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Three aspartic residues in membrane-spanning regions of Na^+/H^+ antiporter from *Vibrio alginolyticus* play a role in the activity of the carrier

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Abstract

The Na^+/H^+ antiporter gene from *Vibrio alginolyticus* restores the growth of an *nhaA*-defective strain of *Escherichia coli*, NM81, in a high NaCl medium (Nakamura, T., Komano, Y., Itaya, E., Tsukamoto, K., Tsuchiya, T. and Unemoto, T. (1994) Biochim. Biophys. Acta 1190, 465–468). This gene, named *nhaAv*, allowed the *nhaA*-defective *E. coli* strains, NM81(*ΔnhaA*) and RS1 (*ΔnhaA*, *chaA*$^-$), to extrude Na^+ at alkaline pH. The extrusion of Na^+ occurred against its chemical gradient in the presence of membrane-permeable amine. Thus, the *nhaAv* gene product is functional as an electrogenic Na^+/H^+ antiporter in *E. coli* cells. The NhaAv protein has only four acidic amino acid residues in the putative membrane-spanning regions, that is, Asp-57, Asp-125, Asp-155 and Asp-156, and these Asp residues are conserved in NhaA from *E. coli*. Asp-111, which is predicted to be in a loop region between the transmembrane segments is also conserved in NhaA. Thus, each conserved Asp residue was replaced with asparagine by a site-directed mutagenesis. *E. coli* NM81 cells containing a plasmid harboring the *nhaAv* gene mutated at Asp-125, -155 or -156 could neither grow in a high NaCl medium nor extrude Na^+ at alkaline pH against its chemical gradient. These results show that Asp-125, -155 and -156, but not Asp-57 and -111, play a role in the activity of the Na^+/H^+ antiporter, NhaAv.

Keywords: Sodium ion/proton antiporter; *nhaA* gene; Aspartic acid residue; Marine bacterium; (*V. alginolyticus*)

1. Introduction

Na^+/H^+ antiporters are integral membrane proteins that transport Na^+ and H^+ in opposite directions across cytoplasmic membranes and are widely distributed in virtually all cell types [1,2]. These antiporters play a variety of physiological functions in each cell type. For example, they are required for the extrusion of Na^+ from bacterial cells and for the intracellular alkalization in eukaryotic and acidification in prokaryotic cells [3].

Most characterized bacterial Na^+/H^+ antiporters are NhaA from *Escherichia coli*. The *nhaA* gene was deleted from *E. coli*, and the strain NM81 (*ΔnhaA*) [4] and RS1 (*ΔnhaA*, *chaA*$^-$) [5] were constructed. In bacteria, six Na^+/H^+ antiporter genes have been sequenced [6–11] including *chaA* from *E. coli* [11]. There are no good homologies among them [2]. Recently, we cloned and sequenced an Na^+/H^+ antiporter gene from the marine *Vibrio alginolyticus*, which restored the growth of *E. coli* NM81 in the LB medium containing 0.5 M NaCl at pH 7.5 [12]. This gene, named *nhaAv*, had a moderate homology with *E. coli nhaA* gene. The deduced amino acid sequence was 58% identical with *E. coli* NhaA. Thus, the comparison of amino acid sequence between NhaAv and NhaA gave a chance to evaluate essential residues.

In the NhaA protein of *E. coli*, His-226 has been assigned to be a part of pH sensor [13]. However, the residues essential for the transport activity have not been identified. It is reasonable to assume that negatively charged amino acid residues localized in the membrane-spanning regions are important for binding or translocation of cations like H^+ and Na^+. There are 9 aspartic acids and 13 glutamic acids in NhaAv protein. Of 22 negatively charged residues, only 4 amino acids are predicted to be in

Abbreviations: EIPA, *N*-ethyl-*N*-isopropylamiloride; EPPS, *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

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membrane-spanning regions and all of them are aspartic acid [12]. These 4 amino acids are conserved in NhaA of *E. coli* [6] and *Salmonella entiritidis* [8]. Furthermore, Asp-111, which is predicted in a loop region between the transmembrane segments is also conserved in NhaA. Therefore, we considered that these 5 conserved amino acids may be related to the transport activity of Na^+/H^+ antiporter. We introduced a mutation of each conserved aspartic acid to asparagine by a site-directed mutagenesis. Of 5 aspartic acid residues, Asp-125, Asp-155 and Asp-156, but not Asp-57 or Asp-111, were found to be essential for the Na^+/H^+ antiport activity of NhaAv.

