A Point Mutation (G338S) and Its Suppressor Mutations Affect Both the pH Response of the NhaA-Na⁺/H⁺ Antiporter as Well as the Growth Phenotype of *Escherichia coli*

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Abraham Rimon, Yoram Gerchman, Zehavit Kariv, and Etana Padan‡

From the Division of Microbial and Molecular Ecology, The Hebrew University of Jerusalem, 91904 Jerusalem, Israel

pH controls the activity of the NhaA Na⁺/H⁺ antiporter of *Escherichia coli*. In the present work we show that replacement of glycine 338 of NhaA with serine (G338S) alleviates the pH control of the antiporter. Monitoring Na⁺-dependent collapse of ΔpH, to assess antiporter activity in isolated membrane vesicles, shows that the mutant protein is practically independent of pH, between pH 7 and 9, and even at pH 6 is 70% active. Similarly the purified reconstituted mutant protein catalyzes pH-independent passive efflux of ²²Na from proteoliposomes as well as ΔpH-driven influx. Whereas the native NhaA in isolated membrane vesicles is exposed to digestion by trypsin only above pH 7, the mutated protein is degraded already at pH 6.5. ΔnhaAΔnhaB cells transformed with a plasmid encoding the pH-independent antiporter are sensitive to Na⁺ but not to K⁺ at alkaline pH, while growing in the presence of both ions at neutral pH. Several possibilities that could explain the Na⁺ sensitivity of the mutant at alkaline pH were excluded; Western analysis and measurement of Na⁺/H⁺ antiporter activity in membrane vesicles, isolated from cells shifted to the non-permissive growth conditions, showed neither reduced expression of G338S-NhaA nor defective activity. The finding that the mutated protein is electrogenic led to the retraction of the idea that the protein is active in *vivo* but not in *vitrō* at alkaline pH, when only Δφ exists in the cells. The Na⁺ concentration needed for half-maximal activity of G338S in isolated everted membrane vesicles is similar to that of the wild type. Therefore an increase in intracellular Na⁺ due to a reduced antiporter affinity could not explain the results. It is suggested that the loss of growth at alkaline pH in the presence of Na⁺ is due to the loss of the pH control of the mutated NhaA. Indeed, in the four mutations suppressing G338S phenotype, growth at alkaline pH was restored together with the pH regulation of NhaA. Three of the four suppressor mutations cluster in helix IV, whereas the original mutation is in helix XI, suggesting that the two helices interact.

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

*Escherichia coli* has two antiporters, NhaA (5) and NhaB (6), that specifically exchange Na⁺ or Li⁺ for H⁺ (4). nhaA is indispensable for adaptation 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 that have been purified to homogeneity and reconstituted in a functional form in proteoliposomes (12–14). The H⁺/Na⁺ stoichiometry of NhaA is 2H⁺/Na⁺ and that of NhaB 3H⁺/2Na⁺. NhaB but not NhaA is sensitive to amiloride derivatives, and the rate of activity of NhaB but not of NhaA is drastically dependent on pH, changing its Vₘₐₓ over 3 orders of magnitude from pH 7 to pH 8 (12).

Interestingly, a strong pH sensitivity is characteristic of antiporters as well as other transporters and proteins involved in pH regulation (15). Identifying the amino acid residues involved in the pH sensitivity of these proteins is important for understanding the mechanism of pH regulation.

None of the eight histidines of NhaA were found essential for the Na⁺/H⁺ antiporter activity of NhaA (16). However, replacement of histidine 225 by Arg (H225R) suggested that His-225 has an important role in the pH sensitivity of the antiporter (16). Whereas the activation of the wild-type NhaA occurs between pH 7.5 and pH 8, that of H225R antiporter occurs between pH 6.5 and pH 7.5. In addition, whereas the wild-type antiporter remains almost fully active, at least up to pH 8.5, H225R is reversibly inactivated above pH 7.5, retaining only 10–20% of the maximal activity at pH 8.5 (16). Furthermore, replacement of His-225 with either cysteine (H225C) or serine (H225S) but not alanine (H225A) yielded an antiporter with a wild-type pH-sensitive phenotype, implying that polarity and/or hydrogen bonding, the common properties shared by His, Cys, and Ser, are essential at position 225 for pH regulation of NhaA (17).

Recently, we have found that NhaA undergoes a conformational change upon its activation by pH which can be probed by trypsin. Thus both the native NhaA as well as H225R, the mutant with a pH profile shifted toward acidic pH, are susceptible to trypsin in isolated membrane vesicles only at the pH range where they are active and reflecting the level of activity (18).

