed in membrane vesicles isolated from EP432/pYG2, similar in magnitude and pattern to those observed in membrane vesicles isolated from EP432/pGM36 (Fig. 4). The maximal activity attained by NhaA in both membranes was also similar and the degree of activation by pH was the same (Figs. 4 and 5). However, the actual pH values at which H226R is activated changed dramatically with respect to the wild-type protein. Whereas the latter is activated mainly between pH 7 and pH 8 and hardly changes above this pH range (up to pH

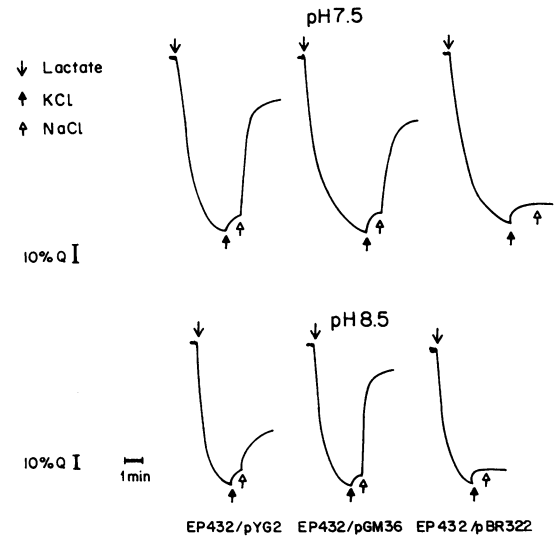

FIG. 4. Na^+/H^+ antiporter activity of the H226R-NhaA in everted membrane vesicles. Membrane vesicles were prepared from EP432/pYG2, EP432/pGM36, and EP432/pBR322 cells grown in LBK (pH 7.5). ΔpH was monitored with acridine orange in medium containing 140 mM choline chloride, 10 mM Tricine (titrated with Tris or HCl to the indicated pH), 5 mM $MgCl_2$, acridine orange (0.5 μM), and membrane vesicles (50 μg of protein). At the onset of the experiment Tris–D-lactate (5 mM) was added and the fluorescence quenching (*Q*) was recorded. KCl (10 mM, closed arrow) and NaCl (10 mM, open arrow) were then sequentially added and the new steady state of fluorescence obtained (dequenching) after each addition was monitored.

8.5), the main activation of H226R occurs at a more acidic pH, between pH 6.5 and 7.5. Furthermore, above pH 7.5 the activity of the H226R protein decreases and at pH 8.5 it is only 10–20% of its maximum (Fig. 5).

To rule out the possibility that pH above 7.5 irreversibly inactivates the H226R protein in isolated membrane vesicles, we incubated membranes isolated from H226R cells at pH 8.5 for 20 min and then assayed their activity at pH 7.5; their activity was identical to that of membrane vesicles which had not been exposed to alkaline pH (data not shown). Therefore the inactivation of the Na^+/H^+ antiporter activity of the H226R mutant at alkaline pH is reversible.

It was previously shown that pH affects the apparent K_m of the Na^+/H^+ antiporter activity in isolated membrane vesicles, suggesting that protons compete with Na^+ directly at the active site of the antiporter (12). The concentrations of Na^+ required for half-maximal effect on acridine orange fluorescence in membrane vesicles isolated from EP432/pYG2 were

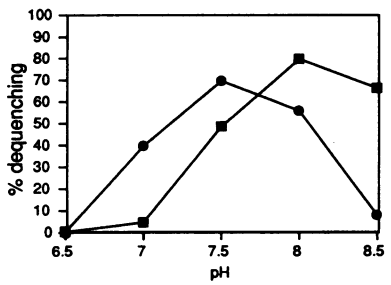

FIG. 5. pH dependence of the Na^+/H^+ antiporter activity of H226R-NhaA and wild-type NhaA. Everted membrane vesicles were prepared from cells of EP432/pGM36 (■) and EP432/pYG2 (●) and the Na^+/H^+ antiporter activity was determined as described for Fig. 4 except that the reaction mixture contained 140 mM KCl instead of choline chloride. The percent of dequenching observed following addition of 10 mM NaCl is shown versus pH of the assay.