To measure the transport activity of Na^+/H^+ antiporter, it is important to directly detect Na^+ extrusion from cells. The extrusion of Na^+ from Na^+-loaded cells carrying Na^+/H^+ antiporter genes has not been shown in the absence of K^+. In this report, we will show the uphill Na^+ extrusion by NhaAv induced by a membrane-permeable amine.

Table 1
Mutagenic primers used for site-directed mutagenesis

Mutation	Primer sequences	Plasmids
D57N	5'-CAGCCATGAGCCC*GTTA*TTAATCC-3' (BanII)	pYS1D57N
D111N	5'-AATCGCCTCCGGA*TTG*TTGGCATT-3' (BspEI)	pYS1D111N
D125N	5'-AGCAAAAGC*AATATT*TGTCGCCGC-3' (SspI)	pYS1D125N
D155N	5'-ACCACACCTAGGTC*ATT*GATGATA-3' (AvrII)	pYS1D155N
D156N	5'-AACCACACCTAGG*TTA*TCGATGAT-3' (AvrII)	pYS1D156N
DDNN	5'-CCACACCTAGG*TTATTG*ATGATAG-3' (AvrII)	pYS1DDNN

Mutations were constructed in *nhaAv* gene of pYS1. Primers were constructed to the opposite direction of *nhaAv* gene. The site changed are indicated by *, that contain silent mismatches. New restriction sites are underlined. The italic letter sequence is the site of amino acid residue changed.

2. Materials and methods

Materials. $^{22}NaCl$ (NEN, USA) and [α-^{32}]PdCTP (Hungarian Academy of Sciences, Hungary) were used. The Sequenase Ver. 2.0 and *Bca*BEST™ Dideoxy Sequencing Kit were purchased from Amersham (UK) and Takara (Tokyo, Japan), respectively. *N*-Ethyl-*N*-isopropylamiloride (EIPA) was purchased from Molecular Probes (USA). All other materials were commercial products of the highest grade available.

Bacterial strains and plasmids. Escherichia coli TG1 (Amersham) was used for in vitro mutagenesis and the preparation of single-stranded DNA. Na^+/H^+ antiporter mutants of *E. coli* NM81 (*ΔnhaA*) [4] and RS1 (*ΔnhaA* and *chaA*$^-$) [5] were supplied by Dr. Etana Padan (Hebrew University) and Dr. Hiroshi Kobayashi (Chiba University), respectively. The plasmid pYS1 containing *nhaAv* gene [12] was prepared from pHG165 [14]. For the expression of mutated NhaAv protein, pT7-5 [15] and BL21(DE3)pLysE (Novagen) were used.

Growth conditions. E. coli cells were grown in the LB medium (pH 7.0) containing a suitable concentration of drugs. The salt tolerance was determined by the ability to grow in the LB medium containing 0.5 M NaCl at pH 7.5 (LB-NaCl medium). For the growth in the medium M9 [16], 100 μg threonine/ml for NM81 and 100 μg proline/ml for RS1 were added.

Preparation of Na^+-loaded cells. Na^+-Loaded/K^+-depleted cells were prepared by using a membrane-permeable amine as described previously [17]. Briefly, *E. coli* cells were suspended in 0.15 M NaCl containing 50 mM diethanolamine-HCl (pH 9.3). By this treatment, cellular K^+ was replaced with diethanolamine by the function of K^+/H^+ antiporter [18]. The amine-loaded cells were washed with 0.15 M NaCl containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid (EPPS)-NaOH buffer (pH 8.5). At this step, cell interior was acidified due to the release of diethanolamine and then Na^+ was loaded in exchange for H^+ via Na^+/H^+ antiporters [19].

