Identification of Membrane Domains of the Na\(^+/\)H\(^+\) Antiporter (NhaA) Protein from Helicobacter pylori Required for Ion Transport and pH Sensing*

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The Na\(^+/\)H\(^+\) antiporter from Helicobacter pylori (HP NhaA) is normally active within the pH range 6.0–8.5. In contrast, the NhaA from Escherichia coli (EC NhaA) is active only within the alkaline pH range 7.5–8.5. We studied structures of HP NhaA involved in ion transport and pH sensing by analyzing mutants with defects in NhaA activity. The 36 mutants were classified into three types. The first type exhibited very low or null activity at all pH levels and had amino acid substitutions in the transmembrane segments (TM) 4, 5, 10, and 11, implicating these TMs in ion transport. The second type, which had amino acid substitutions at Met-138, Phe-144, and Lys-347 in TM 4 and 10, exhibited very low antiporter activity at acidic pH but had significantly higher activity at alkaline pH. These results imply that TM 4 (Met-138 and Phe-144) and 10 (Lys-347) are involved in supporting transport activity at acidic pH, in addition to their essential role in the overall transport mechanism. The third type of mutant exhibited very low antiporter activity at alkaline pH but relatively normal activity at acidic pH and had amino acid substitutions in loop 7 (a hydrophilic region between TM 7 and 8) as well as in TM 8, suggesting that these regions are involved in antiporter activation at alkaline pH. Three revertants that suppress a Lys-347 mutation were identified. Two of three suppressor mutations were located in loops 2 and 4, suggesting a functional interaction between these regions (loops 2 and 4 and TM 10). Thus, HP NhaA activity may be modulated by two independent factors that are dependent on pH: an activation mechanism at acidic pH, which is regulated by residues within TM 4 and 10 and another mechanism functioning at alkaline pH regulated by residues within loop 7 and TM 8.

Na\(^+/\)H\(^+\) antiporters are ubiquitous membrane proteins found in cytoplasmic and organelle membranes of many organisms from bacteria to humans (1–5). They play a primary role in the regulation of intracellular pH and cellular Na\(^+\) concentrations by exchanging Na\(^+\) for H\(^+\). In *Escherichia coli*, three Na\(^+/\)H\(^+\) antiporters (EC NhaA,\(^3\) NhaB, and ChaA) are known, and their functional characteristics have been well described (5–9). Among the three antiporters, EC NhaA plays a major role in regulating intracellular Na\(^+\) concentrations. Its activity is dependent on environmental pH and increases markedly as pH is elevated from neutral to alkaline. At pH 7.0, EC NhaA has negligible antiporter activity, while at pH 8.5 it is enhanced by three orders of magnitude (8). The NhaA gene has been cloned (5), and essential residues for ion transport have been deduced on the basis of its primary structures (10, 11). The topological arrangement of twelve putative transmembrane domains (TM) has been modeled on the basis of its secondary structure using phoA fusion experiments (12) and also on the basis of its tertiary structure using crystallographic analyses at 7 Å resolution (13). For *E. coli* NhaA, three Asp residues in TM 4 and 5 are known to be essential for ion transport (10), while His-225 (11, 14) and Gly-338 (15) play important roles in pH sensing. Although extensive studies have been performed, the structure-function relationships for mechanisms of ion transport or pH sensing are not fully understood.

We have previously shown that NhaA from *Helicobacter pylori* (HP NhaA) functions in *E. coli* where it exhibits a very different profile of pH sensing. In *E. coli* HP NhaA is active within the pH range 6.0–8.5 (16). This is in contrast to EC NhaA, which functions at alkaline pH (7.5–8.5). We have analyzed the molecular basis underlying this difference in HP and EC NhaA pH sensitivity in an effort to elucidate the mechanisms of pH sensing by antiporters. We have previously shown that a chimera antiporter with 8 TMs (1–8) from HP NhaA and 4 TMs (9–12) from EC NhaA, exhibited an intermediate antiporter profile in terms of pH dependence (17). Based on this observation and other related results (15), we hypothesized that a region responsible for pH sensing must lie in a structure formed by the interaction between the N-terminal eight domains (TM 1–8) and C-terminal four domains (TM 9–12), rather than in an unconserved unique sequence found in the loop structure between TM 8 and 9 (loop 8) of HP NhaA.

