Revealing the Ligand Binding Site of NhaA Na⁺/H⁺ Antiporter and Its pH Dependence*

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Background: Cell pH and Na⁺ homeostasis requires Na⁺/H⁺ antiporters such as NhaA. pH and Na⁺ homeostasis in all cells requires Na⁺/H⁺ antiporters. In most cases, their activity is tightly pH-regulated. NhaA, the main antiporter of Escherichia coli, has homologues in all biological kingdoms. The crystal structure of NhaA provided insights into the mechanism of action and pH regulation of an antiporter. However, the active site of NhaA remained elusive because neither Na⁺ nor Li⁺, the NhaA ligands, were observed in the structure. Using isothermal titration calorimetry, we show that purified NhaA binds Li⁺ in detergent micelles. This interaction is driven by an increase in enthalpy (ΔH of −8000 ± 300 cal/mol and ΔS of −15.2 cal/mol/degree at 283 K), involves a single binding site per NhaA molecule, and is highly specific and drastically dependent on pH; Li⁺ binding was observed only at pH 8.5. Combining mutational analysis with the isothermal titration calorimetry measurements revealed that Asp-163, Asp-164, Thr-132, and Asp-133 form the Li⁺ binding site. The pH-dependent NhaA-ligand binding is an insight into the mechanism of activity of NhaA and possibly other antiporters.

Living cells are critically dependent on processes that regulate intracellular pH, Na⁺ content, and volume. Na⁺/H⁺ antiporters play a primary role in these homeostasis mechanisms (Ref. 1; see recent reviews in Refs. 2–4). Accordingly, Na⁺/H⁺ antiporters are found in the cytoplasmic and organelle membranes of cells throughout the biological kingdoms and have long been drug targets in humans (5). They were classified in a huge super-CPA (cation/proton antiporter) family that includes CPA1 and CPA2 (Transporter Classification data-base). Ec-NhaA (henceforth, NhaA) is the main Na⁺/H⁺ antiporter that is responsible for pH and Na⁺ homeostasis in Escherichia coli (2). It belongs to the CPA2 family with many homologues in enterobacteria and orthologues in eukaryotes including humans (6). NHA2 has been suggested to play a pivotal role in human essential hypertension (7).

NhaA is the most extensively studied Na⁺/H⁺ antiporter. Several biochemical characteristics of NhaA underpin its physiological roles: very high turnover (8), electrogenicity with a stoichiometry of 2H⁺/Na⁺ (2, 9), and a dramatic pH dependence, a property it shares with many prokaryotic (2, 8) and eukaryotic (see recent review in Ref. 10) Na⁺/H⁺ antiporters. NhaA is not active below pH 6.5 and increases its rate over 3 orders of magnitude between pH 7.0 and pH 8.5 (2, 8).

The crystal structure of the acid pH-down-regulated NhaA (11) (Fig. 1A) has provided structural insights into the mechanism of antiport and pH regulation of a Na⁺/H⁺ antiporter (3). The NhaA structure, consisting of 12 TMs with the N and C termini pointing into the cytoplasm, revealed a novel fold (11, 12); TMs III, IV and V are topologically inverted with respect to TMs X, XI, and XII. In each repeat, one TM (TM IV/XI assembly) is interrupted by an extended chain in the middle of the membrane (Fig. 1, A–C). The short interrupted helices are denoted as follows: p, at the periplasmic side, and c, at the cytoplasmic side (Fig. 1). Furthermore, because helices Ivc and Xlc are oriented in an antiparallel manner, their partially positive N termini face each other, and the charge is compensated by Asp-133 (Fig. 1C). The negative partial dipoles of the C termini of the two other small helices (IVp and Xlc) are compensated by Lys-300 (Fig. 1C). Hence, TMs IV/XI assembly creates a delicately balanced electrostatic environment in the middle of the membrane and plays a critical role in the cation exchange activity of the antiporter (11, 13). Structures of other bacterial ion-coupled secondary transporters, which share little or no sequence homology, have been determined since the structure of NhaA was determined. Their structural fold also includes inverted

