The Monoclonal Antibody 1F6 Identifies a pH-dependent Conformational Change in the Hydrophilic NH₂ Terminus of NhaA Na⁺/H⁺ Antiporter of Escherichia coli

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One of the most interesting properties of the NhaA Na⁺/H⁺ antiporter of Escherichia coli is the strong regulation of its activity by pH. This regulation is accompanied by a conformational change that can be probed by digestion with trypsin and involves the hydrophilic loop connecting the transmembrane helices VIII–IX. In the present work we show that a monoclonal antibody (mAb), 1F6, recognizes yet another domain of NhaA in a pH-dependent manner. This antibody binds NhaA at pH 8.5 but not at pH 4.5, whereas two other mAbs bind to NhaA independently of pH. The epitope of mAb 1F6 was located at the NH₂-terminus of NhaA by probing proteolytic fragments in Western blot analysis and amino acid sequencing. The antibody bound to the peptide HLHR-FFSS, starting at the third amino acid of NhaA. A synthetic peptide with this sequence was shown to bind mAb 1F6 both at acidic and alkaline pH suggesting that this peptide is accessible to mAb 1F6 in the native protein only at alkaline pH. Although slightly shifted to acidic pH, the pH profile of the binding of mAb 1F6 to the antiporter is similar to that of both the Na⁺/H⁺ antiporter activity as well as to its sensitivity to trypsin. We thus suggest that these pH profiles reflect a pH-dependent conformational change, which leads to activation of the antiporter. Indeed, a replacement of Gly-338 by Ser (G338S), which alleviates the pH dependence of both the NhaA activity as well as its sensitivity to trypsin, affects in a similar pattern the binding of mAb 1F6 to NhaA. Furthermore, the binding site of mAb 1F6 is involved in the functioning of the antiporter as follows: a double Cys replacement H3C/H5C causes an acidic shift by half a pH unit in the pH dependence of the antiporter; N-ethylmaleimide, which does not inhibit the wild-type protein, inhibits H3C/H5C antiporter to an extent similar to that exerted by mAb 1F6.

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⁺ for Li⁺ for H⁺ (3). 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).

NhaA is an electrogenic antiporter that has been purified to homogeneity and reconstituted in a functional form in proteoliposomes (11–12). Its H⁺/Na⁺ stoichiometry is 2H⁺/Na⁺ (12) and its activity drastically depends on pH, changing its $V_{max}$ over 3 orders of magnitude from pH 7 to pH 8 (12). A strong pH sensitivity is characteristic for antiporters as well as for other transporters that are involved in pH homeostasis (reviewed in Ref. 4). Identifying the amino acid residues and the protein domains involved in the pH sensitivity of these proteins is important for understanding the mechanism of pH regulation.

Out of the eight histidines of NhaA only His-225 was found to be essential for the Na⁺/H⁺ antiporter activity of NhaA (13). Whereas the activation of the wild-type NhaA occurs between pH 7.5 and pH 8, replacement of His-225 by Arg (H225R) yields an antiporter that is activated between pH 6.5 and pH 7.5. In addition, although the wild-type antiporter remains almost fully active, at least up to pH 8.5, H225R becomes inactive above pH 7.5, retaining only 10–20% of the maximal activity at pH 8.5 (13). Replacement of His-225 by either cysteine (H225C) or serine (H225S), but not alanine (H225A), yielded an antiporter with a wild-type pH sensitivity, implying that polarity, size, and/or hydrogen bonding, the common properties shared by His, Cys, and Ser, are essential at position 225 for pH regulation of NhaA (14). Changes of Gly-338 also affect the pH dependence of NhaA activity; its replacement by serine (G338S) produced a transporter whose activity is independent of pH; it is active throughout pH 6.5 to 8.5 (15).

Recently, we have also found that NhaA undergoes a conformational change upon its activation by pH which can be probed by trypsin (16, 17). In everted membrane vesicles at acidic pH, the protein is completely resistant to trypsin, whereas at alkaline pH it is digested with a similar pH dependence as the antiporter activity. Furthermore, two variants with a modified pH dependence of activity are susceptible to trypsin, in isolated membrane vesicles, only at the pH range where they are active and reflecting the level of activity (16, 17); H225R, the variant with a pH profile of activity shifted toward acidic pH, is digested at that pH; the G338S variant, which lost pH control, is active and exposed to trypsin throughout the entire pH range.
Monoclonal antibodies (mAbs) have been raised against numerous membrane proteins, and they are a versatile tool for their biochemical and structural characterization. Most importantly, if they recognize a native epitope of a protein, they can be used to probe conformational changes in this epitope (18, 19). Modifying the standard ELISA (His-tag ELISA) we have recently isolated four NhaA-specific mAbs (20). In this assay the antigen is presented in its native form when bound on Ni\(^2+\)-NTA-coated polystyrene plates via its His tag in the presence of the detergent LM and under physiological conditions. Three of the mAbs obtained recognize only the native antiporter and are Western blot-negative (20). The fourth mAb (1F6), which is Western blot-positive, showed a unique behavior. In His tag ELISA it gave rise to much higher signals at alkaline pH as opposed to acidic pH. In this work we show that mAb 1F6 recognizes the \( \text{NH}_2 \) terminus of NhaA which appears to be exposed at alkaline pH but not at acidic pH.