Measurement of Na^+/H^+ antiporter activity. The Na^+-loaded cells were equilibrated with $^{22}Na^+$ (1 kBq/ml) in a medium containing 0.15 M NaCl and 20 mM EPPS-NaOH buffer (pH 8.5) for a few hours on ice [20]. Immediately after the addition of 0.2% glucose, the cells were transferred to 25° C. The extrusion of Na^+ from the cells was induced by the addition of 50 mM diethanolamine-HCl (pH 8.5) [21]. At time intervals, the cells were collected by filtration on membrane filter (OE67, Schleicher & Schull) and washed with 1.5 ml of 0.15 M choline chloride containing 10 mM Bis-Tris-HCl (pH 7.3). Radioactivities on the filters were determined by a liquid scintillator (Beckman).

Protein was determined by the method of Lowry et al. [22] with bovine serum albumin as a standard.

Site-directed mutagenesis. Mutagenesis was performed by the oligonucleotide-directed site-specific mutagenesis method according to the manual of a Sculptor™ in vitro mutagenesis system (Amersham, UK). The primers used for site-directed mutagenesis listed in Table 1 were synthesized with a Cyclone Plus DNA Synthesizer (MilliGen Biosearch Co., USA). Each of the mutagenic primers

contained an additional silent mismatch to make a new restriction site [23]. The mutation was first constructed in the *nhaAv* gene of pYN1 [12]. pYN1 is a pUC118-based plasmid. The single-stranded DNA was obtained from pYN1. The mutated plasmid was detected by the appearance of a new restriction site. The mutated plasmid was cut by *Sac*II and *Sal*I, and the 446 bp fragment containing the mutation point was exchanged with the same site of pYS1 [12]. The newly constructed plasmid was described as pYS1D(number)N as shown in Table 1. The entire DNA sequence of the exchanged fragment was sequenced by using the denatured double-stranded plasmid DNA as a template [16].

Expression of mutated NhaAv proteins in E. coli. The plasmid containing *nhaAv* gene, pYS1, was cut by *Bcl*I and *Xba*I, and then the 1.4 kb fragment was ligated between *Bam*HI and *Xba*I sites of pT7-5 [15] to produce pTYS1. To construct pTYS1 plasmid carrying mutated *nhaAv* gene, the *Sac*II and *Xba*I fragment (1.2 kb) was excised from pTYS1 and then replaced with the equivalent fragment from the mutated pYS1. *E. coli* BL21(DE3)pLysE cells carrying the mutated pTYS1 were grown in the M9 medium containing 34 μg chloramphenicol/ml and 75 μg ampicillin/ml with or without 0.4 mM isopropylthio β-D-galactoside (IPTG) for the induction of T7 RNA polymerase according to the manual of pET systems (Novagen). After the growth with shaking at 37° C for 2 h, 200 μg rifampicin/ml were added to inhibit the bacterial RNA polymerase. After 30 min incubation, 111 kBq [^{35}S]methionine/ml were added for the labeling of newly synthesized proteins. After incubation for another 30 min, the cells were collected by centrifugation and resuspended into 100 μl of the medium containing 1 mM EDTA, 1 mg lysozyme/ml and 10 mM Tris-HCl (pH 7.5). After 5 min on ice, proteinase inhibitors, 6-amino-n-hexanoic acid, benzamidine and phenylmethylsulfonyl fluoride, 1 mM each, were added before sonication [24]. Then, 100 μl of 130 mM Tris-HCl (pH 6.8) containing 2% 2-mercaptoethanol, 20% glycerol and 4% SDS were added. Samples were boiled for 3 min and centrifuged at 18000 × *g* for 30 s. An aliquot (60 μl) was loaded onto SDS-polyacrylamide gel (12.5%) electrophoresis according to Laemmli [25]. The gel was Coomassie brilliant blue stained, destained, treated with Amplify (Amersham), dried, and then exposed to autoradiography using Fuji RX film.