In the present study, we surveyed residues in HP NhaA required for antiporter activity and pH sensing by analyzing mutants with altered antiporter activities. Mutants were classified into three phenotypes based on the localization of their respective mutations to separate domains, and residues implicated in ion transport and pH sensing in HP NhaA were identified.

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1 The abbreviations used are: EC NhaA, *E. coli* Na\(^+/\)H\(^+\) antiporter A; TM, transmembrane segment; HP NhaA, *H. pylori* Na\(^+/\)H\(^+\) antiporter A; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ACMA, 9-amino-6-chloro-2-methoxyacridine.

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The mutated sequence at the target codon is shown by bold letters.

- **Table I**
  Oligo DNAs used in this study

| Mutation | Sequence (5' → 3') | Codon change |
|----------|--------------------|--------------|
| M138C    | GGGATCCCTGGCAGGGAGA| ATG → TTG    |
| M138A    | GGGATCCCTGGCAGGGGA| ATG → TCG    |
| F144C    | GGGATCCCTGGCAGGGAGA| ATG → TTC    |
| K347C    | TTGCTGGGAGGCCTTTAG| AAA → TGG    |
| K347E    | TTGCTGGGAGGCCTTTAG| AAA → CAG    |

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents—**Restriction endonucleases, T4 DNA ligase, Taq, and KOD DNA polymerases were purchased from Toyobo Co. Oligonucleotides were synthesized by Invitrogen Co. Other reagents and materials were of the highest commercially available grade.

**Bacterial Strains and Culture Conditions—** E. coli strains KNabc (Δ nhaA::Km', Δ nhaB::Em', Δ nhaA::Cm', supE, hsdS, thi, Δ (lac-proAB::F' tRNA3'4, proAB::lacI lacM15)) (18) and JM109 (19) were used to express HP NhaA mutant molecules and construct various plasmids, respectively. Cells were cultured in L broth (LB) (20) containing 87 mM KCl instead of NaCl (LBK). For growth on solid plates, agar (1.5%, w/v) was added to the medium. Transformants were selected using an appropriate antibiotic. For analysis of salt tolerance in KNabc transformed with various plasmids, different concentrations of NaCl or LiCl were added to LB plates containing 87 mM NaCl. The plates and broth cultures were incubated at 37 °C.

**Construction of Expression Plasmids—**Construction of HP and EC NhaA expression plasmids derived from pBR322 (pBR-HP and EC) has been described previously (17). Membrane vesicles from membranes of NhaA expression plasmids with the introduced mutated residues was verified by Western blot analysis using antibodies directed against the FLAG epitope tag attached to the C-terminal end of NhaA. A gene was amplified from the pBluescript vector with a FLAG epitope tag at the 3'-end of the coding region and under the control of the tet promoter. NhaA expressed from these vectors contain an additional sequence, GMQ, at the junction of the FLAG tag sequence (DYKDDDDK). A CTT codon was substituted for the GCT codon in control membranes from host E. coli nhaA in order to avoid retardation of host cell growth, as described previously (21).

**Identification of Altered Residues—** Random mutagenesis was introduced into HP nhaA by PCR (23) as follows. The FLAG-tagged HP NhaA gene was amplified from the pBluescript vector with Taq polymerase (TAKARA Co.) and M13 forward and reverse primers. A limited concentration of dATP or dTTP (0.4 μM) was present in the reaction for five cycles (denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and elongation at 70 °C for 30 s), followed by 25 cycles with additional dATP or dTTP (200 μM), as described previously (11). 1-μl aliquots of the above sample were taken, and the mutagenized nhaA was re-amplified by KOD DNA polymerase (TOYOBO) together with T7 and T3 primers for eight cycles (denaturation at 94 °C for 15 s, annealing at 50 °C for 10 s, and elongation at 70 °C for 30 s). The samples thus obtained were digested with EcoRI and Sall, and this fragment was ligated back into the corresponding sites of pBR-HP. After transformation of KNabc, ampicillin-resistant transformants were isolated, and their growth was examined on LB plates containing 0.65 mM NaCl (pH 8.0) or 0.15 mM LiCl (pH 7.5). After incubation for 24 h at 37 °C, clones that did not grow or grew more slowly than the wild type were designated as Na'- or Li'-sensitive mutants. The plasmids carried by the mutant were isolated and the mutated nucleotide(s) verified by DNA sequencing.