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4 The abbreviations used are: TM, transmembrane segment; ITC, isothermal titration calorimetry; DDM, n-dodecyl-β-D-maltopyranoside; LB, L broth; BTP, 1,3-bis(tris(hydroxymethyl)methylamino)propane.
Here, we used isothermal titration calorimetry (ITC) combined with mutagenesis and biochemical analysis to explore the binding of Na\(^+\) / Li\(^+\) to wild type NhaA and NhaA variants purified in detergent micelles. We revealed the thermodynamics of Li\(^+\) binding to NhaA; the high specificity, stoichiometry (1 Na\(^+\) / 1 Li\(^+\) / NhaA), and drastic pH dependence of binding; and that Asp-163, Asp-164, Thr-132, and Asp-133 form the binding site, whereas Lys-300 plays a major role in the pH regulation of NhaA.

**EXPERIMENTAL PROCEDURES**

**Plasmid, Bacterial Strains, and Culture Conditions**—TA16 (melBlid, ΔlacZY, thr\(_1\), and lacI\(^Q\) (8)) and EP432 (melBlid, ΔnhaA1::kan, ΔnhaB1::cat, ΔlacZY, and thr\(_1\) (21)) are *E. coli* K-12 derivatives. Cells were grown either in L broth (LB) or in modified L broth in which NaCl was replaced with KCl (LBK (22)). The medium was buffered with 60 mM BTP, and 100 μg/ml ampicillin was added. For plates, 1.5% agar was used. To test the resistance to Li\(^+\) and Na\(^+\), EP432 cells transformed with the respective plasmids were grown on LBK to A\(_{600}\) of 0.5. Samples (2 μl) of serial 10-fold dilutions of the cultures were spotted onto agar plates containing the indicated concentrations of NaCl or LiCl at the various pHs and incubated for 2 days.

Plasmids pAXH (23) and pAXH3 (24) are pET20b (Novagen) derivatives encoding His-tagged NhaA. The plasmids pAXH-D163C, pAXH-D164C, and pAXH-D133 (25) and pAXH-T132C (20) encode the NhaA mutations D163C, D164C, D133C, and T132C, respectively. pAXH3 lacks the BglII site at position 3382 (20) and contains a BstXI silent site at position 248 in nhaA (26). All plasmids carrying mutations are designated by the name of the plasmid followed by the mutation.

**Site-directed Mutagenesis**—Site-directed mutagenesis was conducted following Ref. 27 with pAXH3 as a template.

**Isolation of Membrane Vesicles and Assay of Na\(^+\)/H\(^+\) Antiporter Activity**—EP432 cells transformed with the respective plasmids were grown, and everted vesicles were prepared and used to determine the Na\(^+\)/H\(^+\) or Li\(^+\)/H\(^+\) antiporter activity as described (28, 29). The assay of antiporter activity was based upon the measurement of Na\(^+\) - or Li\(^+\) -induced changes in the ΔpH as measured by acridine orange, a fluorescent probe of ΔpH. The fluorescence assay (see Figs. 2 and 3) was performed with 2.5 ml of reaction mixture containing 50–100 μg of membrane protein, 0.5 μM acridine orange, 150 mM choline chloride, 50 mM BTP, 5 mM MgCl\(_2\), and the pH was titrated with HCl. After energization with d-lactate (2 mM), quenching of the fluorescence was allowed to achieve a steady state, and then either Na\(^+\) or Li\(^+\) was added. A reversal of the fluorescence level (dequenching) indicates that protons are exiting the vesicles in antiporter with either Na\(^+\) or Li\(^+\). As shown previously, the end level of dequenching is a good estimate of the antiporter activity (30), and the concentration of the ion that gives half-maximal
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**TABLE 1**