**Experimental Procedures**

**Bacterial Strain, Culture Conditions, and Plasmids**-

**EP342** is an *E. coli* K12 derivative which is mBl21ΔΔ, S80C, Na\(^+\)/K\(^+\) pump, nhaA, DnaB, MarZ, thr(11). TA16 is nhaA nhaB lacI\(^q\) and otherwise isogenic to EP342 (12). DH5\(^\alpha\) (U. S. Biochemical Corp.) and JM109 were used as hosts for construction of plasmids. Cells were grown in modified LB broth (Amersham Pharmacia Biotech) in which NaCl was replaced by KCl (87 mm, pH 7.5, (7)). For overexpression cells were grown in minimal medium A without sodium citrate (21) with either glycerol (0.5%) or melibiose (10 mM) as a carbon source. Thiamine (2.5 \( \mu \text{g/ml} \)) was added to the minimal medium. For plates, 1.5% agar was used. The concentrations of antibiotics were 100 \( \mu \text{g/ml} \) ampicillin and/or 50 \( \mu \text{g/ml} \) kanamycin and/or 12 \( \mu \text{g/ml} \) chloramphenicol and/or 12.5 \( \mu \text{g/ml} \) tetracycline. Resistance to Li\(^+\) and Na\(^+\) was tested as described previously (13).

**Plasmid pPAH**,

which encodes Xa-His-tagged NhaA, was constructed previously (22) and contains nhaA fused at its 5' end to the tac promoter for overexpression. At its 3' end to the coding sequence tandem two factor Xa cleavage sites followed by six His residues for affinity purification of the protein on Ni\(^2+\)-NTA column (22). The plasmid pPAH is a PBR322 derivative that bears nhaA and most of the nhaB gene.

**Mutagenesis**-

Site-directed mutagenesis was conducted following a polynucleotide chain reaction-based protocol (15). DNA of pGM100 was used as a DNA template with the forward mutagenic primer 5'-GTC AAA TGT CTC TGC AGA TTC TTT AGC-3' and the reverse mutagenic primer 5'-GCA CCT TCG AGA TTC GGC CAT CAC-3'.

**Isolation of Membrane Vesicles, Assay of NHa+ Antiporter Activity**-

Assays of Na\(^+\)H\(^+\) antiporter activity were conducted on everted membrane vesicles (22). The assay for antiporter activity was based upon the measurement of Na\(^+\)- (or Li\(^+\)-) induced changes in the \( \Delta pH \) as described (5, 22).

**Overproduction and Affinity Purification of His-tagged Antiporters**—To overproduce the wild-type and the mutated antiporters, plasmids leading to overproduction of the His-tagged proteins in TA16 cells were used. The transformed cells were grown in minimal medium to \( A_{600} = 0.6 \), induced with 0.5 mM isopropyl-1-thio-\( \beta \)-galactopyranosidase, grown for 2 h to \( A_{600} = 1-1.2 \), harvested, and used for preparation of membranes either after storage overnight at 4 °C or after freezing at \(-20\) °C (11). His-tagged NhaA and the mutant His-tagged G338S were affinity purified on Ni\(^2+\)-NTA-agarose column (Qiagen, Hilden, Germany) as described (22).

**Digestion by Trypsin**—Purified antiporter was subjected to trypsin digestion in a 30-\( \mu \)l reaction mixture containing 10 \( \mu \)g of antiporter protein, 30 \( \mu \)g of trypsin (type III from bovine pancreas, Sigma T-8253), 0.1% LM (Calbiochem), 8.3 mm potassium acetate, 200 \( \mu \text{M} \) KCl, 6.5% glycerol, 0.7 mm NaEDTA, 20 mm Hepes/Tris (pH 8, if not indicated otherwise), and 1 mm CaCl\(_2\). The mixture was incubated at 37 °C for 30 min (short treatment) or 60 min (long treatment). The reaction was terminated by the addition of 100 ng of trypsin inhibitor (type II-S from soybean, Sigma T-9128). To determine the extent of digestion samples of 5 \( \mu \text{g} \) of protein were subjected to SDS-PAGE (15).