**Site-directed Mutagenesis—** A site-specific mutation was introduced into HP nhaA by PCR. pBluescript II KS+ containing a DNA encoding a FLAG-tagged HP nhaA was used as a DNA template, and the primers used are listed in Table I. The samples thus obtained were digested with EcoRI and Sall, and this fragment was ligated back into the corresponding sites of pBR-HP. The DNA sequence of the constructed plasmids with the introduced mutated residues was verified by sequencing.

**Isolation of Suppressor Mutations—** The expression plasmids harboring a mutation (M138K, F144L, K347E, or K347C) were introduced into KNabc. The transformed cell suspension was spread onto LB plates containing 0.65 mM NaCl (pH 8.0) or 0.15 mM LiCl (pH 7.5) and incubated at 37 °C for 1 day. Clones that grew on the selection plate were designated as revertants.

**Immunological Detection of FLAG-tagged NhaAs—** Expression of HP NhaA and its mutated derivatives was detected by Western blot analysis as described previously (17).

**Preparation of Membrane Vesicles and Na'/H' Antiporter Assay—** Membrane vesicles from E. coli cells transformed with various expression plasmids were prepared as described previously (17). Membrane vesicles (200 μg) were resuspended in 2 ml of assay buffer (10 mM Tricine and 140 mM KCl adjusted to the desired pH with KOH) as described previously (10). Proton flux was measured by monitoring 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching after the addition of potassium lactate (5 mM, pH 7.0) as a substrate of the electroneutral antiport activity. Fluorescence was measured with a fluorospectrophotometer (JASCO FP-750).

**DNA Preparation and Manipulation—** Preparation of plasmids, digestion by restriction endonucleases, and ligation by T4 DNA ligase of the DNA fragments, and other techniques related to handling of DNA were performed according to published procedures (22). The nucleotide sequences of DNA fragments cloned into the various expression plasmids in this study were determined using an automatic sequencer (PE Biosystems Co.).

**RESULTS**

**Isolation of Mutants Defective in HP NhaA Activity and Identification of Altered Residues—** To identify residues responsible for ion translocation and pH-dependent antiporter activities of HP NhaA, we introduced amino acid substitutions into the entire gene of NhaA by causing replication errors during PCR gene amplification. E. coli strain KNabc lacking Na'/H' antiporter genes (18) was transformed by expression of vectors encoding mutant forms of NhaA. 229 independent clones that were sensitive to higher concentrations of NaCl and/or LiCl were isolated from 4258 antibiotic-resistant clones. These mutants showed retarded cell growth on selection plates, which contained high concentrations of NaCl (0.65 mM, pH 8.0) or LiCl (0.15 mM, pH 7.5). The presence of NhaA in transformed cells was tested by Western blot analysis using antibodies directed against the FLAG epitope tag attached to the C-terminal end of NhaA. 59 of the salt-sensitive clones expressed NhaA, and, upon further analysis, NhaA was found in the cytoplasmic membrane fractions of 36 of these clones. The plasmid DNA was isolated from each mutant clone, and the nucleotide sequence of NhaA was determined. Accordingly, mutated base(s) were identified for each mutant (Table II). 21 clones carried single base substitutions, while the rest of the mutants had two or more substitutions. Further analysis revealed that in those mutants with a single base substitution, these mutations occurred within three independent domains of NhaA (Fig. 1). The first, second, and third groups of mutations were found in TM 4 and 5; loop 7 (between TM 7 and 8) and TM 8; and TM 10 and 11, respectively. Most mutants exhibited extensive growth retardation on the agar plates containing 0.65 mM NaCl or 0.15 mM LiCl, strongly suggesting that mutations in NhaA create a defect in antiporter activity (Table II). However, two mutants (T235M, K347T) exhibited very low, but significant, levels of cell growth on the plates, suggesting that these mutations caused defects in transport activity, but to a lesser extent than in the other mutants. It should be noted that F144L, L241P, K347E, and S389P mutations were each found independently on two separate occasions. This suggests that mutagenesis may have been close to saturation in the present survey.