3. Results and discussion

3.1. Measurement of Na^+/H^+ antiporter activity

We previously reported that, in the absence of K^+, the extrusion of Na^+ from the Na^+-loaded cells of *V. alginolyticus* is induced by the addition of diethanolamine at alkaline external pH, which is mediated by an electrogenic

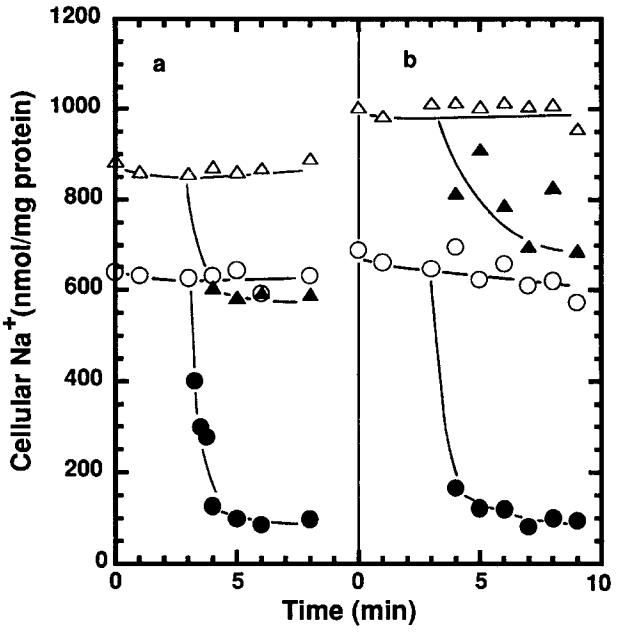

Fig. 1. Extrusion of Na^+ from the Na^+-loaded *E. coli* cells induced by diethanolamine. *E. coli* NM81 (a) or RS1 (b) were transformed with the control plasmid pHG165 (triangles) or pYS1 (circles) that contains *nhaAv* gene. The Na^+-loaded cells were suspended in the medium containing 0.15 M NaCl, 0.2% glucose and 20 mM EPPS-NaOH (pH 8.5) at 25° C. The cellular Na^+ was determined as described in Materials and Methods (open symbols). At 3 min, 50 mM diethanolamine-HCl (pH 8.5) was added and the cellular Na^+ was determined (filled symbols).

Na^+/H^+ antiporter [21]. In the absence of K^+ and at alkaline pH, the Na^+-loaded cells maintain a high inside-negative membrane potential ($\Delta\Psi$) and an inside-acidic H^+ gradient (ΔpH). By the addition of diethanolamine, a membrane-permeable amine, ΔpH transiently decreases and then returns to the original value. Under these conditions, the cellular Na^+ is extensively released from the cells in the presence of electrogenic Na^+/H^+ antiporters.

E. coli has two Na^+/H^+ antiporters, NhaA and NhaB. NhaA was shown to exchange 2 H^+ for each Na^+ [26]. Although the stoichiometry of NhaB was determined to be $3H^+/2Na^+$, NhaB was suggested to be unable to maintain a significant Na^+ gradient at alkaline pH [27]. Therefore, the measurement of antiporter activity at alkaline pH is appropriate for the detection of electrogenic NhaA activity. Since ChaA, a Ca^{2+}/H^+ antiporter, has been reported to have the capacity to extrude Na^+ at alkaline pH [28], we utilized two *E. coli* mutants, NM81 (*ΔnhaA*) and RS1 (*ΔnhaA*, *chaA*$^-$) for the expression of *nhaAv* gene from *V. alginolyticus*.

For the replacement of cellular cations with Na^+, we employed diethanolamine [17] as briefly described in Materials and methods. The cells of *E. coli* NM81 and RS1 transformed with the control plasmid, pHG165, were loaded with Na^+ at the level of 850 to 1000 nmol/mg protein, whereas the cells transformed with pYS1 carrying *nhaAv* gene were loaded with Na^+ at the level of 610 to 700 nmol/mg protein (Fig. 1). Since the loading of Na^+

proceeds in exchange for cellular H^+ [17,19], the presence of electrogenic Na^+/H^+ antiporter will cause a decrease in the extent of Na^+-loading.