**Separation and Isolation of NhaA Tryptic Fragments and Determination of the Amino Acid Sequence of Their NH2 Terminal**—A protein sample (50 \( \mu \text{g} \)) was resuspended in SDS-PAGE sampling buffer and loaded on 15% for fragments larger than 10 kDa or 20% for smaller ones) isopropyl-galactoside gel. After precipitation the peptides were transferred (400 mA for 60 min) to polyvinylidene difluoride type (Millipore Immobilon-P) transfer membranes in transfer buffer containing 25 mm Tris/HCl, 192 mm glycine, 10% methanol, and 0.025% SDS, pH 8.4. The filters were then washed in distilled water, stained for 5 min in 0.1% Coomassie Blue (R-250) in 50% methanol, destained for 5–10 min in 10% acetic acid in 50% methanol, and washed for 5–10 min in distilled water. The stained bands were cut and subjected to NH2-terminal sequencing using a Perkin-Elmer (Applied Biosystem Division) 492 (Procie) Microsequencer System.

**Production and Purification of mAbs**—The production and purification of mAb 1F6, 2C5, and 5H4 have been described previously (20).

**His Tag-tag ELISA and Competition Assay**—The His tag-mediated ELISA was conducted practically as described previously (20). His-tagged NhaA was bound to Ni\(^2+\)-NTA-coated 96-well microtiter plates (Qiagen) using a final concentration of 12 \( \mu \text{g/ml} \) in coating buffer (20 mm Tris/HCl, pH 7.5 (if not indicated otherwise), 500 mm NaCl, 0.1% (w/v) LM), and gently shaking the plates at room temperature for 45 min. The plates were washed three times with 20 mm Tris/HCl, pH 7.5, 500 mm NaCl, 0.03% (w/v) LM, and 0.1% (w/v) BSA. Ni\(^2+\)-NTA-coated plates need no blocking since the peptides are pre-blocked by the manufacturer. The washing buffer was also used for dilution and subsequent incubation of purified mAbs on the plates. All other steps were performed as described in the standard ELISA protocol (20, 23). The competition assay was performed by incubating mAb solutions at a final concentration of 12 \( \mu \text{g/ml} \) with different concentrations of either synthetic peptide, wild-type His-tagged NhaA, or His-tagged G338S-NhaA in a volume of 200 \( \mu \text{l} \) overnight at 4 °C. The mixtures were then used to probe Ni\(^2+\)-NTA plates, which were previously coated with His-tagged NhaA (12 \( \mu \text{g/ml} \)). The subsequent steps were as described for the His tag-tagged ELISA.

**SDS-PAGE, Western Blot, and Protein Determination**—SDS-PAGE was done according to Ref. 24. For Western blot analysis (25) proteins were transferred onto a nitrocellulose membrane using a semi-dry blotting apparatus by applying 50 mA/gel for 1 h at 4 °C. After transfer, the membrane was blocked overnight in phosphate-buffered saline buffer containing 3% (w/v) BSA, 0.1% (w/v) Triton X-100. All washing steps were carried out in phosphate-buffered saline buffer containing 0.05% (w/v) Triton X-100. Incubation with primary antibodies, blood sera, and secondary antibody (anti-mouse IgG alkaline phosphatase-conjugated), the probes were diluted 1:1000 in blocking buffer. The nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate color reaction was performed in 20 mm Tris/HCl, pH 9.8, 500 mm NaCl, 0.5 mm MgCl\(_2\). Protein concentrations were determined according to Ref. 26.

**Gel Filtration Chromatography**—Gel filtration chromatography was carried out using a Superose 6 column (Amerham Pharmacia Biotech), at a flow rate of 50 \( \mu \text{ml/min} \) at 4 °C on a SMART System (Amerham
mAb 1F6 Probes a pH-induced Conformational Change in NhaA

Fig. 1. mAb 1F6-NhaA complex is formed at pH 8 but not at pH 4.5. A, gel filtration chromatography: mAb 1F6 (20 μg) and NhaA (20 μg) were mixed and incubated for 30 min in 100 mM KCl, 5 mM MgCl₂, 7.5% glycerol, 0.03% LM either at pH 8 (- -) or at pH 4.5 (—) using 25 mM potassium acetate buffer. The mixtures were then separated on Superose 6 gel filtration column by high pressure liquid chromatography at the respective pH values. The elution profiles are shown together with the various peaks (A–E). The molecular weight standards are as follows: thyroglubulin (669 kDa); fer, ferritin (450 kDa); cat, catalase (240 kDa); ald, aldolase (160 kDa).