**Characterization of Defects in Mutant Antiporter Activity—** To elucidate the functional consequences of the identified mutations, antiporter activity in inverted membranes prepared from mutant cells was measured under different pH conditions (Fig. 3). As shown previously (17), wild-type HP NhaA antiporter exhibited high activity at all pH conditions tested (Fig. 2a and Fig. 3a), while EC NhaA was activated at alkaline pH. Control membranes from host E. coli without NhaAs showed no antiporter activity (Fig. 3a). The profiles of pH-dependent activities in the mutants were classified into three types (Table III). The first type had both Na'/H' and Li'/H' antiporter activities that were lower than 20% of the wild-type activity at any pH tested. For example, the D172G mutant exhibited null
antiporter activity at all pH levels (Figs. 2b and 3b), and ten other mutants were also classified as this type (Table III). The M138K mutation and two other mutations (Fig. 2c, Table III, and Fig. 3c) induced inhibitory effects on antiporter activity at acidic pH, but increased activity at alkaline pH (type II). Finally, the H233R mutation greatly decreased antiporter activity at alkaline pH but did not affect activity at acidic pH (type I). Other mutants were also classified as type III (Table III).

To exclude the possibility that low antiporter activity may reflect decreased membrane expression of NhaA, representative mutants from each of the three groups were subjected to immunoblot analysis of mutant NhaA in membrane vesicles (Fig. 4). Similar amounts of NhaA were detected for each of these mutations (Fig. 4) and also for several other mutations described in Table III (data not shown). Thus, the low or null antiporter activity in all mutation types must be due to a defect in the mechanism of antiporter activity, rather than to decreased expression of membrane NhaA.

Notably, mutated residues were localized to specific regions of NhaA for each of the three types of mutations (Fig. 1). All type I and type II mutations were found in TM 4, 5, 10, and 11, but not in loop 7 or the TM 8 region, with the exception of S230R. Type III mutations appeared in loop 7 and TM 8. These results suggested that TM 4, 5, 10, and 11 play important roles in basic or regulating antiporter activity. More specifically, the results implied that TM 4 and 10 may be involved in maintaining high antiporter activity at acidic pH, whereas loop 7 and TM 8 appear to be involved in enhancing its activity at alkaline pH.

Eight double mutants that contained amino acid substitutions at two independent residues were also identified. We separated double mutations into single for D171N/I176N, D171N/E347K, and L167F/I347N, and analyzed the phenotype for each constituent mutation. The double mutant with D171N and I176N mutations showed null antiporter activity at all pH values and was phenotypically similar to a type I mutant. Asp-171 corresponds to Asp-163 in EC NhaA, which is essential for antiporter activity (10). The single D171N mutation produced a total loss of antiporter activity, whereas I176N had essentially no effect (data not shown). Therefore, the phenotype of D171N/I176N could be explained mostly by the D171N mu-
The phenotype of the double mutation F144L (TM 4)/I421N (TM 12) was similar to the profile of a type II mutation (data not shown). This phenotype was explained by F144L mutation alone, since the I421N single mutation did not cause any change in the pH sensitivity profile of antiporter activity.