In the present work we found that glycine 338 affects the pH response of NhaA, and its replacement with serine (G338S in TMS¹ XI) produced a transporter which in contrast to the

¹ The abbreviations used are: TMS, trans-membrane segment; DM, n-dodecyl β-D-maltoside; MOPS, 3-(N-morpholino)propanesulfonic acid; bp, base pair(s); DTT, dithiothreitol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)]ethyl]glycine.
wild-type protein lacks pH control; it is active between pH 6.5 and 8.5 and accordingly is exposed to trypsin digestion throughout this pH range. As a result of the loss of pH control, the mutant cells become sensitive to alkaline pH in the presence of Na⁺. Four second-site suppressor mutations, restoring growth at alkaline pH, were isolated. Three of these mutations cluster in TMS IV, but in all of them the pH response of NhaA was restored.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—E.P432 is an E. coli K12 derivative, which is melBLid, ΔnhaA1-kan, ΔnhaB1-cat, ΔlacZY, thr1 (11). TA16 is nhaA- nhaB lacI and otherwise isogenic to E.P432 (12). DH5α (U. S. Biochemical Corp.) was used as a host for construction of plasmids. Cells were grown either in L broth (LB) or in modified L broth (LBK) in which NaCl was replaced by KCl (7) (87 mM, pH 7.5). The plasmids. Cells were grown either in L broth (LB) or in modified L broth with either glycerol (0.5%) or melibiose (10 mM) as a carbon source. Thiamine (2.5 μg/ml) was added to all minimal media. For plates, 1.5% agar was used. Antibiotics were 100 μg/ml ampicillin, and/or 50 μg/ml kanamycin, and/or 12 μg/ml chloramphenicol, and/or 12.5 μg/ml tetracycline. Resistance to Li⁺ and Na⁺ was tested as described previously (16).

Plasmids—pGM36 is a PBR322 derivative that carries wild-type nhaA (20). pAR100 carries nhaA and C terminus truncated nhaR. It was constructed by exchanging the EcoRI-Sph fragment (2220 bp) of pGM36 with the EcoRI-Sal fragment (414 bp) of pACYC184. pAR1 to pBR6 are six pBR10 derivatives bearing mutated nhaA. Both the wild-type and nhaA2 and three of its mutants were also constructed as PBR322 derivative, pGMAR100 and pGMNR1 to pGMNR3, respectively, by exchanging KpnI-MluI fragment (1806 bp) of pNR1, -2, -3 or pAR100 with the respective restricted fragment (3356 bp) of pGM36. Plasmid encoding His-tagged antiporter was constructed by mutating the MluI-Nhel fragment (881 bp) of pGMNR1 with the respective fragment (881 bp) of pAXH (previously named pYG10 (22)) and designated pAXH-G338S. In addition, 2 mM MgSO4, 10 mM Tris-Cl, pH 7.5. The assay itself (13) was conducted at room temperature by dilution of 4 μl of proteoliposomes into 500 μl containing 150 mM choline chloride or NaCl (control without ΔpH) and in addition, 2 mM MgSO4, 10 mM Tris/Hepes, at the indicated pH and 50 μM NaN3. The reaction was stopped by addition of 2 μl of the above ice-cold solution without sodium, filtered on 0.2-μm Schleicher & Schuell filters, and washed with an additional 2 ml. Radioactivity on the filter was measured in a counter. Zero times were established by addition into NhaA (no ΔpH) and the values were subtracted from all points. Experiments were done in triplicate, unless noted otherwise in the figure legend.

RESULTS

Isolation of nhaA Mutants Sensitive to Na⁺—Random mutagenesis was conducted in vitro by hydroxylamine/hydrochloride on plasmids pAR100 or pGMNR1 as described (17). The mutagenized plasmids were transformed into E.P432 or TA16 and plated on LBK, the grown colonies were picked, and the DNA of the colony (0.6 M) and Li⁺ (200 mM) at alkaline pH. The products were analyzed by PhosphorImager were as described (18). The mutant plasmids were transformed into TA15 cells carrying pGP1-3 and either of the plasmids pAXH, pAXH-G338S. Proteolytic digestion of NhaA in right-side-out and inverted membrane vesicles was similar (17), we used sonicated membrane vesicles but the pressure used was 20,000 p.s.i. (French pressure cell press). Protein was determined according to Ref. (24).

