Lysine 300 is essential for stability but not for electrogenic transport of the *Escherichia coli* NhaA Na\(^+\)/H\(^+\) antiporter

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\(\text{Na}^+/\text{H}^+\) antiporters are located in the cytoplasmic and intracellular membranes and play crucial roles in regulating intracellular pH, \(\text{Na}^+\), and volume. The NhaA antiporter of *Escherichia coli* is the best studied member of the \(\text{Na}^+/\text{H}^+\) exchanger family and a model system for all related \(\text{Na}^+/\text{H}^+\) exchangers, including eukaryotic representatives. Several amino acid residues are important for the transport activity of NhaA, including Lys-300, a residue that has recently been proposed to carry one of the two \(\text{H}^+\) ions that NhaA exchanges for one \(\text{Na}^+\) ion during one transport cycle. Here, we sought to characterize the effects of mutating Lys-300 of NhaA to amino acid residues containing side chains of different polarity and length (*i.e.* Ala, Arg, Cys, His, Glu, and Leu) on transporter stability and function. Salt resistance assays, acridine-orange fluorescence dequenching, solid supported membrane-based electrophysiology, and differential scanning fluorometry were used to characterize \(\text{Na}^+\) and \(\text{H}^+\) transport, charge translocation, and thermal stability of the different variants. These studies revealed that NhaA could still perform electrogenic \(\text{Na}^+/\text{H}^+\) exchange even in the absence of a protonatable residue at the Lys-300 position. However, all mutants displayed lower thermal stability and reduced ion transport activity compared with the wild-type enzyme, indicating the critical importance of Lys-300 for optimal NhaA structural stability and function. On the basis of these experimental data, we propose a tentative mechanism integrating the functional and structural role of Lys-300.

Living cells are critically dependent on processes that regulate intracellular pH, \(\text{Na}^+\), and volume. \(\text{Na}^+/\text{H}^+\) antiporters, playing a primary role in these homeostatic processes, are located in the cytoplasmic and intracellular membranes of cells (1, 2). Certain human \(\text{Na}^+/\text{H}^+\) antiporters have long been drug targets (3) because they are involved in cardiac failures and other disorders (4). Homologues of EcNhaA\(^{4}\) (herein, NhaA), the main *Escherichia coli* \(\text{Na}^+\), \(\text{Li}^+/\text{H}^+\) antiporter, have recently been implicated in the virulence of pathogenic bacteria (5, 6) and in human essential hypertension (7) as well as diabetics (8).

NhaA is characterized by exceptionally high transport activity (9), a stoichiometry of 2H\(^+\)/Na\(^+\) (10), and a strong pH dependence (9), a property shared with other prokaryotic (11) and eukaryotic Na\(^+\)/H\(^+\) antiporters (12–15). It is a dimer (16–18), but its functional unit is the monomer (19).

The crystal structure of the NhaA monomer at acidic pH 4 (20) (Fig. 1A) shows that the protein is made up of 12 transmembrane helices (TM). Six of these TM form a highly conserved core domain composed of two structurally related helix bundles (TMs III, IV, V and TM 6) that are topologically inverted with respect to each other (Fig. 1A) (20). The partial positive dipoles of the N termini and the partial negative dipoles of the C termini of the short helices face each other and were suggested to be electrically compensated by Lys-300 and Asp-133, respectively (Fig. 1B) (20). This non-canonical TM assembly, termed the NhaA fold (21), is a unique fold that creates a delicately balanced electrostatic environment in the middle of the membrane at the ion-binding site (20, 22). The number of secondary transporters known to share the NhaA fold is steadily increasing (23–28).