Growth and membrane phenotype of NhaA variants

For characterization of the mutants, EP432 cells transformed with the plasmids carrying the indicated mutations were used. Growth experiments were conducted on agar plates with high Na⁺ (0.6 M) or high Li⁺ (0.1 M) at the pH values indicated in parentheses. (+ + +), the number and size of the colonies after 24 h of incubation at 37 °C was identical to that of the wild type; (+), the number of colonies was much lower than that of the wild type; (−), no growth. Na⁺/H⁺ and Li⁺/H⁺ antiport activity at pH 8.5 was determined with 10 mM NaCl or LiCl. The activity (maximal level of dequenching) is expressed as the percentage of the positive control, EP432/pAXH3. EP432/pBR322 served as a negative control. The experiments were repeated three times with essentially identical results. The data are taken from Ref. 17 (*) or from Ref. 20 (**).

| Mutation | Expression | Growth | Activity | Apparent $K_m$ |
|----------|------------|--------|----------|----------------|
|          |            | Na⁺ (7.0) | Na⁺ (8.3) | Li⁺ (7.0) | Na⁺ Li⁺ | Na⁺ Li⁺ |
| K300R    | 60         | + + +  | -        | + + +    | 36 | 93 | 21.8 | 0.8 |
| k300H    | 64         | + + +  | +        | + + +    | 52 | 88 | 7.5 | 0.24 |
| K300C    | 50         | -      | -        | -        | -  | -  | -   | - |
| K300E*   | 15         | -      | -        | -        | -  | -  | -   | - |
| D163C    | 90         | -      | -        | -        | -  | -  | -   | - |
| D164C    | 90         | -      | -        | -        | -  | -  | -   | - |
| T132C**  | 95         | + + +  | + + +    | + + +    | 90 | 95 | 12.4 | 0.7 |
| D133C**  | 98         | + + +  | + + +    | + + +    | 91 | 84 | 3.6 | 1.24 |
| pAXH3 (WT) | 100      | + + +  | + + +    | + + +    | 100| 100| 0.2 | 0.02 |
| pBR322   | -          | -      | -        | -        | -  | -  | -   | - |

Dequenching is a good estimate of the apparent $K_m$ of the antiporter (30, 31). The concentration range of the cations tested was 0.01–100 mM at the indicated pHs, and the apparent $K_m$ values were calculated by linear regression of a Lineweaver-Burk plot.

Overexpression and Purification of NhaA Protein Variants—Overexpression of the NhaA variants (32) and affinity purification (Ni²⁺-nitrilotriacetic acid-agarose, Qiagen) (33) were performed as described previously, but the protein was eluted in a buffer containing 300 mM imidazole, 25 mM citric acid, 100 mM KCl, 5 mM MgCl₂, and 0.015% n-dodecyl β-D-maltopyranoside (DDM) (final pH was 7.9). After the addition of sucrose (10%) to the eluted protein solution, the protein solution was dialyzed (DDM) (final pH was 7.9). After the addition of sucrose (10%) to the eluted protein solution, the protein solution was dialyzed overnight at 4 °C in acidic elution buffer (23) containing 10% sucrose, 100 mM KCl, 25 mM citric acid, 5 mM MgCl₂, 0.015% DDM (pH 4.0) and frozen at −80 °C.

Detection and Quantification of NhaA and Its Variants in the Membrane—Total membrane protein was determined according to Ref. 34. The expression level of His-tagged NhaA variants was determined by resolving the Ni²⁺-nitrilotriacetic acid purified proteins on SDS-PAGE, staining the gels by Coomassie Blue staining, and quantifying the band densities by Image Gauge (Fuji) software (23).

**ITC**—The NhaA variants were prepared for ITC by melting the respective frozen protein (about 0.7 ml containing ~650 μg of protein) at 4 °C, concentrating it 2.5-fold by filtration (Amicon Ultra, 30K), and washing three times with the original volume of the reaction buffer containing 50 mM BTP, 150 mM choline chloride, 5 mM MgCl₂, 10% sucrose (pH 8.5). These washing cycles yielded a reaction mixture for ITC, containing 40–50 μM of the protein and 0.04% DDM. ITC experiments were performed using the microcalorimeter ITC200 (MicroCal, GE Healthcare). A reaction mixture for ITC (300 μl) of wild type NhaA or its variants was loaded into the sample cell. For titration, 40 mM LiCl (dissolved in the ITC reaction buffer) was loaded into the injection syringe. Before data collection, the system was equilibrated to 10 °C with the stirring speed set to 500 rpm. Titration curves for binding Li⁺ were initiated by injection of 0.8 μl followed by successive 2-μl injections of the ligand every 200 s. Injections of ligands into reaction buffer without protein or reaction buffer into reaction buffer were performed to determine background corrections. The integrated heats from each injection, normalized to the moles of ligand per injection, were fit to a single-site binding isotherm using ORIGIN 7. The integrated peak of the first injection was excluded from the fit due to the large errors in the first step.