Probing the Conformation of NhaA in Membrane Vesicles with Trypsin—Since the pH profile of trypsinolysis of NhaA in right-side-out and inverted membrane vesicles was similar (17), we used sonicated membrane vesicles containing 10 mg of protein to 3 ml to yield 47 mM choline chloride, 3.3 mM Tris-Cl, pH 7, 83 mM sucrose, 20% glycerol, 100 mM MOPS, pH 7, and 1% DM. The suspension was gently mixed for 15 min at room temperature followed by 30 min incubation at 4 °C and then centrifuged (Beckman model TLA100, 245,000 × g, 20 min, 4 °C). All subsequent steps were performed at 4 °C. The supernatant was mixed with 1 ml of prewashed resin (Ni⁺2-NTA-agarose, Qiagen, Hilden, Germany). Prewashing was conducted by adding 8 ml of double distilled H2O, 12 ml of the binding buffer (modified Qiangen protocol: 5 mM imidazole, pH 7.9, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9, 0.1% DM). For loading of the resin with His-tagged antiporter, the mixture was incubated for 1 h with gentle mixing and then reapplied to the column. The loaded resin was washed in 20 mM binding buffer containing 12 mM of wash buffer (300 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9, 0.1% DM, 10% glycerol). Elution was conducted with 3.5 ml of elution buffer (300 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9, 0.1% DM, 10% glycerol). The fraction containing the protein (1 ml) was dialyzed first for 1 h and then overnight, each time in 50 ml containing 25 mM acetic acid, pH 5, 300 mM KCl, 7.5% glycerol, and 0.1% DM. The protein was either used directly or stored at −70 °C after increasing the glycerol concentration to 20%.

Reconstitution of Proteoliposomes—Reconstitution was performed essentially as described (12). A mixture (300 μl) containing 26.7 mg/mEq E. coli phospholipids, 1.2% octyl glucoside, 100 mM MOPS, pH 7, was presoaced in a bath type sonicator (G1128PT, Laboratory Supplier Co., New York) until clear and mixed with 400 μl of a solution containing 5% octyl glucoside, 0.5 mg/ml phospholipids, 1 mg DTT, and 50 mM MOPS, pH 7. The mixture was sonicated again briefly and rapidly diluted (36-fold) into the appropriate dilution buffer. After 20 min at room temperature, proteoliposomes were pelleted by centrifugation at 257,000 × g for 1 h, resuspended in 100 μl of the dilution buffer, frozen in liquid nitrogen, and stored at −70 °C. Frozen proteoliposomes were used for all transport assays. When assayed, the suspension was thawed at room temperature and sonicated briefly until clear.

Sodium Efflux in Proteoliposomes—Proteoliposomes were made by dilution into a buffer containing 150 mM NH4Cl, 1 mM DTT, and 15 mM Tris-Cl, pH 7.5. The assay itself (13) was conducted at room temperature by dilution of 4 μl of proteoliposomes into 500 μl containing 150 mM choline chloride or NH4Cl (control without ΔpH) and in addition, 2 mM MgSO4, 10 mM Tris/Hepes, at the indicated pH and 50 μM NaN3 (1 mM/ml). The reaction was stopped by addition of 2 μl of the above ice-cold solution without sodium, filtered on 0.2-μm Schleicher & Schuell filters, and washed with an additional 2 ml. Radioactivity on the filter was measured in a counter. Zero times were established by addition into NhaA (no ΔpH) and the values were subtracted from all points. Experiments were done in triplicate, unless noted otherwise in the figure legend.

Quantitation of NhaA and Its Mutated Protein in the Membrane—Quantitation of the Na⁺/H⁺ antiporter activity was based upon the measurement of Na⁺ (or Li⁺)-induced changes in the ΔpH as described (5, 24).

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

Protein Determination—Protein was determined according to Ref. (25).

DNA Sequence—Sequencing of DNA was conducted by an automated DNA sequencer (ABI PRISMTM 377, Perkin Elmer).

Quantitation of NhaA and Its Mutated Protein in the Membrane—Quantitation of the NhaA and its mutated derivative in membranes was determined by Western analysis as described previously (17).
Point Mutation and Its Suppressor Mutations Affect pH Response

**FIG. 1.** A mutant of NhaA that grows in the presence of Na\(^+\) at neutral p[H] but not at alkaline p[H]. \(\Delta nhaA\) nhaB cells (EP432) carrying a \(nhaA\) mutant on plasmid (pNR1) that confers growth on agar plates, in the presence of Na\(^+\) (0.6 M) at pH 7, but not at pH 8.3, were grown in LB liquid medium under similar conditions or in the presence of 0.6 M KCl at pH 8.3. The growth of the wild-type (EP432/pAR100) under identical conditions is also shown. [A] pH 7.0, 0.6 M NaCl, [B] pH 8.3, 0.6 M NaCl, [C] pH 8.3, 0.6 M NaCl.