Recently, a new structure of NhaA has been determined also at acidic pH 3.5 (24) which showed the NhaA dimer with monomers very similar to those already known (20). It revealed the details of the dimer interface and that TM X is located one helix turn toward the cytoplasm with respect to its location in the original structure. Furthermore, Lys-300 forms a salt bridge with one (Asp-163) of the two aspartate residues (Asp-163, Asp-164) of the cation binding site (Fig. 1C). These aspartates are the most evolutionary conserved and absolutely essential

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\(4\) The abbreviations used are: EcNhaA, NhaA Na\(^+\)/H\(^+\) exchanger of *E. coli*; TM, transmembrane segment; DSF, differential scanning fluorometry; F350/330, fluorescence emission ratio at 350 and 330 nm; SSM, solid-supported membrane; Bis-tris propane, 1,3-bis(tris(hydroxymethyl)methylamino) propane.
residues (29–31). It is still debatable whether the reason for the difference between the two structures is a wrong helix assignment in the original structure or that the two structures represent different conformations. In any event it led to an interesting proposal for a functional role of Lys-300 (24) in NhaA, a close homologue of EcNhaA (37), in the former by genetically suppressed both in E. coli and Helicobacter pylori NhaA, a close homologue of EcNhaA (37), in the former by genetically suppressed both in E. coli and Helicobacter pylori NhaA, a close homologue of EcNhaA (37), in the former by genetically suppressed both in E. coli and Helicobacter pylori

NhaA, a close homologue of EcNhaA (37), in the former by mutations in TM IV (36) and TM II and in the latter by mutation in TMIII (30, 38). In this study our aim was to investigate the role of Lys-300 in the NhaA antiporter mechanism. Does it only have a structural role

Table 1

| Mutation | Expression | Growth | Activity | Apparent $K_m$ |
|----------|------------|--------|----------|----------------|
|          | pH 7.0     | pH 8.3 | pH 7.0   | Na⁺ | Li⁺ | Na⁺ | Li⁺ |
| WT       | 100        | + + +  | + + +    | + + | 100 | 100 | 0.5 | 0.02 |
| K300R⁵   | 60         | + + +  | + + +    | + + | 36  | 93  | 21.8 | 0.8 |
| K300H⁵   | 64         | + + +  | + + +    | + + | 52  | 88  | 7.5  | 0.24 |
| K300C⁵   | 50         | -      | -        | -   | 0   | 44  | ND   | 4   |
| K300A⁵   | 75         | +      | -        | -   | 0   | 30  | ND   | 1.3 |
| K300L⁵   | 18         | -      | -        | -   | 0   | ND  | ND   | ND  |
| K300C⁶   | 15         | -      | -        | -   | 0   | ND  | ND   | ND  |

* Data are taken from Kozachkov et al. (36).

Figure 1. NhaA structure. A crystal structure of NhaA at pH 4 (PDB entry 1ZCD), as reported in Hunte et al. (20). Roman numerals denote transmembrane helices. Helices IV and XI (in green) are interrupted by an unwound portion and are thus split into a cytoplasmic (c) and periplasmic (p) segment. Red lines indicate the cytoplasmic and periplasmic funnels, respectively. B, Lys-300 compensates for the partial negative charges (5⁻) of TMVlp and Xlc of the NhaA structure. The same structure as panel A is shown, with TMVs other than IV, X, and XI removed for clarity. C, the salt bridge (dashed line) formed by Lys-300 and Asp-163 of NhaA, as seen in the structure (PDB entry 4AU5) reported in Lee et al. (24). The perspective is rotated by −180° on the y axis relative to panels A and B.
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as suggested by Hunte et al. (20), is it primarily relevant for the
NhaA transport mechanism as put forward by (24), or possibly
both? We, therefore, constructed a number of NhaA variants
where Lys-300 was replaced by amino acid residues having a basic
(Arg and His), non-polar (Ala, Leu), polar (Cys), or acidic (Glu)
side chain and tested the thermal stability and transport activity of
these mutants. Based on these experimental results, conclusions
are drawn about the role of Lys-300 in the structural and func-
tional properties of the E. coli NhaA Na\(^+\)/H\(^+\) exchanger.

