Replacements of Histidine 226 of NhaA-Na\(^+\)/H\(^+\) Antiporter of Escherichia coli

CYSTEINE (H226C) OR SERINE (H226S) RETAIN BOTH NORMAL ACTIVITY AND pH SENSITIVITY, ASPARTATE (H226D) SHIFTS THE pH PROFILE TOWARD BASIC pH, AND ALANINE (H226A) INACTIVATES THE CARRIER AT ALL pH VALUES*

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We have previously shown that replacement of His-226 in the NhaA Na\(^+\)/H\(^+\) antiporter of Escherichia coli to Arg (H226R) shifts the pH profile of the antiporter toward acidic pH and as a result a ΔnhaANhaB strain bearing this mutation is Na\(^+\) sensitive at alkaline pH (Gerchman, Y., Olami, Y., Rimon, A., Taglicht, D., Schuldiner, S. and Padan, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1212–1216).

In the present work the role of His-226 in the response of NhaA to pH has been studied in detail. The Na\(^+\) sensitivity of the ΔnhaANhaB mutant bearing the H226R-NhaA plasmid at alkaline pH provided a very powerful tool to isolate revertants and suppressants of H226R growing on high Na\(^+\) at alkaline pH. With this approach cysteine (H226C) and serine (H226S) replacements were found to efficiently replace His-226 and yield an antiporter, which like the wild-type protein, is activated by pH between pH 7 and 8. These results imply that polarity and/or hydrogen bonding, the common properties shared by these amino acid residues, are essential at position 226 for pH regulation of NhaA.

This suggestion was substantiated by site-directed mutagenesis of His-226 either to alanine (H226A) or aspartate (H226D). Whereas H226A-NhaA shows very low activity which is not activated by pH, H226D-NhaA is active and regulated by pH. The pH profile of H226D is shifted by half a pH unit toward alkaline pH, as opposed to the previously isolated mutant H226R which has a pH profile shift, to the same extent, but toward acidic pH. It is suggested that charge modifies the pH profile but is not essential for the pH regulation of NhaA.

Sodium proton antiporters are ubiquitous membrane proteins found in the cytoplasmic and organelle membranes of cells of many different origins, including plants, animals, and microorganisms. They are involved in cell energetics, play primary roles in signal transduction and in regulation of intracellular pH, cell Na\(^+\) content, and cell volume (for reviews, see Refs. 1–4).

Escherichia coli has two antiporters, NhaA (5) and NhaB (6), which specifically exchange Na\(^+\) or Li\(^+\) for H\(^+\) (4). NhaA is indispensable for adaption to high salinity, for challenging Li\(^+\) toxicity, and for growth at alkaline pH (in the presence of Na\(^+\)) (7). Accordingly expression of nhaA which is dependent on NhaR, a positive regulator, is induced by Na\(^+\) in a pH-dependent manner (8–10). nhaB by itself confers a limited sodium tolerance to the cells, but becomes essential when the lack of NhaA activity limits growth (11).

Both the NhaA and NhaB are electrogenic antiporters which have been purified to homogeneity and reconstituted in a functional form in proteoliposomes (12–14). The pH at which half-maximal V\(_{\text{max}}\) of NhaA is highly dependent on pH, changing more than 1000-fold over the pH range between 7 and 8 (12). This pH dependence of NhaA is expressed also in isolated membrane vesicles (15) and is unique to NhaA. The V\(_{\text{max}}\) of NhaB is pH independent (7, 13).

The steep pH dependence of NhaA is expected from its proposed role in pH homeostasis of the cytoplasm at alkaline pH (3, 16). 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 a self regulated mechanism (4). This pattern of a "pH meter" and "titrator" in the same molecule also exists in other transporters involved in pH regulation which show very little sequence similarity with NhaA: in the mammalian Na\(^+\)/H\(^+\) antiporter (Nhd1) (17), and in the non-erythroid C1/HCO\(_3^-\) exchanger (18). The pH at which half-maximal activity (the set point) of the human protein (nhd1) is observed seems to be regulated by hormones and environmental conditions such as osmolality (19). In the eukaryotic systems none of the protein resides involved in pH sensing are known.