phases (8.3) but not at neutral pH would be impaired in amino acid residues of NhaA which are involved in its response to pH. To screen for such mutants we used randomly mutagenized plasmid pAR100 (harboring \(nhaA\)) transformed into a strain (EP432) lacking both Na\(^+\)-specific Na\(^+\)/H\(^+\) antiporters (\(\Delta nhaA\) nhaB). This strain is highly sensitive to Na\(^+\) at all pH values, unless transformed with a plasmid harboring wild-type \(nhaA\). Hence it has proven very useful to identify new mutations of \(nhaA\) impaired in either its activity or pH regulation (15). Mutations of \(nhaA\), rendering the cells Na\(^+\)-sensitive at alkaline pH but not at neutral pH, were identified by replica plating of the cells bearing the randomly mutagenized plasmid on medium containing 0.6 M NaCl, either at pH 7 or pH 8.3. Six independent plasmidic mutants (pNR1 to pNR6), allowing growth in the presence of high Na\(^+\), only at the neutral pH, were isolated. The growth phenotype of EP432/pNR1 in liquid culture is shown in Fig. 1. At neutral pH both the wild type and the mutant grew at the same rate in the presence of high Na\(^+\) or high K\(^+\) (not shown). In contrast to the wild type that grew in the presence of both ions at the alkaline pH, high Na\(^+\) but not high K\(^+\) totally inhibited the growth of the mutant at alkaline pH. A similar phenotype was obtained when the mutated gene was cloned into plasmids, differing in copy number, pBR322 or pACYC184.

Expression of Mutated \(nhaA\)—The amount of the mutated NhaA in membrane vesicles isolated from cells grown under the permissive growth conditions, pH 7 (LBK), was measured by Western analysis (Table I). As compared with pAR100, the wild-type plasmid, all mutants were expressed to at least 30% of the wild-type plasmid, pACYC184 derivatives. To compare the level of expression, densitometry was used; the developed blots were scanned and analyzed. pAR100 which harbors wild-type \(nhaA\) and pNR1 (G338S) to pNR6 which harbor mutated \(nhaA\) are pACYC184 derivatives. pGMAR100 which harbors wild-type \(nhaA\), pGMNR1 which carries G338S mutation and plasmids, pSup1, pSup2, pSup10, and pSup150 carrying its suppression mutations are pBR322 derivatives.

| Plasmid | Expression | Plasmid | Expression |
|---------|------------|---------|------------|
| pAR100  | 100        | pGMAR100| 100        |
| pNR1    | 43         | pGMNR1  | 113        |
| pNR2    | 29         | pSup1   | 112        |
| pNR3    | 38         | pSup2   | 77         |
| pNR4    | 40         | pSup10  | 110        |
| pNR5    | 41         | pSup150 | 99         |
| pNR6    | 58         |         |            |

\(\Delta nhaA\) nhaB cells transformed with the indicated plasmids were grown to an OD\(_{600}\) = 0.7 in LBK. The membrane fraction, prepared by sonication (17), was treated with 0.6 M NaCl and then used for Western analysis. To compare the level of expression, densitometry was used; the developed blots were scanned and analyzed. pAR100 which harbors wild-type \(nhaA\) and pNR1 (G338S) to pNR6 which harbor mutated \(nhaA\) are pACYC184 derivatives. pGMAR100 which harbors wild-type \(nhaA\), pGMNR1 which carries G338S mutation and plasmids, pSup1, pSup2, pSup10, and pSup150 carrying its suppression mutations are pBR322 derivatives.

The concentration of Na\(^+\) required for half-maximal activity (at pH 8.1) of the pH-insensitive mutant was found to be 0.095 M as compared with 0.21 M (26) of the wild type. The Li\(^+\) specificity of the mutant, at neutral pH, was very similar to that of the wild type (not shown). G338S—The mutant encoding the pH-insensitive NhaA was chosen for further study. To identify the location of the mutation in the \(nhaA\) gene, a BglII-MluI fragment of the mutated \(nhaA\) was exchanged with the respective fragment in the wild-type \(nhaA\) of plasmid pGMAR100. The mutated phenotype was transferred by this exchange. Determination of the DNA base pair sequence of the fragment, carrying the mutation, identified the mutation as a single replacement of glycine 338 with serine (G338S).