B, SDS-PAGE of the fractions obtained by the gel filtration chromatography. Samples (5 μl) of each peak fraction were run on SDS-PAGE. lane 1, sample from peak E; lanes 2–5, samples from peak D, as explained in the text; lane 6, peak A; lane 7, peak B containing the 1F6-NhaA complex; lane 8, peak C. The gel was silver-stained. Since the protein samples were not heated up to 95 °C to avoid aggregation of NhaA, the bands observed at higher molecular weights are due to incomplete denaturation of the antibody molecules.

Pharmacia Biotech. To assess molecular weights a calibration curve was calculated based on purified proteins (aldolase, catalase, ferritin, and thyroglubulin, Sigma). The running buffer contained 100 mM KCl, 5 mM MgCl₂, 7.5% (w/v) glycerol, 0.03% (w/v) LM, and either 25 mM potassium acetate, pH 4.5, 25 mM Bis-Tris/HCl, pH 6, 25 mM MOPS/NaOH, pH 7, or 25 mM Tris/HCl, pH 8. For each run mAb 1F6 and His-tagged NhaA (20 μg each), were mixed in the respective buffers and incubated for 30 min at 4 °C.

Peptide Synthesis on Cellulose Membranes—The general protocol has been described previously (27). Membranes were obtained from Abimed (Langenfeld, Germany); Fmoc amino acids and N-hydroxybenzotriazole were obtained from Nova Biochem (Laufenfingen, Switzerland). The ASPage robot (Abimed) was used for the coupling steps. Two hundred and eight overlapping decapetides frameshifted by two residues, representing the entire NhaA, were synthesized on cellulose membrane. All peptides were acetylated at their NH₂ termini. After the peptides were assembled, the side chain protecting groups were removed by trifluoroacetic acid treatment.

Assay for mAb 1F6 Interaction with Cellulose-bound Peptides—The technique was performed as described previously for epitope analysis (27). The saturated membrane was incubated with 12 μg/ml mAb 1F6 solution in the blocking buffer (Genosys Biotechnologies, UK) for 2 h at room temperature. Bound mAb 1F6 was detected by incubation of the membrane at 25 °C for 30 min in a 1:3000 dilution of an anti-mouse IgG (whole molecule) conjugated to alkaline phosphatase (Sigma) and subsequent addition of a phosphatase substrate (5-bromo-4-chloro-3-indole phosphate and 5-bromo-4-chloro-3-indole phosphate). A blue precipitate on the spot indicated mAb binding.

Synthesis of Peptide HLHRFFSS—The 8-mer peptide (NH₂-COOH) HLHRFFS (-COOH) was synthesized by Fmoc solid-phase synthesis on a Labortec peptide synthesizer (model SFP40, Bubendorf, Switzerland). The peptide was deprotected and released from the resin by trifluoroacetic acid treatment in the presence of appropriate scavengers. The peptide was lyophilized, and its purity was assessed by high pressure liquid chromatography.

Binding of mAb 1F6 to Peptide HLHRFFSS—The peptide was dissolved in distilled water to a final concentration of 50 μg/ml, activated, and bound to the Covalink NH plates, according to the Covalink NH manual (Nunc). Then the plates were washed in 500 mM NaCl, 0.1% BSA, 0.03% LM, and either 20 mM Tris/HCl, pH 8, or 20 mM potassium acetate, pH 4.5, and probed in the respective solutions with mAb 1F6 (12 μg/ml), according to the His tag ELISA.

Immunopurification Column—For construction of the column (2 ml) the ImmunoPure Protein A IgG Plus Orientation kit from Pierce was used. 3 mg of purified mAb 1F6 were bound to the protein A and cross-linked with disuccinimidyl suberate. The column was washed in Tris buffer containing 20 mM Tris/HCl, pH 8, 300 mM KCl, 5 mM MgCl₂, 7.5% glycerol, and 0.03% LM. Purified His-tagged NhaA (800 μg) was loaded onto the column in the same buffer, and the column was washed in 5 column volumes of the buffer. His-tagged NhaA was eluted in 2 column volumes of the binding buffer containing 25 mM potassium acetate, pH 4.5, instead of Tris/HCl.