**RESULTS**

In this study, we identified the binding site of NhaA Na⁺/H⁺ antiporter using mutagenesis combined with ITC measurements.

Construction of Mutants Impaired in the Putative NhaA Binding Site—Several previously isolated mutants were suggested to be impaired in the cation binding site of NhaA on the basis of their compromised functionality, location in the pH 4.0 crystal structure, evolutionary conservation analysis, and computation. These were Cys replacements: D163C, D164C (18, 20), T132C and D133C (20), and K300C (17) (Table 1). To test the expression and antiporter activity, all NhaA mutations were transferred to pAXH3 to avoid effects due to different vectors and were characterized in EP432, an *E. coli* strain that lacks the two specific Na⁺/H⁺ antiporters (NhaA and NhaB) (21). This host strain can therefore grow on the nonselective medium (LBK, LB in which NaCl is replaced by KCl) but cannot grow on high salt-selective media (0.6 M NaCl at pH 7.0 or pH 8.3, or 0.1 M LiCl at pH 7.0) unless transformed with wild type NhaA. The Na⁺/H⁺ and Li⁺/H⁺ antiport activity of all variants was determined in everted membrane vesicles isolated from the respective transformants using acidine orange, a fluorescent probe of ΔpH. EP432 transformed with plasmid pAXH3 encoding wild type NhaA (Fig. 2, WT and Table 1) or with pBR322, the vector plasmid (data not shown), served as positive and negative controls, respectively. The apparent $K_m$ values for Na⁺ and Li⁺ at pH 8.5 and the extent of activity (maximal dequenching) at pH 8.5 were determined for each mutant (Table 1).

Variants D163C and D164C allowed growth of EP432 only on the nonselective medium (18, 20) (Table 1). In addition, they did not exhibit any Na⁺/H⁺ antiporter activity in everted membrane vesicles (Table 1). Variants T132C and D133C allowed growth on the salt-selective media but showed a much higher apparent $K_m$ for Li⁺ (0.7 and 1.24 mM, respectively) and for Na⁺ (12.4 and 3.6 mM, respectively) antiporter activity as compared
with the WT (0.02 and 0.2 mM, respectively) (20) (Table 1) in isolated membrane vesicles. The substantial antipporter activity implies that the mutants maintain the NhaA structure. These variants were well expressed in the membrane (Table 1), so we could easily purify the proteins for the ITC measurements.

With respect to Lys-300, we could not use the previously isolated mutants (K300E and K300C (17)) because the expression of the former was very low (Table 1) and the apparent $K_m$ for Li$^+$ of the latter was very high (4 mM, Table 1), reducing the possibility to get significant ITC signals. We therefore constructed novel Lys-300 mutants, K300H and K300R, and characterized them biochemically (Table 1).

**Growth and Membrane Phenotypes of Variants K300H and K300R—**EP432 transformed with either K300R and K300H grew on both Na$^+$-selective and Li$^+$-selective agar plates at pH 7.0, but only K300H grew on the Na$^+$-selective agar plates at pH 8.3, albeit less efficiently than the wild type (Table 1). The Na$^+$/H$^+$ and Li$^+$/H$^+$ antipporter activity of these variants is shown in Fig. 2 and summarized in Table 1. The apparent $K_m$ values of K300H and K300R with both substrates were higher by an order of magnitude as compared with Lys-300 (7.5 and 21.8 mM versus 0.2 mM with NaCl; 0.2 and 0.8 mM versus 0.02 mM with LiCl, respectively, Table 1). These results show that the residue at position 300 of TM domain X is important for the native NhaA antipporter activity.