\(\Delta p\)H-driven \(Na^+\) Uptake by Purified His-tagged G338S Antipporter Reconstituted in Proteoliposomes—To study the properties of G338S protein, it was fused in frame to polyhistidine. The resulting His-tagged G338S was affinity purified on a Ni-NTA column, with an efficiency similar to that of the His-tagged wild-type protein (22). Table II summarizes an experiment measuring \(\Delta p\)H-driven \(^{22}\)Na\(^+\) uptake, at various pH values, in reconstituted proteoliposomes of His-tagged G338S and the His-tagged wild-type proteins. The wild-type derivative shows the same dependence on pH as shown previously (12); it was almost completely shut off below pH 7, 30% active at pH 8, and maximally active at pH 8.6. The maximal activity of the His-tagged G338S was also observed at pH 8.6; but it was about 60% that of the wild type. His-tagged G338S on the other hand was much less sensitive to pH. As compared with pH 8.6, its rate was hardly changed at pH 8, reduced to 66% at pH 7.5, and at pH 7 when the wild-type protein had only 3.5% of its maximal activity, the mutant was still 50% active. It should be noted, however, that since a \(\Delta p\)H (acidic inside) is the driving force, the reduction in activity at neutral pH and below may be ascribed to both a reduction in the driving force as well as a pH effect.
The activity of purified His-tagged G338S antiporter in proteoliposomes and the effect of pH

His-tagged wild-type NhaA and its mutant G338S were purified from the transformants TA16/pAXH and TA16/pAXH-G338S, respectively; proteoliposomes were prepared and either ΔpH-driven $^{22}\text{Na}^+$ uptake, or $\text{Na}^+$ efflux was measured as described under “Experimental Procedures” and expressed as percent of maximal activity shown in parentheses (nmol/mg protein/min).

| pH | ΔpH driven Na$^+$ uptake | Passive Na$^+$ efflux | ΔpH-driven Na$^+$ uptake | Passive Na$^+$ efflux |
|----|--------------------------|----------------------|--------------------------|----------------------|
| 6.5 | 0.6 | 72.8 | 40 | 40 |
| 7  | 3.5 | 5.8 | 50 | 72.8 |
| 7.5 | 7 | 23.5 | 66 | 85.7 |
| 8  | 30 | 58 | 100 | 97.1 |
| 8.6 | 100 (700) | 100 (1700) | 100 (400) | 100 (700) |

The pH Dependence of His-tagged G338S—We have previously shown (12) that the pH profile of the activity of the purified antiporter is best measured when both components of the driving force, $\Delta p\text{H}$ and $\Delta \psi$, are cancelled and the turnover of the antiporter is mediated by a $\text{Na}^+$ gradient, directed outward. We therefore measured $\text{Na}^+$ efflux from His-tagged wild-type and His-tagged G338S proteoliposomes, under conditions in which $\Delta p\text{H}$ is cancelled by acetate (100 mM) and $\Delta \psi$ by K$^+$ (100 mM) in the presence of valinomycin (Table II). The results show that similar to the $\Delta p\text{H}$-driven $^{22}\text{Na}^+$ uptake, the maximal efflux activity of the purified His-tagged mutant is less than that of the His-tagged wild-type protein. Remarkably, in contrast to the drastic pH dependence of the wild-type protein, the mutant protein is hardly dependent on pH, and it is still significantly active even at pH 6.5, when the wild-type protein is practically shut off.

His-tagged G338S Protein Is Expressed and Active Even After Shifting the Cells to Alkaline pH in the Presence of Na$^+$—The significant $\Delta p\text{H}$-driven Na$^+/\text{H}^+$ antiporter activity at alkaline pH both in membranes and proteoliposomes was puzzling in view of the lack of growth of the mutant at that pH, in the presence of Na$^+$. We therefore considered the possibility that either the level of G338S in the membrane is reduced, following the shift to alkaline pH in the presence of Na$^+$, or that the protein is inactive under these conditions.

To test these possibilities, cells were grown in LBK, pH 7, washed, and incubated for 1 h in LB, pH 7, in the presence of 0.6 M KCl to allow for adaptation to the osmolarity change and then shifted to pH 8.3 in the presence of Na$^+$ (0.6 M). The cells stopped growing immediately following the pH shift, but their colony forming capacity remained constant for at least 2 h. Cell samples were withdrawn, membrane vesicles isolated, and both antiporter level (by Western analysis) as well as activity (at different pH) were measured. No change in these parameters were found following the 2-h shift to the non-permissive growth conditions (not shown). Hence the lack of growth of mutant G338S at alkaline pH, in the presence of Na$^+$, cannot be ascribed to either reduced expression or impaired activity of the antiporter.

His-tagged G338S Protein Is Electrogenic—Loss of electrogenic property of NhaA could also explain lack of growth of G338S at alkaline pH since under these conditions only $\Delta \psi$ exists across the membrane of E. coli cells (27).