In Escherichia coli His-226 has been shown to be involved in the pH sensitivity of NhaA (20). Site-directed mutagenesis of each of the eight histidines of NhaA showed that none are essential for Na\(^+\)/H\(^+\) antiport activity of NhaA. However, the replacement of His-226 by Arg (H226R) markedly changed the pH dependence of the antiporter. A strain deleted of both antiporters genes, nhaA and nhaB, transformed with multi-copy plasmid bearing wild-type nhaA, exhibits both Na\(^+\) and Li\(^+\) resistance, throughout the pH range of 6–8.5. In marked contrast, transformants of plasmid bearing H226R-nhaA are resistant to Li\(^+\) and 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 8, that of H226R antiporter occurs between pH 6.5 and 7.5. In addition, while the wild-type antiporter remains almost fully active, at least up to pH 8.5, H226R is reversibly inactivated above pH 7.5, retaining only 10–20% of the maximal activity at pH 8.5 (20).

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In the present work the role of His-226 in the response of NhaA to pH has been studied in detail. Both histidine and arginine potentially bear a positive charge, we tested the effect of a negative charge or no charge, by site-directed mutagenesis of His-226 to aspartate (H226D) or alanine (H226A), respectively. Furthermore, the Na⁺ sensitivity of the ΔnhaAΔnhaB mutant bearing the H226R-NhaA plasmid at alkaline pH provided a very powerful tool to isolate first-site revertants and suppressants of H226R growing on high Na⁺ at alkaline pH. Combining both approaches we found that positively charged or polar residues, histidine, cysteine, and serine, allow the increase in activity of NhaA at alkaline pH. Aspartate shifts the pH profile toward alkaline pH. Alanine, however, drastically reduces the activity of the antiporter at any pH.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions—**EP432 is an E. coli K12 derivative, which is mbl1Δ, nhaA1, ΔnhaB, kan, cam, lacZΔ, thr1 (11). CC130 mutD-S was used for in vivo mutagenesis. It is a derivative of MC1000 which is Δara-leu, thi, galE, galK, rpsL. (StrpΔ), ΔlacZΔΔ, (pH226R-proc), tac::Tn5 (KanR) kindly provided by Etian Babi, Weizmann Institute of Science, Israel. Cells were grown either in L broth (LB) or 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 BTP, and pH was titrated with KOH. For plates the growth medium was solidified with 1.5% Difco agar. For selection, kanamycin (50 μg/ml), ampicillin (100 μg/ml), and chloramphenicol (20 μg/ml) were used.

Plasmids—All plasmids are pBR322 derivatives. pGM36 carries wild-type nhaA (21). pyG2 which carries the H226R mutation in nhaA was constructed previously (20) and occasionally denoted pH226R after the mutation. This nomenclature is used here for all mutated nhaA bearing plasmids constructed as described below. Thus pH226R, pH226A, pH226C, and pH226S carry position 226, instead of the histidine codon, codons of aspartate, alanine (Table I), cysteine, or serine (Table II), respectively.

Construction of Mutations in Histidine 226 of NhaA—Site-directed mutations for NhaA mutations H226D and H226C were obtained following a polymerase chain reaction based protocol (22). DNA of pGM36 was utilized as a DNA template. The end primers and the mutagenic primers are described in Table I. In both cases, the resulting mutagenized DNA (1450 base pairs) was digested with BglII-Mul, yielding a fragment of 682 base pairs, which was ligated to the 6.2-kilobase pair BglII-Mul fragment of pGM36, to yield plasmids pH226D and pH226A, respectively. In each case, the entire fragment originated by polymerase chain reaction and placed in the recombinant plasmid was sequenced to verify the accuracy of mutagenesis. Sequencing of double stranded DNA was conducted on a midi preparation (Plasmid Midi Kit, Qiagen, Germany) using the Sequenase kit (version 2.0, U. S. Biochemical Corp.).

Random mutagenesis of H226R-nhaA was conducted either in vitro, by chemical mutagenesis of pH226R utilizing hydroxyamine (23), or in vivo, by proliferation of pH226R in mutD strain (24, 25). For the chemical mutagenesis, pH226R (5 μg) was incubated in 1 ml with 50 mM sodium phosphate (pH 6), 1 mM sodium EDTA, and 0.4 M hydroxyamine hydrochloride (pH 3) for 3 h at 70 °C, the DNA purified on Jensen (Genomed) and transformed into EP432 by electroporation (Bio-Rad version 1.0). The transformants were grown on LBK plates and the grown colonies screened for Na⁺ resistance (at alkaline pH) containing 700 mEq NaCl (pH 8.3).