4 mM at pH 7.5 and 0.4 mM at pH 8.5, which are very similar to those obtained for EP432/pGM36, 3 mM and 0.6 mM, respectively. Therefore, although these measurements yield values only indirectly related to the K_m of the antiporter, it appears that His-226 is not responsible for the effect of pH on the K_m for Na^+. Therefore, we suggest that a pH-sensitive site regulates the V_{max} of the NhaA antiporter and that His-226 is involved in the pH "sensing" capacity of this site.

DISCUSSION

In this study we found that none of the histidines in NhaA are essential for the Na^+/H^+ antiporter activity of the NhaA protein. However, the replacement of His-226 by Arg markedly changes the previously described unique activation of the antiporter by alkaline pH (5, 11, 12). Thus, while the wild type and all histidine mutants, except H226R, confer upon NM81 and EP432 resistance to Na^+ up to pH 8.5 and also resistance to Li^+ (tested at pH 7), cells bearing the H226R mutation (pYG2) are resistant to Li^+ at pH 7 and to Na^+ below pH 7.5, but they become Na^+ sensitive above pH 7.5.

The Na^+-sensitive phenotype conferred by H226R at alkaline pH is not due to the slightly reduced amount of H226R-NhaA in the membrane, since these levels are much higher than the level detected in membranes isolated from the wild-type strain, TA15, which contains only a single copy of *nhaA* (5, 6). Furthermore, membrane vesicles derived from EP432/pYG2 or EP432/pGM36 permitted the comparison of the Na^+/H^+ antiporter activity of the mutant protein with that of the wild type. These membranes are devoid of NhaB activity and, in the presence of K^+ at a high concentration, which saturates the K^+/H^+ antiporter, display only the NhaA Na^+/H^+ antiporter activity. This comparison shows that the sensitivity to Na^+ of H226R-bearing cells at alkaline pH is due not to a change in the maximal level of activity the H226R protein can attain, but rather to a change in the pH dependence of this activity. Whereas the drastic activation by pH of wild-type NhaA occurs between pH 7 and 8, that of the H226R antiporter occurs between pH 6.5 and 7.5. Furthermore, while the wild-type antiporter remains almost fully active at least up to pH 8.5, the activity of the mutated one peaks at 7.5, and it decreases to only 10–20% of the maximal activity at pH 8.5.

Since the H226R mutation affects not the maximal activity of the antiporter but only its pH dependence, we suggest that a pH-sensitive site exists on NhaA which regulates its activity. Although His-226 is part of this site, which we call the "pH sensor," it is most likely that other amino acids are involved in this sensor since, albeit abnormally, the mutated protein still reacts to pH. If protonation of His-226 or Arg-226 is involved in the reactivity of the protein to pH, we must conclude that either both or one of them has a pK in the protein which is different from its pK in solution.

The setpoint of the pH sensor of NhaA is around pH 7.5, the homeostatic value of the intracellular pH of *E. coli* (13). An increase in intracellular pH will cause an accelerated activity of NhaA, which results in acidification of the cell cytoplasm back to the homeostatic value. This sensor is essential, as shown by the fact that H226R, which cannot regulate its activity and displays only 10% of the maximal activity at pH 8.5, cannot grow under these conditions.

Most interestingly, this kind of pH sensor has been described in other proteins which are hypothesized to play a role in pH regulation. The animal Na^+/H^+ antiporter (Nhe1) is also regulated by pH, but its dependence on pH is a mirror image of that of NhaA. As expected from its role as the main acid extrusion device in these cells, it is activated at acidic pH and is inactivated above pH 7.2–7.3 (15, 17). The nonerythroid Cl^-/HCO_3^- exchanger seems to respond to pH in a manner similar to NhaA (16). It will be most interesting to compare the "pH sensors" of these proteins.

Even though the observation is not directly related to the role of the histidines, we found that the 14 N-terminal amino acids of NhaA can be replaced with 17 different amino acids with no effect on the phenotype displayed by the cell, suggesting that the N terminus is not necessary for expression, insertion, or activity of the NhaA protein.

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