The activity of an electrogenic antiporter involves the electrokinetic movement of permeant ions, to compensate for charge translocation. Hence, the presence of such ions may determine the turnover rate of electrogenic transporters. K$^+$ in the presence of the ionophore valinomycin has been often used as a counter ion in reconstituted proteoliposomes. To test the effect of a counter ion on the transport rate of His-tagged G338S NhaA, we measured $\Delta p\text{H}$ (acid inside)-driven uptake of $^{22}\text{Na}^+$ into proteoliposomes, in the presence of 10 mM KCl, with or without valinomycin (5 μM). The results summarized in Fig.
3 show that the initial rate of transport was stimulated 3–5-fold upon addition of K⁺ in the presence, but not in the absence, of valinomycin. The extent of stimulation was very similar to that of the wild-type protein (Fig. 3) (12, 14). It thus appears that the mutant protein G338S is electrogentic.

As Probed by Trypsin Digestion of Isolated Membrane Vesicles—His-tagged G338S Lost the pH-induced Conformational Change, Characteristic of the Native Protein—The activation of NhaA by alkaline pH is accompanied by a conformational change which was identified by probing membrane vesicles, containing labeled antiporter, with trypsin. The wild type was not digested by trypsin at acidic pH, even after 4 h exposure, but degraded at alkaline pH (18) (Fig. 4). In marked contrast to the wild-type protein, 89% of the G338S protein was digested by trypsin in 2 h at pH 6.5 and 93% at pH 8.5 (Fig. 4). Hence the residue Gly-338 is crucial in the pH-dependent conformational change of NhaA.

Suppression Mutations Restoring Growth of G338S at Alkaline pH in the Presence of Na⁺—Suppression mutations restoring growth of G338S mutant at pH 8.3, in the presence of Na⁺ (0.6 M), were obtained following random mutagenesis of pGMNR1 with hydroxylamine. The mutagenized plasmid was transformed into EP432, and transformants were replicated on the selective medium as described for the isolation of pNR1. Four plasmids (pSup1, pSup2, pSup10, and pSup15) restoring growth of G338S were obtained, and the DNA fragment containing the phenotype was sequenced. Each of these plasmids encodes, in addition to G338S, replacements in the protein as follows: pSup1, A127V; pSup2, A130T; pSup10, P129L; and pSup15, A349V, P129L, and Cys → Thr base transition at ~30, with respect to the first base of the initiation codon. The latter mutation maps within the cis regulatory sequences of nhaA, recognized by NhaR, the positive regulator of nhaA (26), and therefore if at all, it can only have an effect on expression of the protein. Indeed we have constructed plasmid pSup15 which does not carry this mutation, by replacing Nhel-MflI fragment (8796 bp) of the wild-type gene (in pGMAR100) with that of pSup15. The phenotype conferred by pSup15 was indistinguishable from that of pSup15. The growth rate of each suppressor mutant is shown in Table III.

The results summarized in Table I show that all antiporters bearing suppressor mutations were very well expressed.

The Na⁺/H⁺ Antiporter Activity in Everted Membrane Vesicles Derived from Mutants Bearing the Original Mutation (En-

![Image](85x565 to 261x729)

**FIG. 4.** Probing membrane vesicles of G338S by digestion with trypsin. Membrane vesicles (50 μg protein/30 μl) obtained by sonication and containing radioactively labeled (20,000 cpm/30 μl) His-tagged derivatives of G338S-NhaA or wild-type (wt) were prepared and subjected to digestion by trypsin (0.4 μg/30 μl for 4 h or 0.1 μg/30 μl for 2 h at pH 6.5 and 8.5, respectively) as described under “Experimental Procedures.” The digested products were resolved on SDS-polyacrylamide gel electrophoresis (12.5%) and exposed to Phosphorimager. Samples (50 μg of proteins) in lanes 1, 3, 5, and 7 obtained the trypsin inhibitor (type II S, Sigma) at zero time, whereas the reaction mixtures applied in lanes 2 and 4 were incubated in the presence of trypsin for 4 h at pH 6.5 and those applied in lanes 6 and 8 for 2 h at pH 8. The reactions were terminated by the addition of the trypsin inhibitor. Purity of the radioactivity in the protein, the autoradiograms were scanned and the intensities expressed in % (100% = the density observed at zero time for each respective treatment).