By the addition of 50 mM diethanolamine at pH 8.5, *E. coli* NM81 and RS1, that are defective in NhaA, released about 250 to 300 nmol Na^+/mg protein, but the cellular Na^+ never decreased less than about 600 nmol/mg protein. As calculated from the cellular water space of 4.1 μl/mg protein, which was determined by the method described previously [20], the cellular Na^+ corresponded to 146 mM. Thus, the efflux of Na^+ observed here was according to the downhill release of Na^+ from the cells. On the other hand, the introduction of *nhaAv* gene to these cells allowed the extensive release of Na^+ from the cells against its chemical gradient (Fig. 1). At a new steady-state, the cellular Na^+ reached about 100 nmol/mg protein. Significant difference was not observed between NM81 and RS1. Thus, NhaB and ChaA hardly contributed to the extensive release of Na^+ under the experimental conditions. When the *nhaA* gene from *E. coli* was introduced to NM81, essentially the same results were obtained (data not shown).

Fig. 2 shows the effects of NaCN and *N*-ethyl-*N*-isopropylamiloride (EIPA) on the efflux of cellular Na^+. The addition of 10 mM NaCN, a respiratory inhibitor, or 1 mM EIPA, an amiloride analog, prevented the uphill extrusion of Na^+ from the Na^+-loaded cells carrying *nhaAv* gene (NM81/pYS1). These inhibitors, however, showed no significant effect on the downhill extrusion of Na^+ from NM81/pHG165. Since the addition of 10 mM NaCN abolished the generation of $\Delta\Psi$, inside negative, the extrusion of Na^+ by NhaAv was apparently dependent on the $\Delta\Psi$. The extrusion of Na^+ from NM81/pYS1 was not inhibited by amiloride up to 20 mM (data not shown). Although most prokaryotic Na^+/H^+ antiporters were re-

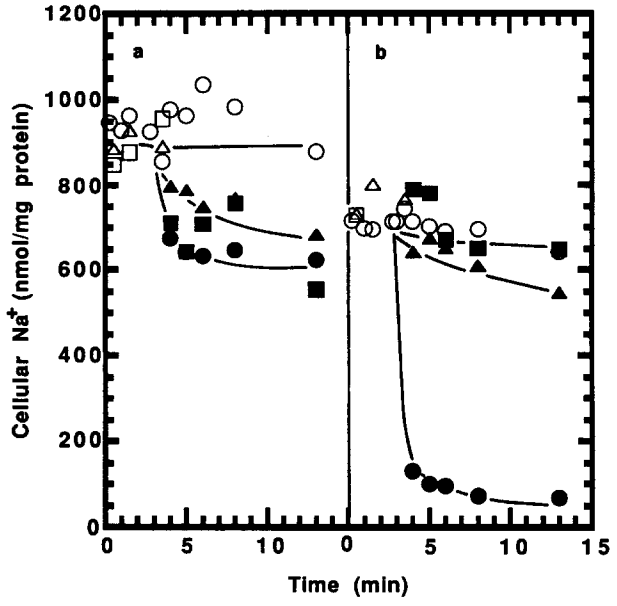

Fig. 2. Effect of NaCN and EIPA on the extrusion of Na^+ from the Na^+-loaded cells. The Na^+-loaded cells of NM81/pHG165 (a) or NM81/pYS1 (b) were suspended in the same medium as in Fig. 1 in the absence (circles), or the presence of 10 mM NaCN (triangles) or 1 mM EIPA (squares). After pre-incubation for 10 min at 25° C, the cellular Na^+ was determined (open symbols). At 3 min, 50 mM diethanolamine-HCl (pH 8.5) were added and the cellular Na^+ was determined (filled symbols).