**The Effect of the NhaA Variants on the pH Regulation of NhaA—**We have previously defined two types of mutations with respect to the pH response of NhaA (20, 35). When measured at saturating concentrations of substrate, the first type of mutation shows a pH dependence very similar to that of the wild type. In contrast, the second type of mutation retains an abnormal pH dependence of the Na$^+$/H$^+$ or Li$^+$/H$^+$ exchange activity, even at saturating concentrations of the ion. It has been shown previously that variants T132C and D133C show a pH dependence similar to that of the wild type (20). The pH dependence of the antipporter activity of variants K300H and K300R with 10 and 100 mM of both substrates is shown in Fig. 3. As expected from its high apparent $K_m$, the pH profile of the membrane activity of K300H at saturating Na$^+$ (100 mM NaCl) was similar to that of the wild type. Hence, the pH profile of K300H is hardly affected by the His replacement. Remarkably, in contrast to both Lys-300 and K300H, the pH profile of K300R was shifted dramatically to the alkaline side by one pH unit (Figs. 2 and 3) in the presence of both 10 and 100 mM NaCl. Hence, Lys-300 plays a role in both the antipporter activity and the pH response of NhaA.

**Thermodynamics and Stoichiometry of Li$^+$ Binding to Wild Type NhaA—**We measured the thermodynamics of Li$^+$/Na$^+$ binding to purified wild type NhaA in DDM micelles using ITC. This technique allows the direct measurement of changes in free energy ($\Delta G$), enthalpy ($\Delta H$), entropic free energy component ($\Delta S$), and heat capacity ($\Delta C_P$). To minimize the potential for artifacts in our binding assays arising from contaminating Na$^+$ co-purified with NhaA, we extensively washed the protein in the reaction buffer, which is practically Na$^+$-free (<1 mM Na$^+$ as determined by atomic absorption).

Li$^+$ binding to the wild type NhaA at 10 °C is shown in Fig. 4A. The ITC binding isotherms fit best to a single-site model with a fixed protein-to-Li$^+$ stoichiometry $n = 1$. This resulted in the dissociation constant, $K_d$, of 1.2 ± 0.1 mM, enthalpy ($\Delta H$) of $-8.0 \pm 0.3$ kcal/mol, and entropy ($\Delta S$) of $-15.2$ cal/mol/degree (Table 2). Hence, Li binding to NhaA is thus driven entirely by $\Delta H$. Very similar results were obtained at 25 °C (data not shown).

**Thermodynamic binding models of higher complexity describing two sites random or sequential binding modes yielded poorer fits to the data, with either high $\chi^2$ values or nonconverging parameters. The data are consistent with a single-substrate binding site per NhaA molecule. However, we have no way to directly determine the percentage of active/available protein for ion binding.**

Unfortunately, we did not get ITC signals with Na$^+$, suggesting that the binding affinity of Li$^+$ to NhaA is much tighter than that of Na$^+$ in the detergent micelles. This difference is in line with the 10-fold difference in apparent $K_m$ for the cations (0.2–
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FIGURE 4. NhaA binds specifically to Li$^+$ in a pH-dependent manner. ITC was conducted with WT NhaA purified in DDM and prepared for ITC as described under “Experimental Procedures.” The reaction mixture of 300 μM wild type NhaA protein (30–40 μM, 0.04% DDM, 50 mM BTP, 150 mM choline chloride, 5 mM MgCl$_2$, 10% sucrose at the indicated pHs. The injection steps were 2 μL every 200 s. Top panels show the heat change during injection of 40 mM LiCl into a reaction mixture containing 40 mM KCl, implying that NhaA is highly specific to Li$^+$ and not to K$^+$.