To verify that the resistance is conferred by a mutation in the plasmid, plasmids were isolated from the Na⁺-resistant colonies, retransformed into EP432, and rescreened on the high Na⁺/high pH plates.

To locate the mutation, the BglII-Mul fragment of the plasmid was excised and cloned into a BglII-Mul fragment of the wild-type plasmid (pGM36) as described above. If the Na⁺ resistance was retained, both strands of the entire cloned fragment were sequenced.

For the in vivo random mutagenesis, pH226R was transformed into CC130 mutD5(24), grown for 1 h in LBK (20 μg/ml thymidine), and then for an additional 12 h in the presence of ampicillin and kanamycin, plasmids were isolated and transformed by electroporation into EP432. To locate for mutants resistant to Na⁺ at alkaline pH and the identification of the mutations were conducted as above.

**Isolation of Everted Membrane Vesicles and Assay of Na⁺ / H⁺ Antiporter Activity—**Assays of Na⁺ / H⁺ antiporter activity were conducted on everted membrane vesicles prepared from cells grown in LBK at pH 7.5 (26). The assay of antiporter activity was based upon the establishment of a ΔpH (transmembrane pH gradient) by addition of o-lactate and then the partial abolition of that ΔpH upon the subsequent addition of NaCl or LiCl. The ΔpH was monitored with acridine orange as a probe (5, 27) at an excitation wavelength of 490 nm and emission wavelength of 530 nm.

Quantitation of NhaA in the Membranes—For quantitation of the Na⁺/H⁺ antiporter activity, cells were grown in LBK up to OD600 0.9, washed, and concentrated 50-fold by centrifugation (5 min at 7,000 × g), in buffer containing 150 mM NaCl, 20 mM Tris-Cl (pH 7.5), 1 mM 1,4-dithiothreitol, and protease inhibitors benzamidine, phenylmethylsulfonyl fluoride, ε-amino-n-carboxylic acid, 1 mM each. The suspension was sonicated (level 3, Heat System-Ultrasonic W385) 3 times for 20 s. Whole cells were removed by centrifugation for 3 min at 18,000 × g and the supernatant was centrifuged at 245,000 × g for 20 min. The membranes (1 mg) were resuspended in 200 μl containing 6 mM urea, 10 mM Tris-HCl (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% urea, 6.75% acrylamide, and 0.21% bis-acrylamide and transferred to polyvinylidene difluoride membranes (Millipore), prior to reaction with an antibody directed against a synthetic peptide based on the C-terminal sequences of NhaA (20). Detection was made using alkaline phosphatase coupled to anti-rabbit IgG (29) and quantitated by densitometry. Linearity between the amount of NhaA and the recorded density was confirmed by conducting the Western analysis at various dilutions of the antigen. Protein was determined according to Bradford (30).

**RESULTS**

To further explore the pH-sensitive domain of NhaA we used the previously isolated mutant H226R, which shows a pH optimum of activity shifted toward acidic pH and as a result is inactive at alkaline pH (20). When transformed into a strain lacking both Na⁺/H⁺ antiporters nhaA and nhaB (EP432), this antiporter mutant, as opposed to the wild type, renders the cells Na⁺ sensitive at alkaline pH (20). Hence this sensitivity to Na⁺ at alkaline pH affords a very powerful tool to select for Na⁺-resistant bacteria at alkaline pH created by reversion of H226R and/or either first-site or second-site suppression in the pH-sensitive domain of NhaA.

To increase the mutation frequency in H226R-nhaA, pH226R was treated in vitro by hydroxyamine or propagated in a mutD host. The randomly mutagenized plasmids were transformed into ΔnhaAΔnhaB host, plated at pH 8.2 in the presence of 700 mM Na⁺ and the Na⁺-resistant clones isolated. To verify that the mutations are plasmidic, the plasmids were isolated and shown to confer Na⁺ resistance at alkaline pH upon retransformation into ΔnhaAΔnhaB. To locate the mutation, a fragment (BglII-Mul), bearing codon 226, of the mutated nhaA was cloned instead of an identical fragment of the wild-type nhaA of pGM36. After verification that this DNA fragment is responsible for the Na⁺ resistant property at alkaline pH, the cloned fragment was sequenced to identify the mutation.