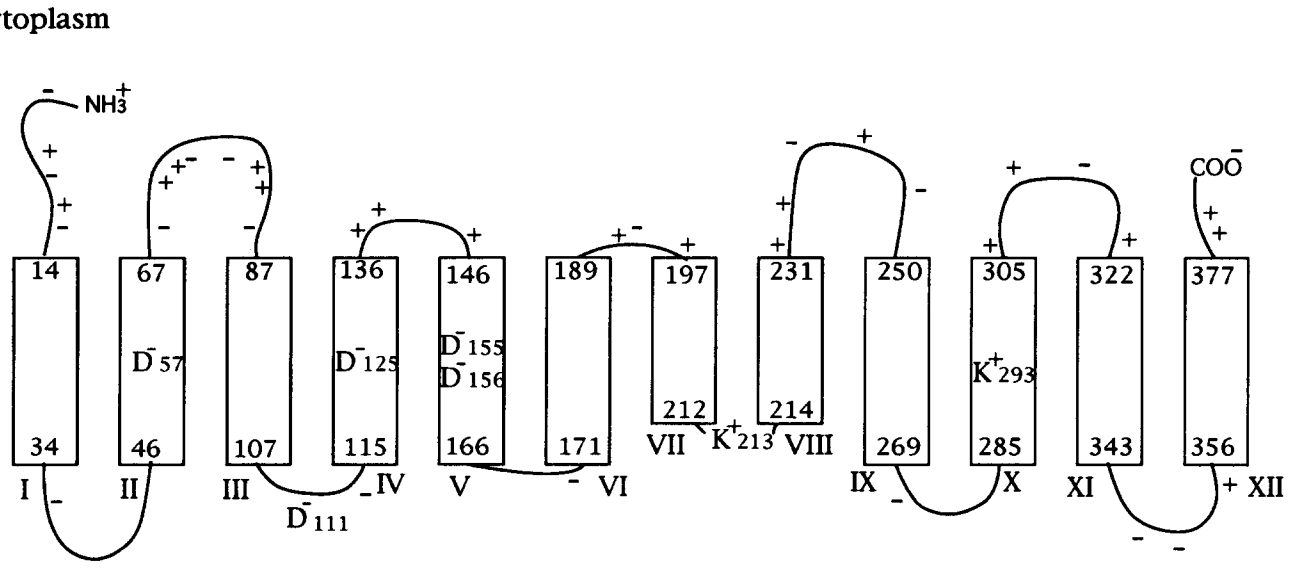

Fig. 3. Secondary structure model of NhaAv showing the positions of negative and positive charges. Predicted transmembrane helices (I–XII) are enclosed by boxes with the number of start and end residues. The single-letter amino acid code is used. The approximate sites of acidic or basic amino acid residues in the loop regions are indicated by − or + sign, respectively.

ported to be insensitive to amiloride and its derivatives [1,27], the activity of NhaAv was suggested to be sensitive to EIPA.

3.2. Secondary structure model of NhaAv

From the hydropathy of the deduced amino acid sequence of NhaAv protein, we previously predicted 11 putative membrane-spanning segments [12]. The number of putative transbilayer segments is not clear, especially at the region from Leu-197 to Leu-231. Although NhaA protein from *E. coli* was predicted to have 11 segments [13], we formulated a secondary structure model of NhaAv to have 12 segments (Fig. 3). NhaAv has 22 negative and 22 positive charges. Among them, only 4 negative and 1 positive charges were located in hydrophobic segments, and all these residues were conserved in NhaA from *E. coli* [6] and *S. entiritidis* [8]. In this model, 11 negative and 19 positive charges were located in the cytoplasmic, and 7 negative and 2 positive charges in the periplasmic face of membranes. Out of 18 negative and 21 positive charges in the loop regions, 11 negative and 13 positive charges were not conserved in NhaA of *E. coli*. From the conserved charged residues, we selected Asp-57, Asp-125, Asp-155 and Asp-156 in the hydrophobic regions, and Asp-111 in the loop region as a target of site-directed mutagenesis.