**TABLE III**

| Second site mutations | Doubling time (min) |
|-----------------------|---------------------|
|                       | 0.6 μM NaCl (pH 7.2) | 0.6 μM NaCl (pH 8.3) |
| pGMNR100 (WT)         | 50                  | 80                  |
| pGMNR1 (G338S)        | 60                  | 0                   |
| pSup1                 | A127V               | 60                  | 170                 |
| pSup2                 | A130T               | 60–65               | 80                  |
| pSup10                | A349V               | 60–65               | 75                  |
| pSup15                | P129L               | 60–65               | 85                  |

**FIG. 3.** **His-tagged G338S is electrogentic.** His-tagged wild-type NhaA (□) protein and its mutant G338S (▲) were overexpressed, purified, and assayed for pH-driven 22Na⁺ uptake as described under "Experimental Procedures," but 10 mM KCl was added to all reaction mixtures. Where indicated (filled symbols) valinomycin was added to 5 μM.
Point Mutation and Its Suppressor Mutations Affect pH Response

FIG. 5. Na\(^+\)/H\(^+\) antiporter activity of mutants containing the original, first-site, and the second-site suppressor mutation. Everted membrane vesicles were prepared from EP432 transformed with plasmids harboring the indicated plasmids, and the Na\(^+\)/H\(^+\) antiporter activity was determined as described in Fig. 2. □, pGMAR100, wild-type nhaA; □, pGMNR1, (G338S); ▲, G338S/A127V; ●, G338S/A130T; △, G338S/A349V; ○, G338S/P129L; ▽, P129L.

between pH 7.5 and 8.

Remarkably restoration of the wild-type growth rate correlated with the capacity to regain pH control and shut off the antiporter at acidic pH; Sup1 regained only very slight pH control and even at pH 6 retained 50% of its activity (Fig. 5). Accordingly, its growth rate was very low (Table III). Sup2, Sup10, and Sup150 regained a significant pH control together with the capacity to grow at a growth rate similar to that of the wild type (Fig. 5 and Table III).

The strongest suppression effect was observed in Sup150. It was therefore chosen to test what is the phenotype of the suppressor mutation alone and in the absence of G338S mutation. For this purpose the Eco88I-BamHI fragment (956 bp) of pGMAR100 was replaced with that of pSup150. The new plasmid pP129L conferred resistance upon EP432, ΔnhaAΔnhaB strain, with respect to both Na\(^+\) (0.6 M) and pH (8.3). Most interestingly, in contrast to the Na\(^+\)/H\(^+\) antiporter activity of P129L/G338S which shuts off only at pH 6 and below, P129L protein was completely shut off up to pH 7.5 and exhibited a pH profile markedly shifted to alkaline pH, even with respect to the wild-type protein (Fig. 5).

DISCUSSION

One of the most interesting characteristics of NhaA, the main Na\(^+\)/H\(^+\) antiporter of E. coli, is its drastic dependence on pH. In the present work we found that replacement of Gly-338 of NhaA with serine (G338S) produced an antiporter, which, to a large extent, lost its pH regulation. In contrast to the wild-type antiporter, which shuts off at pH 6.5 and increases activity by 3 orders of magnitude with increasing pH to pH 8.5, G338S is hardly affected by pH values between pH 7 and 9 and even at pH 6 is 70% active. This property of G338S was observed in both isolated everted membrane vesicles as well as proteoliposomes, containing purified His-tagged derivative of G338S. For unknown reasons, the mutated His-tagged protein was less active (by about 40–50%), as compared with the His-tagged wild-type protein. Since similar reduction in activity was observed in everted membrane vesicles harboring the His-tagged mutated protein, it cannot be ascribed to any of the reconstitution steps.

We have previously suggested (18) that the activation of NhaA by pH is accompanied by a conformational change which can be detected, using trypsin digestion as a probe. Thus both the wild-type NhaA as well as its mutant H225R, with a pH profile shifted by half a pH, are susceptible to proteolytic digestion, each at the pH range where it is activated, pH 7 to pH 8.5 or pH 6.5 to 8, respectively. The results presented here (Fig. 4) strengthen the suggestion that the two events, exposure to proteolysis and activation, are related; a single replacement, G338S, relieves NhaA of the control by pH and produces a protein that is active between pH 6 and 9. At the same time it renders the protein as susceptible to digestion by trypsin at pH 6.5 as at pH 8.5. Hence it is suggested that when the protein is inactive, the protein adopts a "closed" conformation, which is not susceptible to trypsination, despite the existence of many tryptic cleavage sites. When it is active, the conformation state is open, and more domains are exposed to the enzyme.

The mutation yielding G338S antiporter was obtained by selection for growth at neutral pH but not at alkaline pH (8.3) in the presence of Na\(^+\) (0.6 M). The mutant grew, however, at alkaline pH in the presence of K\(^+\) (0.6 M). Hence, the combination of alkaline pH and high Na\(^+\) harms G338S cells rather than each condition separately or osmolarity.