**TABLE I**

| Mutation | Use         | Location* | Change of codon 226 |
|----------|-------------|-----------|---------------------|
| H226A    | End primer  | 313 → 329 | None                |
| End primer | 1553 → 1529 | None      |                     |
| H226D    | Mutagenic primer | 772 → 799 | GCC → ACC  |
| H226D    | Mutagenic primer | 772 → 799 | GAC → GAC  |

*Location numbers are on the sequence as appeared in Gene Bank access number J03897.

b This mutation added a HindI site.
Table II summarizes the results of the screen which resulted in 45 mutants. Most of the mutants (71%) were revertants of H226R back to His-226. These results re-emphasize the importance of histidine at position 226 in the pH sensing capacity of NhaA. Eleven mutants obtained from the hydroxylamine-treated cells contained cysteine codons, 10 TGC and one TGT. Both at pH 7.5 and 8.5, the growth phenotype of the H226C mutant in the presence of Na+ is very similar to that of the wild type strain (Table III).

The Na+/H+ antiporter activity of the mutant was determined at various pH values in everted membrane vesicles isolated from ΔnhaAΔnhaB transformed with the mutated plasmids. Again this host proves very useful since it has no background of Na+/H+ antiporter activity when transformed with the vector plasmid (pBR322, Fig. 1) but exhibits full activity with the plasmid bearing wild-type nhaA (pGM36, Figs. 1 and 2). The data obtained from the mutant at pH 7.5 and 8.5 is shown in Fig. 1 while the pH profile of the Na+/H+ antiporter activity throughout the pH range from 7 to 9 is summarized in Fig. 2A. For comparison, the Na+/H+ antiporter activity versus pH of pH226R (20) is also shown.

The maximal activity of the Na+/H+ antiporter in isolated membrane vesicles derived from the H226C-mutant is between 60 and 62% of that of the wild type both at neutral and alkaline pH (Fig. 1 and data not shown). However, the pH dependence of this mutant antiporter is identical to that of the wild type activity increasing between pH 7 and 8 (Fig. 2A). These results show that cysteine at position 226 can replace His-226 and confer growth parameters very similar to that of the wild type as well as Na+/H+ antiporter activity which is substantially activated by pH between pH 7 and 8.

Most interestingly, the mutant screen after in vivo mutagenesis in the mutD genotype also produced mainly first-site reversions to His-226. Furthermore, it yielded a new mutation, serine at position 226 (Table II). Both in growth parameters (Table III) and Na+/H+ antiporter activity of everted membrane vesicles (Figs. 1 and 2A) the H226S mutant is very similar to the H226C mutant, and thus to the wild type. To assess the expression of the various mutants we used anti-NhaA antibody and found that both the H226C and H226S mutants are significantly expressed in membranes, 40 and 90%, respectively (Table IV).

Hence, either one or two properties shared by histidine, cysteine, and serine is important for the activation of NhaA by alkaline pH. These properties are polarity and capacity of forming hydrogen bonds. Nevertheless we have previously shown that arginine, which also has these properties but in addition bears an ionizable group with a pK at a more alkaline pH than that of histidine, shifts the pH profile of NhaA toward acidic pH (20). We therefore tested the effect of aspartate, presumably bearing a negative charge, in addition to the other common properties, and alanine with none of these properties, by site-directed mutagenesis of His-226 to H226D or H226A, respectively. The mutated plasmids were transformed into a ΔnhaAΔnhaB strain so that the growth phenotypes conferred by the mutated nhaA could be assessed with no interference of chromosomally encoded Na+/H+ antiporters.

The growth phenotype of the mutants is summarized in Table III. H226D grows at a growth rate very similar to that of the wild type at Na+ concentrations at least up to 400 mM and throughout the pH range between pH 7.5 and 8.5. Growth on solid medium is observed up to 700 mM NaCl at pH range between pH 7 and 8.5 (not shown). At the lower pH H226A also behaves like the wild type. In marked contrast, however, at pH 8.4 and above, H226A does not grow in the presence of 400 mM Na+ (Table III) while no difference was noted in the presence of 400 mM K+ (not shown).