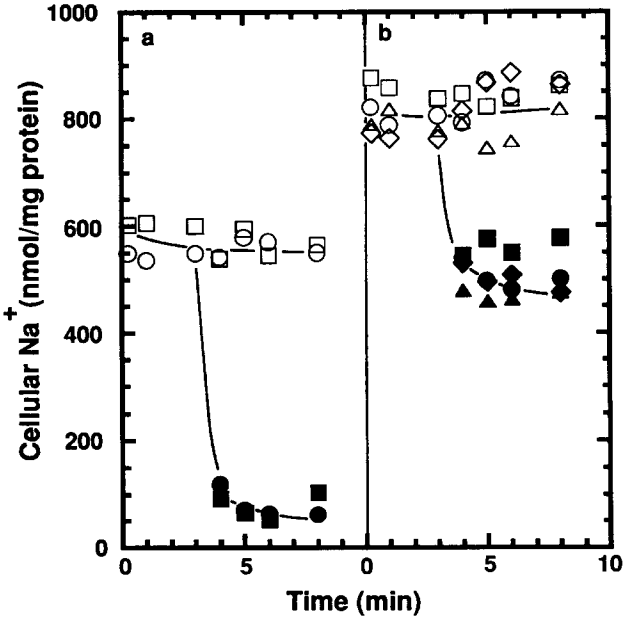

Fig. 4. Effect of diethanolamine on the extrusion of Na^+ from the Na^+-loaded NM81 cells carrying mutated plasmids. Plasmids tested were pYS1D57N (a, circles), pYS1D111N (a, squares), pYS1D125N (b, circles), pYS1D155N (b, squares), pYS1D156N (b, diamonds) and pYS1DDNN (b, triangles). The Na^+-loaded cells were suspended in the same buffer as in Fig. 1. At 3 min, 50 mM diethanolamine-HCl (pH 8.5) was added to induce Na^+ extrusion (filled symbols).

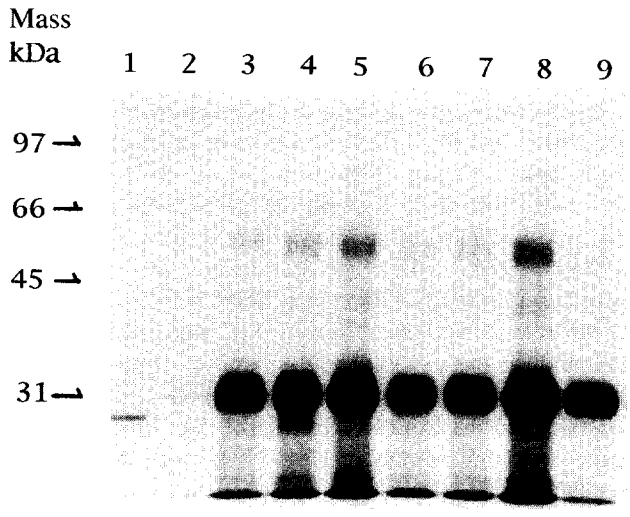

Fig. 5. Autoradiography of ^{35}S-labeled proteins after SDS-PAGE. The expression of NhaAv was performed as described in Materials and Methods. Plasmids tested were pT7-5 (lane 1), pTYS1 (lanes 2 and 3), pTYS1D57N (lane 4), pTYS1D111N (lane 5), pTYS1D125N (lane 6), pTYS1D155N (lane 7), pTYS1D156N (lane 8) and pTYS1DDNN (lane 9). Except for lane 2, T7 RNA polymerase was induced by IPTG. Each lane contains about 15 mg proteins.

3.3. Effect of the replacement of aspartic acid residue by asparagine

An individual mutation of Asp-57, -111, -125, -155 and -156, and a coupled mutation of Asp-155 and -156 to asparagine were performed by site-directed mutagenesis, and mutated plasmids were constructed as described in Table 1. These plasmids were transformed into NM81, and then the growth of the transformed cells in the LB-NaCl medium was examined. NM81 carrying pYS1 (wild type), pYS1D57N or pYS1D111N was able to grow in the LB-NaCl medium. However, NM81 carrying pYS1D125N, pYS1D155N, pYS1D156N or pYS1DDNN was unable to grow in the LB-NaCl medium.

Then, we measured the extrusion of Na^+ from the Na^+-loaded cells carrying each plasmid (Fig. 4). NM81 carrying pYS1, pYS1D57N or pYS1D111N could extensively extrude the cellular Na^+, whereas those carrying pYS1D125N, pYS1D155N, pYS1D156N or pYS1DDNN could not. These results clearly show that the conserved negative charges of D57 and D111 are not important, whereas those of D125, D155 and D156 play an important role in the activity of NhaAv.