**TABLE 2** Thermodynamics of Li$^+$ binding to NhaA and variant D133C

|                | Wild type NhaA | NhaA D133C |
|----------------|----------------|------------|
| $K_r$ (1/mM)   | 790 ± 40       | 160 ± 20   |
| $K_f$ (mM)     | 1.2 ± 0.1      | 6.3 ± 0.8  |
| $\Delta H$ (kcal/mol) | −8.0 ± 0.3   | −9.4 ± 0.7 |
| $\Delta S$ (cal/mol/degree) | −15.2        | −23.2     |
| $\Delta G$ (kcal/mol) | −6.3         | −6.5       |

0.5 mM Na$^+$ and 0.02–0.03 mM Li$^+$) found in isolated membrane vesicles (Table 1) and liposomes (8, 36).

The Cation Specificity of the NhaA Protein—To measure the cation specificity of NhaA, we used the same procedure described in Fig. 4A but with 40 mM KCl (analytically pure) for the binding assay (Fig. 4B). No heat signals were observed with KCl, implying that NhaA is highly specific to Li$^+$.

The pH Dependence of Li$^+$ Binding to NhaA—The Li$^+/Na^+/H^+$ antiport activity of NhaA is tightly pH-regulated, as observed in isolated membrane vesicles and liposomes (reviewed in Ref. 2). NhaA is inactive below pH 6.5 and increases its activity by 3 orders of magnitude between pH 6.5 and pH 8.5 (8). Therefore, the question arises whether the pH dependence of activity is a reflection of the pH dependence of the ligand binding to NhaA or a reaction following binding. To answer this question, we measured the pH dependence of Li$^+$ binding to NhaA in different ITC reaction buffers with pH values of 6.5, 7.0, 7.5 (not shown), 8.0, 8.5, and 9.0 (Fig. 4A, C, and D). However, binding was observed only at pH 8.5 (Fig. 4A). These results imply that ligand binding to NhaA is extremely sensitive to pH.

**Thermodynamic Properties of Li$^+$ Binding to NhaA Variants**—The determination of the experimental conditions suitable for measuring the thermodynamics of Li$^+$ binding to wild type NhaA protein paved the way to use ITC for identifying the NhaA binding site. The Li$^+$ binding capacity of NhaA variants bearing mutations in the putative cation binding site as compared with that of the wild type was determined by ITC. To determine the Li$^+$ binding capacity of the variants proteins (D163C, D164C, T132C, D133C, K300H, and K300R) using ITC, we purified the respective proteins in DDM and obtained a yield at least 60% of that of the wild type (Table 1). The ITC measurements were conducted as described in Fig. 4. The results show that although WT NhaA bound Li$^+$ (Fig. 4A and Table 2), variants D163C (Fig. 5B) and D164C (data not shown) did not show any ligand binding under the same reaction conditions. These results strongly support the contention that residues Asp-163 and Asp-164 are part of the NhaA cation binding site.

Variant D133C bound Li$^+$ (Fig. 5A and Table 2) with the following thermodynamic parameters: $K_r$ of 6.3 ± 0.8 mM, $\Delta H$ of $−9.4 ± 0.7$ kcal/mol, entropy $\Delta S = −23.2$ cal/mol/degree, and $T\Delta S = −6.5$ kcal/mol. Overall $\Delta G$ of the mutant binding is within the experimental error range of the value for the wild type (Table 2). However, the Li$^+$ binding affinity of D133C is 5-fold lower because its entropy change is somewhat higher than that of the wild type, whereas the enthalpy is very similar between the two (Table 2). These differences can account for...
the high apparent $K_m$ for Li$^+$ of D133C as compared with the wild type (Table 1). In contrast to D133C, variant T132C did not show any binding of Li$^+$ in the ITC assay. It is possible that in addition to low binding affinity, the mutation affects the stability of the protein so that when purified from the membrane in DDM, it lost its binding capacity. Unfortunately, we could not test K300H because the protein aggregated. K300R gave just a hint of binding only at pH 9.0, but no quantitative analysis could be done. This is not surprising because of the very high $K_m$ of K300R (Table 1). Taken together, we conclude that the Li$^+$ binding site of NhaA is formed by Asp-163, Asp-164, Asp-133, and Thr-132 and that lys-300 dramatically affects the pH response of NhaA in addition to being involved in its antiporter activity.