The two mutants were expressed significantly but to a different degree (Table IV). In comparison to the wild type, the highest expression was that of H226A amounting to 78% and the lowest expression (24%) was that of H226D.

The maximal Na+/H+ antiporter activity in isolated everted membrane vesicles conferred by pH226D at alkaline pH is lower than that of the wild type (pGM36) by only 25%. However, the mutation shifts the pH profile of the mutated protein by half of a pH unit toward alkaline pH (Fig. 2B); whereas the maximal difference in activity of the wild type occurs between pH 7 and 8, H226D is activated between pH 7.5 and 8.5.

Strikingly, throughout the pH range between 7.5 and 9, H226A is not activated by pH and in contrast to both H226D and the wild type, exhibits very low and constant activity (Figs. 1 and 2B). Below pH 7.5 the very low activity of H226A decreases with lowering of the pH and vanishes at pH 7. Hence a polar group and/or the capacity to form hydrogen bonds, is essential for the activation of NhaA by alkaline pH. A charge shifts the pH profile.

### Table II

| Mutation   | No. of mutants | Percent of mutants | Codon change |
|------------|----------------|--------------------|--------------|
| H226R → H226 | 32 (22 + 10) | 71% | CGC → CAC |
| H226R → H226C | 10 | 22% | CGC → TGC |
| H226R → H226S | 2 | 4% | CGC → AGC |
| Total       | 45 (33 + 12) | 100% | |

### Table III

| Conditions | Doubling time (min) |
|-----------|---------------------|
| pH | NaCl | pGM36 | pH226A | pH226D | pH226C | pH226S | pH226R | pBR322 |
|-------|-------|--------|--------|--------|--------|--------|--------|--------|
| 7.5   | 400   | 43     | 43     | 50     | 45     | 50     | 46     | NG     |
| 8.4   | 400   | 72     | NG     | ND     | 65     | ND     | NG     | NG     |
| 8.5   | 400   | 85     | NG     | 95     | 90     | 90     | NG     | NG     |
FIG. 1. Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity in inverted membrane vesicles of the NhaA mutants at codon 226. Membrane vesicles were prepared from a ΔnhaAΔnhaB E. coli strain, EP432 (11) transformed with plasmid bearing wild-type nhaA, pGM36 (21), pBR322, or plasmids carrying the various mutations in nhaA in which codon 226 was replaced with cysteine (pH226C), serine (pH226S), aspartate (pH226D), or alanine (pH226A). Cells were grown in LBK (pH 7.5) and pH was monitored with acridine orange in medium containing 140 mM KCl, 10 mM Tricine (titrated with Tris or HCl to the indicated pH), 5 mM MgCl\textsubscript{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 (arrows pointing down) and the fluorescence quenching (Q) was recorded. NaCl (10 mM, arrows pointing up) were then added and the new steady state of fluorescence obtained (dequenching) after each addition was monitored.

DISCUSSION

The distinct pH dependence of NhaA Na\textsuperscript{+}/H\textsuperscript{+} antiporter of E. coli, being practically inactive below pH 7.0 and increasing V\textsubscript{max} dramatically between pH 7.0 and 8.0, led us to suggest the existence of a pH-sensitive domain in NhaA (12, 20). The pH sensitivity of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter was first demonstrated in right-side-out membrane vesicles measuring 2\textsuperscript{22}Na efflux driven by imposed artificial ΔpH or Δψ (15) and then in purified protein functionally reconstituted in proteoliposome, measuring passive 2\textsuperscript{22}Na efflux (12).

Since flux of cations via the cation/H\textsuperscript{+} antiporters affects the ΔpH across the membrane, cation induced changes in the fluorescence of acridine orange, and similar probes measuring Δψ, have been proven a fast and reproducible way to monitor the activity of H\textsuperscript{+}/cation antiporters in isolated membrane vesicles (26, 27). Although calculation of the kinetic parameters of an antiporter with this technique is complicated due to the indirect nature of the measurement, when it is conducted at cation concentrations above the K\textsubscript{m} of the antiporter, it most probably reflects the V\textsubscript{max} of the system and is thus most suitable for comparison of antiporters activity (26).