Q229R/G231C mutations decreased antiporter activity at alkaline pH, similar to type III mutations. The mutant containing G231C mutation alone displayed this phenotype, but Q229R did not cause any change in antiporter pH dependence (data not shown). In summary, the analyses of single mutations...
Kinetic Analysis of the Antiporter Activities for the Type II Mutants—To understand the effects of type II mutations on the transport mechanism, we performed a kinetic analysis of antiporter activity. As shown in Table IV, M138K and F144L mutations decreased maximal velocity \( (V_{\text{max}}) \) of Na\(^+\)/H\(^+\) antiporter activity, but their Michaelis constants \( (K_m) \) were similar to that of the wild type (~0.2 mM). In contrast to these mutations, two mutations at Lys-347, K347Q and K347S, which were isolated as revertants, (described below), exhibited different kinetics: both mutations affected the \( K_m \) values. The effect of the K347Q mutation is obvious in terms of increasing \( K_m \), derived from the double mutants identified new type I (D171N) and III (G231C) mutations (Table III).

Site-directed Mutations at Met-138, Phe-144, and Lys-347—The residues of type II mutations, Met-138, Phe-144, and Lys-347 are conserved between H. pylori and E. coli NhaA, and mutations in these residues shifted the pH dependence profiles of the antiporter to the EC NhaA phenotype. We further analyzed the effects of other amino acid substitutions at these two residues. Both F144C, as well as the original F144L mutation, shifted the pH profile to that of EC NhaA (Fig. 5a), suggesting that this residue plays an important role in enhancing antiporter activity at acidic pH. Substitution of Lys-347 to Ala as well as the original K347Q mutation, shifted the pH-dependence profile of antiporter activity to that of the EC type (Fig. 5c). However, the K347R, K347C, and K347E mutations caused extensive loss in the antiporter activity (Fig. 5c), although K347R mutation still had a very low activity. These results suggest that Lys-347 plays important roles not only in regulating the antiporter mechanism at acidic pH but also in the overall transport mechanism.

Fig. 4. Immunological detection of mutated NhaA in membranes. Membrane proteins (2 \( \mu \)g) from the KNabc strain transformed with plasmids expressing FLAG epitope-tagged wild-type and mutant NhaA were subjected to SDS-polyacrylamide gel electrophoresis (12.5% acrylamide). As a negative control, membrane proteins of pBR322 transformants were also analyzed. After electrophoresis, the proteins were transferred to a GVHP filter and probed with the anti-FLAG M2 monoclonal antibody, and immunoreactive bands were visualized (17).

Table III

| Type | Substitution |
|------|--------------|
| I    | D141G        |
|      | D171N*       |
|      | V162D        |
|      | I165N        |
|      | L167P        |
| II   | M138K        |
|      | F144L        |
|      | K347Q        |
|      | G383R        |
|      | G385S        |
|      | G385D        |
|      | S389P        |
| III  | G231C*       |
|      | H233R        |
|      | T235N        |
|      | L241P        |

Table IV

| Mutation | \( K_m \) | \( V_{\text{max}} \) |
|----------|----------|------------------|
| Wild type| 0.2      | 100              |
| M138K    | 0.6      | 52               |
| F144L    | 0.4      | 40               |
| K347Q    | 4.3      | 49               |
| K347S    | 1.3      | 89               |
suggested that this mutation affected the affinity of the mutated NhaA for $\text{Na}^+$. These results imply that type II mutations can be further divided into two subclasses. As a control, we also tested the H233R mutation and found that this mutation decreased $V_{\text{max}}$ but did not affect $K_m$ (data not shown).