We have previously shown that increased antiporter activity is a prerequisite for growth at alkaline pH in the presence of Na\(^+\). Transformation of the ΔnhaAΔnhaB strain with multicopy plasmids bearing nhaA restores growth at alkaline pH in the presence of Na\(^+\). Accordingly, although being fully active at pH 7.5 but inactive at pH 8.5, the H225R antiporter could not support growth of the ΔnhaAΔnhaB mutant at alkaline pH in the presence of Na\(^+\) (16). Based on these data the mutants obtained, with the selection used here, were expected to have low antiporter activity, due to either defects in activity, lack of expression, and/or impaired pH control, failing to activate the antiporter at alkaline pH. Indeed 5 out of the 6 mutants isolated expressed an inactive or defective antiporter (Fig. 2).

However, G338S phenotype, an active antiporter that confers Na\(^+\) sensitivity at alkaline pH, was unexpected and puzzling. It was therefore first considered that at alkaline pH in the presence of Na\(^+\), G338S is not expressed or expressed in an inactive form. These possibilities were ruled out since Western analysis and measurement of the Na\(^+\)/H\(^+\) antiporter activity in membrane vesicles, isolated from cells shifted to alkaline pH in the presence of Na\(^+\), showed that G338S protein is present in the membrane at significant amounts and is active. A third possibility considered was that the G338S protein is electroneutral and therefore is inactive in vivo at alkaline pH due to the lack of ΔpH, the driving force. At alkaline pH in E. coli the only component of the ΔΨ that is maintained by respiration is ΔΨ (27). This possibility was ruled out since G338S was found endowed with a pH profile marked by half a pH, even with respect to the wild-type protein (Fig. 5).
were obtained. Remarkably, in all of them, the growth at alkaline pH in the presence of Na\(^+\) was regained, together with the capacity of pH to control NhaA, and shut down significantly its activity between pH 6 and pH 6.5 (Fig. 5). The possibility that the restoration of these two phenotypes has been a coincidence is highly unlikely, in view of the four independent suppressions obtained. Note, however, that a complete arrest of the Na\(^+\)/H\(^+\) activity was observed only in everted membrane vesicles isolated from Sup150 and at pH 6, half a pH unit more acidic than the pH that shuts off the wild-type protein. Remarkably, the effect of the suppression mutation of Sup150 on the pH regulation of the antiporter was even more pronounced in a wild-type genotype background. The pH profile of P129L protein was shifted toward alkaline pH, with respect to both the wild-type and P129L/G338S proteins (Fig. 5).

Why lack of pH control and sustained high activity of the antiporter at all pH values impair growth at alkaline pH\(_\text{out}\), (in the presence of Na\(^+\)) but not at neutral pH\(_\text{out}\)? One explanation would be over-acidification of the cytoplasm. This possibility, however, is unlikely for two reasons: alkaline pH\(_\text{out}\) should have improved, rather than impaired, growth; and addition of weak bases, a procedure shown to titrate and increase pH\(_\text{in}\) (29), did not rescue G338S.

We have previously shown that at alkaline pH in the presence of high Na\(^+\), E. coli is under energetic stress (27, 30) which is reflected in a growth rate slower than that at neutral pH. This stress would be aggravated by a high turnover of an uncontrolled antiporter, which would consume the already limited energy resources. We therefore suggest that the mutant G338S and its suppression revealed the importance of the pH set point of NhaA activity.

In addition to G338S, His-225 is the only other residue of NhaA that has been shown to affect pH regulation of the antiporter (16, 17, 31). However, these residues differ in various aspects as related to the antiporter structure/function relationship. (a) The topology of the residues within the protein is different. Based on phoA fusions, proteolysis, from either side of the membrane, and epitope mapping, we have previously advanced a putative two-dimensional model of NhaA (32). In this model His-225 is located at the periplasmic edge of TMS VIII and exposed outward. This has recently been proven by probing H225C with membrane-impermeant SH reagents (22). According to this model, Gly-338 is located in the middle of the hydrophobic TMS XI. (b) The residues are involved in different steps of the pH response of NhaA. We did not succeed in obtaining second-site suppressor mutations in codon H225R, and the first-site suppressor mutations maintain the drastic dependence on pH, although within a new range. In contrast to His-225 mutants, the pH control of G338S is alleviated, and we did obtain second-site suppressor mutations to G338S, but none map in H225R codon. Accordingly, as probed by trypsin, replacements in His-225 shift the pH profile of the “open” conformation of NhaA, whereas Gly-338 maintains this conformation at all pH values.

One of the Gly-338 suppressor mutations maps in the loop connecting TMS XI and XII, the other three clusters in TMS IV (32). This clustering is most interesting since Asp-133, in the vicinity, has recently been shown to be involved in the functioning of NhaA (33). The suppression mutations may even indicate that TMS IV and XI interact.

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