RESULTS

Table I summarizes the results of an experiment that led us to consider the possibility that the Western blot-positive mAb 1F6 recognizes a peptide that is specifically exposed in a pH-dependent manner. In this experiment Ni²⁺-NTA ELISA plates were coated with His-tagged NhaA at pH 7.9, washed, and exposed to mAb 1F6 at different pH values. As a control we used mAb 2C5, another monoclonal antibody, which is Western blot-negative and recognizes only a native form of NhaA (20). The results show that, whereas the signal obtained from mAb 2C5 was high and independent of pH, the signal from mAb 1F6

Table I

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was pH-dependent. Increasing the pH from pH 4.5 to 8.3 doubled the signal of mAb 1F6. At pH 9.5 a small decrease in the maximal signal was observed.

To test directly the possibility that the binding of mAb 1F6 to NhaA is pH-dependent, purified NhaA and mAb 1F6 were mixed, incubated at either pH 8 or pH 4.5, and then separated at the respective pH values by gel filtration chromatography (Fig. 1). For control NhaA and mAb 1F6 were also run each separately on this column. Their retention volumes at both pH values were similar, 1.57 and 1.60 ml, respectively (not shown), and thus could not be separated on this column. Calibration of the column with the molecular weight standards indicated in Fig. 1 showed that these retention volumes correspond to 160 and 150 kDa for His-tagged NhaA and mAb 1F6, respectively. The apparent molecular weight observed for NhaA is much higher than expected from its amino acid composition (41 kDa (11)). This is due to the LM micelle and to the dimeric form of NhaA in the LM micelle (20).

At pH 4.5 one main peak (Fig. 1A, peak D) was observed with a retention volume of 1.58 ml and a shoulder at 1.5 ml corresponding to molecular mass of 160 and 300 kDa, respectively. Samples were taken from the shoulder and various fractions of peak D (corresponding to retention volumes of 1.5, 1.6, and 1.7 ml) and analyzed by SDS-PAGE (Fig. 1B). It was evident that the shoulder contained only mAb 1F6 (Fig. 1B, lane 1) which eluted at a retention volume corresponding to an apparent molecular weight higher than expected. This was most probably due to some aggregation of the antibody occurring during preincubation. Fig. 1B, lanes 2–5, shows that most of peak D was composed of a mixture of the free uncomplexed proteins NhaA and mAb 1F6. This conclusion was based on the fact mentioned above that the apparent molecular weights of both proteins when uncomplexed are very similar and do not allow their separation on this column.

At pH 8 three peaks were observed with retention volumes of 0.88, 1.29, and 1.58 ml corresponding to molecular mass of 800, 500, and 160 kDa, respectively (Fig. 1A). SDS-PAGE analysis of the three peaks (Fig. 1B) showed that the material eluting at high molecular weight (Fig. 1A, peak A) consisted of the antibody (Fig. 1B, lane 6). As discussed above this apparent very high molecular weight observed in the gel filtration was most probably due to aggregation. The medium molecular weight peak (Fig. 1A, peak B) contained both NhaA and mAb 1F6 (Fig. 1B, lane 7). Since the apparent molecular weight of this peak is higher than that of either NhaA or mAb 1F6 (see above), it can be concluded that this fraction contains the mAb 1F6-NhaA complex. The low molecular weight peak (Fig. A,}

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**FIG. 2.** Formation of the complex NhaA-1F6 as a function of pH. The experiment was conducted as described in Fig. 1A, but the pH values were changed with the following buffers: pH 4.5, 25 mM potassium acetate; pH 6, 25 mM bis-Tris/ HCl, pH 7, 25 mM MOPS; pH 8, 25 mM Tris/HCl. The results (■) were expressed as percentage of complex formation (100% = the NhaA-1F6 complex peak area at pH 8). For comparison of the pH dependences the figure also shows the activity of the NhaA Na+/H+ antiporter (♣) and the trypsin digestibility of NhaA (●) as a function of pH. The data of both pH profiles were obtained from Refs. 11 and 16, respectively, and are expressed as percentage of each maximum.