**Second Site Mutations Suppress Defective Antiporter Activity in K347E or K347C Mutations**—As shown by analyses of type I and II mutations, residues in TM 4, 5, 10, and 11 were thought to be involved in antiporter activity, and TM 4 and 10 were thought to play an important role in enhancing the activity of HP NhaA at acidic pH. Since these TMs are far apart from each other in the primary structure, we postulated that they might closely associate in the folded protein. To confirm this hypothesis, we surveyed functionally interacting residues in these TMs by analyzing mutations suppressing the defect originally caused by the M138K, F144L, or K347Q mutations. Since the KNabc isolate expressing NhaA with the K347Q mutation grew very slowly on the selection plate containing 0.65 M NaCl, we introduced site-specific mutations at Lys-347 and found that the K347E and K347C mutations resulted in a complete loss of cell growth. From these mutants two independent revertants were found that carried the same K347S mutation, and two other revertants carried second site mutations at Thr-158 (T158N/K347C) or Glu-87 (E87K/K347E) (Table IV). Glu-87 and Thr-158 are located at putative loop 2 between TM 2 and 3, and loop 4 between TM 4 and 5, respectively (Fig. 1). Thus Lys-347 in TM10 is shown to interact functionally with the distant residues. Rather, it seems to be due to the overall protein structure formed by TM 4 and TM 10. For TM 4 it should be noted that for all of the revertants, pH-dependent profiles of antiporter activities were determined as described in the legend to Fig. 2. The percentage fluorescence dequenching observed after addition of 5 mM NaCl is plotted against assay pH. K347E, open circles; K347C/T158N, open squares; K347S, filled squares; K347E, open triangles; K347E/E87K, filled triangles.

**DISCUSSION**

In previous studies, we have investigated the structure-function relationship of the Na$^+$/H$^+$ antiporter NhaA, especially the structure regulating pH-responsive antiporter activity (10, 11, 16, 17, 21). However, the molecular basis for the very different profiles of pH sensing exhibited by NhaA from E. coli and H. pylori (16, 17), despite their highly conserved primary sequences, remain to be elucidated. Here, we introduced extensive mutations into NhaA of H. pylori and surveyed residues that play an essential or important role in antiporter activity. Using this genetic approach, we found that mutations affecting antiporter activity were localized at residues in TM 4, 5, 8, 10, and 11, and in loop 7. Thus, we concluded that functionally essential or important residues are located in these limited regions of NhaA.

Further biochemical analyses of antiporter activities in the everted membrane vesicles of these mutants revealed that the phenotypes of antiporter activities can be classified into three different types. In the first type, mutations result in low or null antiporter activity. These mutations occur in TM 4, 5, 10, and 11, but not in TM 8 or loop 7, with the exception of the S230R mutation. These residues include Asp-141, Asp-171, and Asp-172, corresponding to Asp-133, Asp-163, and Asp-164 of EC NhaA, respectively. These residues in EC NhaA have been shown to be essential for antiporter activity (10). We previously reported that mutations of L302P (HP NhaA Leu-349) or G303R (HP NhaA Gly-350) in TM 10, and mutations of C335P (HP NhaA Ala-382) or S342P (HP NhaA Ser-389) in TM 11 greatly decrease antiporter activity of EC NhaA (11). However, integration of these mutant EC NhaAs into the membranes had not been tested. Firm evidence of the functional importance of residues in TM 10 or 11 was reported only for Gly-338 in TM 11 (15). Thus, our discovery that mutations in TM 10 and 11 cause very low antiporter activity supports the functional importance of TM 10 and 11. The present findings, together with the extensively conserved primary structures of both NhaAs (16), suggest that TM 4, 5, 10, and 11 are important regions for antiporter activity of NhaA in both H. pylori and E. coli.