To examine the expression of *nhaAv* gene and the mutated genes in *E. coli*, these genes were ligated to T7 expression vector pT7-5, and the plasmid pTYS1 and those carrying mutated genes were constructed as described in Materials and Methods. pTYS1 contains a promoter region of *nhaAv* just down stream of T7 promoter gene. The plasmids, pTYS1, pTYS1D57N and pTYS1D111N re-

stored the growth of NM81 in the LB-NaCl medium. On the other hand, pTYS1D125N, pTYS1D155N, pTYS1D156N and pTYS1DDNN could not restore the growth of NM81 in the LB-NaCl medium. The expression of wild and mutated *nhaAv* genes was detected in *E. coli* BL21(DE3)pLysE as described in Materials and Methods. As shown in Fig. 5, all T7 RNA polymerase-induced cells carrying wild or mutated *nhaAv* genes produced a labeled protein with 31 kDa. This protein was not detected from the cells without *nhaAv* gene (lane 1) or the cells where T7 RNA polymerase was not induced (lane 2). The reason for the appearance of a minor band of higher molecular weight in the lanes 4, 5 and 8 is unknown, but several bands of lower molecular weights may be some degradation products from NhaAv. Thus, all the plasmids carrying *nhaAv* genes are able to produce NhaAv proteins in *E. coli*.

When concentrated cell suspensions of *E. coli* NM81 carrying pYS1D125N, pYS1D155N or pYS1D156N were spread on the LB-NaCl agar plates and then incubated at 37° C for overnight, 4, 5, or 4 colonies, respectively, could be detected. Plasmids were isolated from these independent 13 colonies. The isolated plasmids were confirmed to allow the growth of NM81 in the LB-NaCl medium, indicating that they were spontaneous revertants. Then, the nucleotide sequences of all revertants were determined. In all cases, the first position of genetic code for Asn residue converted by the site-directed mutagenesis, that is A, was found to be reversed to G by a point mutation to produce the conversion of Asn to the original Asp. These results further support the importance of Asp-125, Asp-155 and Asp-156 in the activity of the electrogenic Na^+/H^+ antiporter, NhaAv. These residues are conserved in NhaA from *E. coli* [6], *S. entiritidis* [8] and *V. parahaemolyticus* [29]. These acidic residues may be considered as candidates for the cation binding site(s).

Although Na^+/H^+ antiporters generally share very limited homology [2], similar patterns of negative charges were observed in membrane-spanning regions of some antiporters. The Na^+/H^+ antiporter from *Enterococcus hirae* (NapA) has Asp-153 and Asp-154 in the predicted transmembrane helix VI, and Glu-184 in the next helix VII [9]. The Na^+/H^+ antiporter protein that confers Na^+ and Li^+ tolerance in fission yeast (Sod2) has Glu-173 and Asp-178 in helix VI, and Glu-211 in the next helix VII [30]. The NhaB and ChaA of *E. coli* have Glu-85 in helix III [10,11]. The Na^+/H^+ antiporter from *Bacillus firmus* (NhaC) has Asp-86 [7] in helix III. These acidic residues can be related to their antiporter activities.

Besides Na^+/H^+ antiporters, there are several excellent papers that demonstrate the importance of negative charges in the membrane-spanning segments for cation-coupled transporters. They are sarcoplasmic reticulum Ca^{2+}-ATPase [31], metal-tetracycline/H^+ antiporter of encoded by transposon Tn 10 (TetA(B)) [32,33], bacteriorhodopsin that functions as a light-driven H^+ pump in *Halobacterium halobium* [34], melibiose carrier (MelB) that cotransports melibiose and H^+, Na^+ or Li^+ [35,36]. In these transporters, negative charges are considered to construct a cation pathway across the membranes. Some of them (MelB and bacteriorhodopsin) have a distribution pattern of essential negative charges in the transmembrane helices very similar to that observed in NhaAv, that is, one negative charge in one helix and two negative charges in the next helix. Further studies are required to define the specific role of these residues in the electrogenic Na^+/H^+ antiporter, NhaAv and NhaA.

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