**FIG. 3.** NhaA binds at alkaline pH to an affinity column based on mAb 1F6 and elutes at acidic pH. The 2-ml affinity column based on mAb 1F6 was constructed as described under "Experimental Procedures." At each step during the test of the column, samples (5 μl) were taken for SDS-PAGE analysis: lane 1, wash of the column in 2 volumes of binding buffer containing 25 mM Tris/HCl, pH 8, 200 mM KCl, 5 mM MgCl₂, 7.5% glycerol, and 0.03% LM; lane 2, application of 700 μg of NhaA in binding buffer; lanes 3 and 4, wash with 6 column volumes of binding buffer; lane 5, elution with 2 column volumes containing the same ingredients as the binding buffer but 25 mM potassium acetate at pH 4.5 instead of Tris. The gel was silver-stained.
Fig. 2 shows the pH dependence of the formation of NhaA-1F6 complex. It is evident that at pH 4.5 NhaA and 1F6 did not bind, but with increasing pH the complex was progressively formed. At pH 8, very little NhaA was left free (Fig. 1B, lane 8). The apparent molecular weight of the complex is in agreement with a stoichiometry of 2 NhaA dimers per antibody molecule. Based on the pH-dependent interaction between mAb 1F6 and NhaA, we then constructed an affinity column for NhaA. NhaA was expected to bind to this column at alkaline pH and to elute at acidic pH. The purified mAb 1F6 was first bound to protein A immobilized on agarose beads and then covalently linked to the resin via disuccinimidyl suberate. At each step thereafter samples of the column effluent were taken for SDS-PAGE analysis (Fig. 3). The column was washed in binding buffer (Fig. 3, lane 2) and washed twice at pH 8 (Fig. 3, lanes 3 and 4). Elution was conducted at pH 4.5 (Fig. 3, lane 5). It is clear that NhaA bound to immobilized 1F6 at pH 8 and eluted at pH 4.5. Loading of the column with NhaA at pH 4.5 showed no binding to the column since NhaA eluted in the void volume (not shown).

Taken together all these results show that mAb 1F6 recognizes its epitope only at alkaline pH. Possible reasons are as follows: (i) mAb 1F6 or/and NhaA are differently protonated at low or high pH. This effect could lead to the binding at high pH only. (ii) NhaA (or/and mAb 1F6) possesses a different conformation at low or high pH, and the epitope recognized by mAb 1F6 is only accessible at high pH. We therefore started experiments to find out whether alternative i or ii is correct.

We have previously shown that short treatment with trypsin cleaves purified NhaA into two fragments at Lys-249, in loop VIII-IX of NhaA. The resulting fragments are an NH2-terminal and COOH-terminal fragment, 2/3 and 1/3 the size of the intact protein, respectively (Fig. 4A (16, 17)). The time course of the trypsinolysis of NhaA shown in Fig. 4A shows that after 30 min the protein was split quantitatively into two fragments. Thereafter the COOH-terminal fragment (Fig. 4A, L.F.) was not digested any further, whereas the NH2-terminal fragment (Fig. 4A, H.F.) was almost completely degraded during a long treatment of 120 min. The two tryptic peptides (heavy and light, Fig. 4A, H.F. and L.F.) were separated on SDS-PAGE (15%), and binding of mAb 1F6 was investigated by Western blot analysis. The results presented in Fig. 4B show that mAb 1F6 recognizes only the NH2-terminal tryptic fragments of NhaA. The same result was observed after a prolonged proteolysis of NhaA when only the light fragment unrecognized by mAb 1F6 remained (Fig. 4, C and D) together with the small cleavage products of the NH2 terminus (Fig. 4C). These fragments smaller than 6000 Da in size were separated on SDS-PAGE (20%), and binding of mAb 1F6 was tested (Fig. 4D). Four small peptides were recognized by mAb 1F6. The two smallest ones were isolated from the gels and subjected to NH2-terminal amino acid sequencing. The N termini of these two fragments were found to be MKHLHRFFSSDAS, the native NH2 terminus of NhaA (11).

To identify the peptide recognized by mAb 1F6, a peptide library composed of 208 overlapping 10-mer peptides, frame-shifted by two residues, was generated by SPOT synthesis. Binding of mAb 1F6 to the membrane-bound peptides was then

Fig. 4. Western blot analysis of tryptic fragments of NhaA by mAb 1F6. Purified His-tagged NhaA was subjected to trypsin cleavage for various times: A, 15, 30, 60, 120 min, lanes 2–5, respectively, 60 min. B, lane 2, and 120 min. C, lane 2; and D, as described under “Experimental Procedures.” The products were separated on SDS-PAGE 15% (A and B) or 20% (C and D) polyacrylamide. The gels shown in A and C were stained by Coomassie Blue, and those shown in B and D were probed by mAb 1F6 in Western blot analysis. The undigested control reaction mixtures obtained the trypsin inhibitor at time 0 (lanes 1 in A and B). H.F., heavy fragment; L.F., light fragment.

Fig. 5. Reactivity with mAb 1F6 of overlapping decapeptides synthesized according to the amino acid sequence of NhaA. The membrane on which the peptides were synthesized was incubated with 12 μg/ml mAb 1F6 and developed as described under “Experimental Procedures.” The peptide spots are numbered from 1 to 208. The amino acid sequence common to the two positive peptides is shown.