Results

Effect of Lys-300 mutations on E. coli salt resistance

To investigate the role of Lys-300 in NhaA functionality, we
used previously isolated mutants K300C, K300E (36), K300H,
and K300R (22) and constructed K300L and K300A. To char-
terize the mutants with respect to expression, growth, and
antiporter activity in everted isolated membrane vesicles, the
mutant plasmids were transformed into EP432, an E. coli strain
that lacks the two specific Na\(^+\)/H\(^+\) antiporters NhaA and NhaB
(35). This strain neither grows on selective media (0.6 M NaCl at
pH 7/pH 8.3 or 0.1 M LiCl at pH 7.0) nor does it exhibit any
Na\(^+\)/H\(^+\) antiporter activity in isolated everted membrane ves-
icles unless transformed with a plasmid encoding an active anti-
porter. Whereas variants K300R, K300H, K300A, and K300C
were substantially (>50%) expressed (Table 1) as compared
with the level of expression of the WT (100%), mutants K300E
and K300L were expressed to 15 and 18% of the control level,
respectively (Table 1). However, because all variants are
expressed from multicopy plasmids, even a low level of expres-
sion is way above the level expressed from a single chromo-
somal gene, which confers a Na\(^+\) resistance phenotype (39).
Variants K300H and K300R grew similarly to the WT on the
selective agar plates at pH 7 but grew slowly (K300H) or did not
grow (K300R) on the Na\(^+\)-selective medium at pH 8.3 (Table 1).
K300C and K300L did not grow on the selective media, whereas
K300A grew with very small colonies only on high Na\(^+\) at pH 7.

Antiport activity of Lys-300 mutants in isolated membrane
vesicles

Next, everted membrane vesicles were isolated from each
mutant-expressing strain, and the Na\(^+\)/H\(^+\) antiporter activity was
determined. Upon energization by the addition of Tris-D-lactate,
the membranes maintained a ΔpH, acidic inside, and the change of
ΔpH caused by Na\(^+\) or Li\(^+\) addition was monitored using acridine
orange as a probe of ΔpH. Although K300L (Fig. 2) and K300E (36)
(Table 1) were inactive, mutants K300R and K300H showed sub-
stantial Na\(^+\)/Li\(^+\) antiporter activity (22), and mutants K300C
(Table 1) and K300A (Fig. 2) were active only with Li\(^+\). At saturat-
ing cation concentrations, the pH-dependent activity profiles of
mutants K300C, K300A, and K300H were very similar to that of
the WT (36). In marked contrast, the pH dependence of K300R
was shifted to the alkaline side by one pH unit (22).

Electrophysiological characterization of Lys-300 variants

We then tested the electrophysiological behavior of the
mutants using solid-supported membrane (SSM)-based elec-
trophysiology. Here, negative currents were observed after Na\(^+\)
concentration jumps at different pH values in mutants K300R,
K300H, K300C, and K300A, as for the WT (Fig. 3A, Table 2). To
decide whether these currents represent steady-state charge
transport or a presteady-state charge displacement, it is
instructive to compare transient currents from electrogenic
WT NhaA (40) and the electroneutral NhaP Na\(^+\)/H\(^+\) exchang-
ers of Methanocaldococcus jannaschii (MjNhaP1) and Pyrococ-
cus abyssi (PaNhaP) (41, 42). The former is a prototype of
steady-state charge transport, whereas the latter are prototypes of
presteady-state charge displacements. WT NhaA transient
currents show the following characteristics: 1) They decay
slower (~20 ms; Ref. 40) than presteady-state currents (10 ± 1
ms for MjNhaP1; Ref. 41). 2) Their decay time constants
strongly decrease with rising substrate concentration in con-
trast to those of the Mj and Pa exchangers. 3) Unlike the Mj
and Pa exchangers, the currents show alkaline down-regulation,
indicating that H\(^+\) translocation is involved. Overall, the tran-
sient currents of the investigated Lys-300 NhaA variants
behave like WT NhaA with somewhat modified apparent sub-
strate affinities but with all characteristics 1–3 of WT NhaA; e.g.
K300A NhaA currents decay with 19 ± 2 ms, their decay times
depend on the Na\(^+\) concentration (supplemental Fig. S1), and
their amplitudes are down-regulated at alkaline pH (Fig. 3G).
Similar conclusions can be drawn for all active Lys-300 NhaA
variants that, therefore, represent net steady-state transport of
positive charge out of the proteoliposomes (or negative charge
into the proteoliposomes) after a Na\(^+\) concentration jump.
No transporter-specific currents were observed for K300E and K300L at pH 8.5, the recorded traces for these variants being comparable with those recorded for empty liposomes, devoid of protein (Fig. 3A). Furthermore, no transporter-specific currents were recorded for these variants in the entire pH range tested (6.0–9.5). For K300C, whereas no transporter-specific currents were detected at pH 8.5 (Fig. 3A), an increase in pH to 9.5 revealed transporter-specific activity (Fig. 3F).