**DISCUSSION**

**Thermodynamics of Ligand Binding to NhaA and Functional Implications**—The ITC study of ligand binding to purified wild type NhaA in DDM micelles revealed the thermodynamics of Li$^+$ binding to the protein (Fig. 4 and Table 2). The change of free energy of binding ($\Delta G$) is due to an increase in enthalpy ($\Delta H$), which is twice that of the entropic free energy component ($T\Delta S$). The large negative value for $\Delta H$ may suggest tight packing upon ligand binding; a specific conformer of NhaA is selected from the dynamic ensemble of conformations upon ligand binding. Similar binding assays of galactoside-ligands to the symporter LacY gave opposite results (37), almost no ligand binding. Similar binding assays of galactoside-ligands to the symporter LacY gave opposite results (37), almost no ligand binding. Similar binding assays of galactoside-ligands to the symporter LacY gave opposite results (37), almost no ligand binding. Similar binding assays of galactoside-ligands to the symporter LacY gave opposite results (37), almost no ligand binding.

Previous biochemical (8, 36) and electrophysiological (38) transport studies in isolated membrane vesicles and liposomes showed that NhaA is highly specific to Li$^+$ and Na$^+$; no other ions were shown to be transported by NhaA. However, the apparent $K_m$ for Na$^+$ (0.2–0.5 mM) is 10-fold higher than the apparent $K_m$ for Li$^+$ (0.02–0.03 mM) (8, 36) (Table 1). In line with the high specificity of NhaA observed under physiological conditions, the *in vitro* ITC study showed no binding with KCl (concentration range in the reaction mixture of 0.3–6.6 mM), whereas Li$^+$ gave very reproducible signals (Fig. 4). However, we could not observe any ITC signals with Na$^+$ because, unlike in a helix, this oxygen is not fully saturated with internal hydrogen bonds. Therefore, a replacement of such residues may still provide the peptide bond carbonyl oxygen to coordinate Na$^+$.

We suggest that the Cys replacements, T132C and D133C, are such replacement variants most probably providing polar backbone groups to coordinate Li$^+$. Indeed, their Cys replacements still grow on certain selective media and show substantial antiporter activity, although with an apparent $K_m$ about 10-fold higher than that of the wild type. This can be attributed to a structural/functional effect of the new side chain. Although the ITC results with T132C could not identify any Li$^+$ binding signal, most probably due to a high $K_d$ variant D133C showed a clear Li$^+$ binding (Fig. 5A). The $\Delta G$ of Li$^+$ binding to D133C is similar to that of the wild type (Table 2) but with a higher pro...
portion of $\Delta S$, suggesting that the number of conformers for ligand-bound D133C is very likely greater than that for the unliganded protein, resulting in an increase in the entropic free energy component upon ligand binding.

A Positive Charge at Position Lys-300 Is Required for Efficient Antiport Activity—A critical role of the positive charge of Lys-300 in the activity of NhaA has been predicted on the basis of the crystal structure at pH 4.0 (11), evolutionary conservation, mutagenesis (17), and computation (43). Lys-300 resides in the vicinity of the C termini of the interrupted helices, IVp and XIc of the TMS IV XI assembly (Fig. 1), and therefore can compensate for their partial negatively charged dipoles, a characteristic of the C termini of helices. The role of the positive charge of Lys-300 was explored here by replacements K300H and K300R, potentially positively charged residues assumed to have different $p_K_a$ values. This assumption was supported by a recent systematic study of Asp, Glu, Lys, and Arg residues at 25 internal positions in staphylococcal nuclease (44). This study showed that although the $p_K_a$ values of Asp, Glu, and Lys can be highly anomalous and shift as many as 5.7 pH units relative to their $p_K_a$ values in water, Arg residues at the same position exhibit no detectable shifts in $p_K_a$ and remained charged at pH ≤ 10. This unique capacity of Arg side chains to retain their charge in dehydrated environments likely contributes to the important functional roles of internal Arg in situations where a charge is needed in the interior of a protein, in a lipid bilayer, or in similarly hydrophobic environments. On the basis of this analysis, we considered the possibility that the $p_K_a$ values of wild type NhaA (Lys-300) and its variant K300H is in the physiological range, whereas the $p_K_a$ of variant K300R is in the more extreme alkaline range. We therefore compared the physiology of K300H and K300R with known acidic or neutral replacements of Lys-300.