Indeed with this technique we found the same drastic pH dependence of NhaA Na\textsuperscript{+}/H\textsuperscript{+} antiporter as was found by direct flux measurement using 2\textsuperscript{22}Na (20). Furthermore, the experimental system used was inverted membrane vesicles isolated from the ΔnhaAΔnhaB mutant transformed with multicopy plasmid bearing nhaA. This system has proven most suitable to identify (by site-directed mutagenesis) residues in NhaA involved in the pH-sensitive domain of the protein. A mutation can be easily introduced into the plasmidic NhaA and its effect tested both in vitro, in inverted isolated membrane vesicles, and in vivo, in both cases with no background of chromosomal encoded by either NhaA or NhaB Na\textsuperscript{+}/H\textsuperscript{+} antiporters.

With this approach, we found that although none of the eight histidines in NhaA are important for Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity, His-226 is essential for the strong pH dependence of this protein. Thus NhaA bearing the mutation Arg-226 (H226R) has a pH profile shifted to the acidic pH range by half a pH unit and in addition at pH 8.5 is practically inactive rendering cells Na\textsuperscript{+} sensitive at this pH.

In the present study we have further tested the importance of His-226 in the pH sensitivity of NhaA Na\textsuperscript{+}/H\textsuperscript{+} antiporter. In one approach we randomly mutagenized H226R-nhaA, and exploited its Na\textsuperscript{+} sensitive phenotype at alkaline pH to select for mutations, restoring the capacity of the antiporter to function at alkaline pH and promoting growth in the presence of 700 mM NaCl. Such mutations were assumed to occur either in codon 226, the first-site (reversions and suppressions), or in other sites (second-site suppressions) participating in the pH sensor domain of NhaA.

Interestingly, all 45 mutations obtained were in the first-

![Graph showing pH dependence of Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity](Image)

![Table IV: Expression of NhaA mutants](Table)

| Plasmid | Expression |
|---------|------------|
| pGM36   | 100        |
| pBR322  | 0          |
| pH226C  | 40         |
| pH226D  | 90         |
| pH226R  | 50         |
| pH226S  | 24         |
| pH226A  | 78         |

FIG. 2. pH dependence of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter activity of the His-226 mutants. Everted membrane vesicles were prepared and assayed as described in the legend to Fig. 1 at the indicated pH values. A, the data obtained for EP432/pH226 (control), EP432/pH226C (●), and for EP432/pH226S (○), are plotted each, as percent of the respective maximal activity which was 60–62% of the wild-type. B, the percent of dequenching observed following addition of 10 mM NaCl is shown versus pH of the assay. EP432/pH226C (●), EP432/pH226D (▲), EP432/pH226A, EP432/pH226R, copied from Ref. 20.
site, 32 of which reversed back to His-226. Both after chemical mutagenesis of the H226R bearing plasmid with hydroxyamine or after in vivo mutagenesis by its proliferation in a mutD host bacteria, the majority of the first-site mutations reversed back to His-226, although in different proportions. Hydroxyamine acts by deaminating cytosine residues and converting them to thymine (23). This mutagenesis is therefore expected to be biased; cytosine containing codons such as that of H226R (GCG) would be changed back to that of histidine GTG. However, since the mutD dependent in vivo mutagenesis, which is random (25), gave mainly His-226 revertants we suggest that the high frequency of reversions to histidine at position 226 reflects the importance of this residue in the pH sensitivity of NhaA.

Three non-histidine first-site suppressors were obtained by the random mutagenesis approach. The hydroxyamine treatment produced two mutants with cysteine in position 226 (H226C) each encoded by a different codon: TGC and TGT, respectively. The mutD mutagenesis yielded the H226S mutation encoding serine at position 226. These first-site suppressors exhibit an identical phenotype, very similar to that of the wild type both in terms of growth in the presence of Na+ and D, and even binding of water molecules (31–33). It should be emphasized, however, that as long as the structure of the protein is unsolved we cannot exclude short range steric effects or long range conformational effects not related directly to the pH sensor.

It is of interest that the difference between the mutants in the activity of the antiporter at acidic pH is not expressed in growth phenotype. Even H226D with the lowest activity at this pH range grows like the wild type. On the other hand, at alkaline pH growth is performed only by the mutants which are substantially activated by pH, thus H226A stops growing beyond pH 8.4. These results corroborate our previous results with H226R (20) emphasizing the physiological importance of NhaA at alkaline pH in the presence of Na+.

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