(H2N-) HLHRFFSS (-COOH)
tested. Fig. 5 shows that two positive spots were discovered. These two spots contained peptides with a common sequence which is (NH₂)-HLHRFFSS-(COOH). Hence, mAb 1F6 recognizes this peptide that starts at the third amino acid from the NH₂ terminus of the protein (11).

We then synthesized the peptide HLHRFFSS and used it in a competition assay with NhaA for binding mAb 1F6. The antiporter protein was bound to Ni²⁺-NTA-coated ELISA plates. MAb 1F6 and mAb 5H4 (which recognizes only the native form of NhaA (20)) were preincubated separately with various concentrations of the peptide at room temperature for 90 min. The mixtures were then used to probe the Ni²⁺-NTA-bound NhaA. Each value represents the mean ± S.D. of triplicate determinations. Curves represent exponential fittings of the experimental data.

It was therefore interesting to test the binding properties of 1F6 to the G338S-NhaA. Purified His-tagged wild-type NhaA was bound to Ni²⁺-NTA ELISA plates. Then, mAb 1F6 (12 μg/ml) was incubated overnight at 4 °C and at pH 6.5 or 8.3 with various concentrations of either wild-type protein or the G338S-NhaA. These mixtures were then used to test binding to the wild-type NhaA-coated plates at pH 8. The results summarized in Fig. 8 show that, whereas mAb 1F6 bound wild-type NhaA only at pH 8.3, G338S protein was bound at both pH 8.3 and pH 6.5 with a similar concentration dependence. Hence the mutation G338S affects similarly the activity, trypsin digestibility, and accessibility of NhaA to mAb 1F6.

We have previously shown that binding of mAb 1F6 to NhaA in proteoliposomes inhibits the Na⁺/H⁺ antiporter activity (20). It was therefore interesting to investigate whether the NH₂-terminal peptide recognized by mAb 1F6 contains amino acids that play a role in the activity and/or pH regulation of the antiporter. For this purpose an NhaA variant harboring two amino acid replacements, H3C/H5C, was constructed. The plasmid, pH3C/H5C, carrying the mutated gene was transformed into EP432, a ΔnhaAΔnhaB strain lacking both specific Na⁺/H⁺ antiporters, NhaA and NhaB. The Na⁺/H⁺ antiporter activity in membrane vesicles isolated from EP432/pH3C/H5C was then determined (Fig. 9). It is evident that the double mutation had no effect on the maximal activity of the antiporter, but it caused an acidic shift in its pH dependence. Whereas the wild-type NhaA shows a drastic pH-dependent increase in activity between pH 6.5 and 8, the mutant H3C/H5C is mostly sensitive between pH 6 and 7.8. Interestingly, the SH reagent N-ethylmaleimide, which has no effect on the wild-type NhaA (14), inhibited the mutant (Fig. 9).
DISCUSSION

One of the most interesting properties of NhaA Na⁺/H⁺ antiporter of *E. coli* is the dramatic regulation of its activity by pH (2, 3). To understand the mechanism of this regulation, it is essential to identify both the amino acid residues as well as the protein domains involved. In the present work we show that mAb 1F6 recognizes a peptide of NhaA that shows a pH-dependent accessibility. This antibody binds NhaA at pH 8.5, but not at pH 4.5, as observed with NhaA bound to Ni²⁺-NTA-coated plates or by direct separation of the NhaA-mAb 1F6 complex by gel filtration chromatography. Similarly mAb 1F6 covalently linked to agarose beads via protein A bound NhaA at pH 8.5 but not at pH 4.5. Other mAbs, 2C5 and 5H4, that recognize other native epitopes of NhaA showed pH-independent binding.

mAb 1F6 binds to a peptide starting at the third amino acid from the NH₂ terminus of NhaA (HLHRFFSS). There are charged amino acids in the epitope that could account for the effect of pH. We had also to consider the possibility that the pH dependence of mAb 1F6 binding stems from a pH-dependent change in the antibody itself. These two alternatives were ruled out, Western blot analysis conducted both at pH 4.5 and 8.5 showed the same efficiency (data not presented). A synthetic peptide HLHRFFSS containing the 1F6 epitope was shown to bind NhaA in a pH-independent fashion. Hence, mAb 1F6 recognizes a peptide in native NhaA, which either is accessible only at high pH or possesses a conformation at low pH, which is not recognized by mAb 1F6. Furthermore, the NH₂-terminal peptide that binds mAb 1F6 at alkaline pH participates in the pH regulation of the antiporter. We have previously shown that binding of mAb 1F6 to NhaA inhibits the Na⁺/H⁺ antiporter activity in reconstituted proteoliposomes (20). In the present work we show that a double Cys replacement, H3C/H5C in the binding site of mAb 1F6 causes acidic shift, by half a pH unit, in the pH dependence of the antiporter activity, whereas the wild-type protein is inactive at pH 6.5 and progressively acti-
vated by pH to a maximal value at pH 8, the mutant protein is shut off at pH 6 and attains its maximal rate at pH 7.5.