The second type of mutations that we identified reside at Met-138 and Phe-144 in TM 4, or Lys-347 in TM 10 and cause very low antiporter activity at acidic pH. The pH dependence profiles of residual antiporter activity in these mutants are similar to those of wild-type EC NhaA antiporter activity (Fig. 3, a and c). Therefore, these residues are thought to be involved in maintaining high antiporter activity at acidic pH specifically for HP NhaA. While Phe-144 and Lys-347 are conserved for HP and EC NhaA, Met-138 is not. Replacement of Met-138 by Ala, which creates the EC NhaA sequence, did not shift the pH dependence profile to EC type. Thus the high antiporter activity at acidic pH for HP NhaA is not attributable to one of these residues. Rather, it seems to be due to the overall protein primary structure formed by TM 4 and TM 10. For TM 4 it should be noted that the Asp-141 residue, which is essential for ion transport, is located near Met-138 and Phe-144 on the same face of a putative $\alpha$-helix of TM 4, as shown by a helical wheel model of this domain (data not shown). Recently, Galli et al. (24), reported that replacement of Val or Thr for Ala at residue 127 of EC NhaA (corresponding to Gly-135 of HP NhaA) caused an altered pH response of antiporter activity. Since Gly-135 (Ala-127 of EC NhaA) is proximal to Met-138, a crucial structure for HP-specific NhaA antiporter activity at acidic pH may also involve Gly-135 as well as Met-138.
The K347C, K347E, or K347R mutation caused null or very low antiporter activity under all pH conditions tested, while K347Q, K347A, or K347S mutation causes a loss of antiporter activity at acidic pH. These results suggest that, depending on the substituted amino acid residues, two different conformations can exist. One conformation is presumably important for ion transport while the other affects the response of antiporter activity to different pH. Therefore, it is suggested that this residue (Lys-347) plays an important role in both the basic ion transport mechanism and in the putative enhancing mechanism at acidic pH. Kinetic analysis of antiporter activity in the mutant revealed that K347Q and K347S mutations were effective in dictating the antiporter $K_{m}$, while the M138A and F144L mutations significantly decreased its $V_{max}$ value without affecting $K_{m}$. These results imply that type II mutations can be further subdivided into two classes, one being related to both pH dependence and the affinity of NhaA for Na⁺, and the other to pH dependence alone.

The third type of mutation causes very low antiporter activity at alkaline pH but relatively normal activity at acidic pH. These mutations are located in the loop 7 and TM 8 regions, suggesting that these contiguous regions are involved in regulating antiporter activity at alkaline pH. For EC NhaA, substitution of His-225 corresponding to His-233 of HP NhaA in loop 7 has been reported to alter pH sensitivity (11, 14), which is consistent with the present finding concerning the phenotype of the His-233 mutation. Thus, the pH-dependent mechanism of antiporter activity at alkaline pH may be essentially the same for the two NhaAs. Here, we discovered that residues flanking His-233 are also involved in pH sensing. These residues, Thr-235 and Leu-241, may indirectly contribute to the sensing mechanism and may support proper conformation of His-233, allowing it to sense H⁺ concentration.

The present findings clearly show that the pH-independent profile of H. pylori NhaA is due to two factors. One is a mechanism for enhancing antiporter activity at alkaline pH, which is closely associated with loop 7 and TM 8. The second factor is a mechanism that functions to maintain high antiporter activity at acidic pH and is associated with TM 4 and 10. The former factor may be common to both NhaAs, while the latter factor is specific to HP NhaA.

We have previously shown that pH-dependent antiporter activity requires at least the interaction of both of the N-terminal and the C-terminal halves of NhaA, based on experiments using chimeric NhaA from E. coli and H. pylori (17). The chimeras with the N-terminal and C-terminal halves derived from HP and EC, respectively, exhibited an intermediate profile of pH-dependent antiporter activity, which is consistent with the present hypothesis. Further supporting this hypothesis, for EC NhaA suppression, G338S mutations were found at Ala-127, Pro-129, and Ala-130, all of which are located in TM 4 (15). Here, we found that Thr-158 in loop 4 and Glu-87 in loop 2 are functionally related to Lys-347, suggesting that these residues are in close proximity upon protein folding. These results again support the notion that the N-terminal and C-terminal halves are functionally related. To prove this hypothesis, close co-localization of residues in TM 4, 5, 10, and 11 needs to be demonstrated more directly.

Although HP NhaA was expressed in E. coli cells as a heterologous system in the present study, the expression and activity of HP NhaA were relevant to those of EC NhaA in KNabc. Therefore, we believe that comparison of pH-dependent antiporter activity from EC and HP NhaA is valid and significant. We have, therefore, used H. pylori NhaA, rather like a mutant EC NhaA, as a tool to understand the pH-dependent regulatory mechanism of NhaA activity. More detailed study of HP NhaA antiporter activity in H. pylori cells will be required to understand its precise molecular control mechanisms, but this was not the specific goal of the current study.

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