A detailed electrophysiological analysis of the active Lys-300 mutants was then performed (Fig. 3, B–H). For the K300C mutant, as currents could only be recorded at pH 9.5, the full pH-dependent profile could not be determined. However, we could determine the Na\(^+\)–dependent profile of the currents at pH 9.5, resulting in an apparent Na\(^+\) affinity of 71 ± 20 mM (Fig. 3F). This represents a 10-fold decrease in affinity compared with the value of 7.3 mM determined for the WT at pH 9 (34). Furthermore, the maximum current amplitude of the recorded transients was very low compared with the WT (Table 2). This indicates either reduced transport activity in the mutant (also supported by the activity determined in everted membrane vesicles using acridine orange as a probe of pH across the membrane as shown in Table 1) or that the pH optimum of this mutant lies high in the alkaline range.
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Table 2
Electrophysiological characteristics and thermal stability of Lys-300 variants

| Mutation | pHopt | Apparent \( K_{a} \) (pH) | Stability |
|----------|-------|--------------------------|-----------|
|          |       | Na\(^+\) | Li\(^+\) | \( I_{\text{max}} \) | \( T_{m} \) |
| WT\(^a\) | 8.7   | 11 ± 1 (8.5) | 7.3 (8) | 12 ± 2 | 65.9 ± 0.1 |
| K300R    | 9.1   | 78 ± 17 (9) | ND      | 1.6 ± 0.7 | 62.4 ± 0.3 |
| K300H    | 8.9   | 112 ± 21 (8) | ND      | 2.2 ± 1 | 59.9 ± 0.3 |
| K300C    | ND    | 71 ± 20 (9.5) | ND      | 0.28 ± 0.03 | ND |
| K300A    | 8.7   | 17 ± 7 (9) | 1.3 ± 0.6 (9) | 0.9 ± 0.1 | ND |
| K300L    | ND    | ND          | ND      | 0 | ND |
| K300E    | ND    | ND          | ND      | 0 | ND |

\(^a\) Data are taken from Mager et al. (34) and from Zuber et al. (40).

In the case of the K300H, K300R, and K300A mutants, a full electrophysiological characterization was possible. All three mutants displayed maximum current amplitudes far reduced compared with the WT protein (Table 2), with maximum currents recorded increasing on average in the order K300A < K300R < K300H. The pH-dependent profile of K300H is highly similar to that of the WT (Fig. 3B), with a pH optimum of 8.8. The Na\(^+\) affinity was also determined for K300H at two different pH values, 8.0 and 7.5 (Fig. 3C), and competition between Na\(^+\) and H\(^+\) was readily apparent, as is the case for the WT (34).

By comparison, the pH profile of K300R was shifted to the alkaline range compared with the WT or K300H (Fig. 3D), with a maximum at pH 9.1. An alkaline shift of the pH dependence of K300R activity was also observed in everted membrane vesicles (22). The determined apparent affinity for Na\(^+\) (Fig. 3E) of K300R was much lower than that determined for the WT (34) at a similar pH (78 mM versus 7.3 mM).