Interestingly, despite this pH shift, cells bearing the H3C/H5C-NhaA show growth phenotype and salt resistance at alkaline pH (0.6 M NaCl, pH 8.5) similar to that of the wild-type cells (data not shown). We have shown previously that two properties of the antiporter are essential for growth at alkaline pH in the presence of Na\(^+\). One is high rate of antiporter activity at high pH (13); the other is a capacity to shut off at acidic pH (15). Both these properties are exhibited by H3C/H5C-NhaA. Although the maximal activity of the antiporter was not affected by the double Cys replacement, the SH reagent N-ethylmaleimide, which has no effect on the wild-type protein, inhibited the mutant protein. The magnitude of this inhibition (60–70%) was similar to that obtained by binding of mAb 1F6 (20). It is suggested that a certain structure of the NH\(_2\) terminus and/or interaction required for the activity of the antiporter is disrupted by the modification exerted by either N-ethylmaleimide or mAb 1F6.

Fig. 2 shows a comparison of the pH dependences of three characteristics of NhaA, the Na\(^+\)/H\(^+\) antiporter activity, the conformational changes of NhaA as probed by trypsin, and by binding of mAb 1F6. The pH dependences of the digestibility by trypsin and antiporter activity are very similar. However, although the three properties reach almost a maximum at pH 8, the binding of mAb 1F6 decreases rather rapidly with acidification. Whereas at pH 7 the NhaA Na\(^+\)/H\(^+\) antiporter activity and trypsinolysis are very low (less than 5%), 50% of the binding of 1F6 was still observed at this pH. It is possible that the conformational change detected by 1F6 is different from that detected by trypsin. However, it is more likely that the conformational change is identical but that binding of the antibody affects the pH profile; it shifts a pH (toward acidic pH) of a residue involved in an essential conformational change. We prefer the latter alternative for two main reasons: (a) as mentioned above and in Ref. 20, binding of mAb 1F6 to NhaA inhibits the activity of the antiporter in reconstituted proteoliposomes; (b) the mutant G338S, which alleviates the pH control of NhaA and, accordingly, renders the trypsin digestion independent of pH, affects in a similar fashion the binding of mAb 1F6 to NhaA. The antibody binds the mutant protein with the same efficiency both at acidic and alkaline pH.

We thus suggest that the pH-dependent conformational change that is detected by mAb 1F6 and involves the NH\(_2\) terminus of NhaA plays an important role in the pH-dependent activation of the antiporter. The conformational change that is detected by trypsin and involves loop VIII–IX also appears to be crucial for the pH-dependent activation of the antiporter (17). Various mutations in this loop change the pH profile of the antiporter toward acidic pH. Furthermore, recently an NhaA homolog was cloned from Helicobacter pylori (28). In contrast to the E. coli NhaA which is activated at alkaline pH, the H. pylori antiporter is active at acidic and neutral pH. Remarkably, the sequences and the hydrophathy profiles of these antiporters are very similar except for one additional region found in H. pylori protein. The H. pylori antiporter carries an insertion of about 40 hydrophilic amino acid residues next to residue 235 of the E. coli NhaA and includes loop VIII–IX.

Based on the analysis of the hydrophathy profile, phoA fusions, epitope mapping, and proteolysis, a secondary structure model of NhaA with 12 TMS was obtained (29). According to this model both the NH\(_2\) terminus as well as loop VIII–IX are hydrophilic domains that face the cytoplasm. mAb 1F6 does not bind to either everted membrane vesicles or right-side-out membrane vesicles at either acidic or alkaline pH (data not shown). Hence, the peptide recognized by mAb 1F6 is masked either by the membrane or another loop.

In addition to the pH-sensitive domains at these two hydrophilic domains of NhaA, several amino acids have been found to affect the pH regulation of NhaA, His-225 (13, 14) at the periplasmic edge of TMS VIII and Gly-338 (15) in the middle of TMS XI, demonstrating that the pH regulation of NhaA is exerted in a global rather than local manner.
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REFERENCES


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