The variants K300R and K300H retained both Na\(^+\)/H\(^+\) antiporter activity (52 and 36%, respectively) and Li\(^+\)/H\(^+\) activity (88 and 93%, respectively) at pH 8.5 (Table 1), whereas a negatively charged replacement at this position was shown previously to inactivate the antiporter both in E. coli (K300E) (17) and in Helicobacter pylori (HpNhaA K347E) (31). HpNhaA is a close homologue of EcNhaA (45) with a protein sequence similarity of 82% and identity of 49%. Hence, a positive charge at position 300 is essential for the NhaA activity.

Nevertheless, although the maximal rates of the Na\(^+\), Li\(^+\)/H\(^+\) exchange of K300H and K300R variants were similar to the wild type (Figs. 2 and 3) at pH 8.5, their apparent $K_m$ values were 10-fold higher than that of the WT with both substrates (Table 1). Unfortunately, we could not observe Li\(^+\) binding to K300H by ITC because the purified protein in DDM micelles aggregated. In the case of K300R, we observed significant but small signals at pH 9.0 that were not enough for quantification, most probably because of the very high apparent $K_m$ for Li\(^+\) of the variant (Table 1). Taken together, there are further subtleties than just charge $p_e$ at position 300 that are important for the mechanism of the antiporter.

The $p_K_a$ of Lys-300 Is Involved in the pH Dependence of NhaA Ligand Binding—NhaA is strictly regulated by pH in membrane vesicles and proteoliposomes (reviewed in Ref. 2). In the present work, we revealed that the binding of Li\(^+\) to NhaA in detergent micelles is also very sensitive to pH; ITC signals of Li\(^+\) binding to NhaA were observed only at pH 8.5 (Fig. 4), the optimal pH of activity in membrane vesicles and liposomes (8). However, the pH sensitivity of Li\(^+\) binding to pure NhaA in detergent micelles was more drastic than in membranes; no ITC signals were observed below or above pH 8.5, whereas the pH profile in membrane vesicles and liposomes progressively increases between pH 6.5 and 8.5 (Fig. 3) (8). We also revealed here that in contrast to variants T132C, D133C (35), and K300H, which had no effect on the pH response of NhaA in isolated membrane vesicles (Fig. 3), variant K300R caused a dramatic alkaline shift, of one pH unit, in the pH profile of NhaA (Figs. 2 and 3). Furthermore, K300R protein in DDM micelles showed small ITC signals only at pH 9.0 (see above). We therefore suggest that in the membrane, the $p_K_a$ of Lys-300 participates in the pH control of NhaA by affecting ligand binding. Because the $p_K_a$ of an amino acid in a protein is affected by the surrounding residues, it is likely that during purification, this environment is changed, affecting the pH sensitivity of the active site in DDM micelles.

In conclusion, Asp-163, Asp-164, and the backbone of Thr-186 in FhNhaA (HpNhaA K347E) (31). HpNhaA is a close homologue of EcNhaA (45) with a protein sequence similarity of 82% and identity of 49%. Hence, a positive charge at position 300 is essential for the NhaA cation binding site. The positive charge of Lys-300 is essential for the antiporter activity, most likely by affecting ligand binding. Therefore, its $p_K_a$ is an important factor in the pH response of NhaA.

Although the prokaryotic NhaA is evolutionarily remote from the eukaryotic sodium/proton exchangers (NHEs) and NHAAs, we successfully modeled NHE1 and NHA2 on the basis of the crystal structure of NhaA (19, 37). Therefore, the results presented here can guide experiments that would lead to better understanding of the functionality of the human antiporters.

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