K300A is special among the active Lys-300 variants. It has a WT-like pH dependence (Fig. 3G) with maximum activity at pH 8.7, but in contrast to other active variants, its apparent Na\(^+\) affinity is similar to the WT (Table 2). Even more surprising is its extremely high apparent affinity for Li\(^+\), which agrees with the results of the dequenching assay, where activity was found with Li\(^+\) but not with Na\(^+\) (Tables 1 and 2 and Fig. 2). Furthermore, the decay time constants of the Li\(^+\)-induced currents are even larger than those with Na\(^+\) and depend on the Li\(^+\) concentration (supplemental Fig. S1). Li\(^+\)/H\(^+\) exchange of K300A NhaA is, therefore, electrogenic like Na\(^+\)/H\(^+\) exchange. The same applies to WT NhaA (40).

Structural stability of investigated NhaA variants

In the course of the electrophysiological measurements, it became obvious that only the WT protein produced signals that were stable in magnitude over a long time span (>2 h). For all investigated Lys-300 mutants we could observe high decreases (“run-downs”) in the magnitude of the recorded currents even over relatively short periods of time (10–30 min). These were not reproducible and ranged anywhere from a 10% reduction of the transient current amplitude up to a complete loss of the measured currents. Therefore, we employed differential scanning fluorimetry (DSF) in order to quantify the stability of the

Figure 4. Thermal stability of the investigated Lys-300 variants. A, melting curves for the EcNhaA protein variants (WT, K300A, K300C, K300E, K300H, K300L, and K300R) at a concentration of 0.5 mg/ml in solution at pH 4. The ratio between fluorescence emission intensities at 350 and 330 nm, respectively (F350/330) was plotted against the temperature, and any pronounced change in this relationship was associated with a protein unfolding event. These traces are representative from three different measurements. B, first derivative analysis obtained from the melting curves of the investigated variants. Peak values represent the point at which half of the protein population is unfolded (\( T_{m} \)). Colors of traces in panel B are the same as for panel A.

NhaA Lys-300 mutants and to compare it with that of the WT transporter (Fig. 4). Stability of the NhaA variants was determined by following the fluorescence emission intensity of the protein’s tryptophan and tyrosine residues (43) during continuous heating of the protein in solution at pH 4. Thermal unfolding events can be detected thanks to the high sensitivity of these amino acids to changes in their local microenvironment. Every time these residues are exposed to hydrophilic conditions as a consequence of structural changes, their quantum yield decreases, which leads to fluorescence maximum shifts and alterations in fluorescence intensity (43). To account for these two phenomena, the ratio between the emission intensities at 350 nm and 330 nm (F350/330) is plotted against the temperature. The resulting trace is known as melting curve.

A more pronounced inflection point was evidenced in the melting curve for EcNhaA WT in comparison to all Lys-300 mutants (Fig. 4A), indicating that any change in this residue compromises thermal stability. The obtained melting temperature for the WT variant was 65.9 ± 0.1 °C, which is close to that reported by Kohlstaedt et al. (44) using a thermofluor assay, 66.4 ± 0.6 °C at pH 6.

The extent of the shift in the melting temperature (\( T_{m} \)) for the Lys-300 mutants depended on the side-chain nature. When
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Lys-300 was exchanged by a residue with a basic side chain (Arg or His), stability was lower, and the transition temperature $T_m$ dropped to 62.4 ± 0.3 °C in K300R and 59.9 ± 0.3 °C in K300H (Fig. 4). Polar (Cys) and nonpolar (Ala, Leu) side chains yielded irregular melting curves with a shallow progression and/or multiple inflection points (multiple maxima in the first derivative), which indicates that a well defined protein melting transition does not take place. The melting curve for the case where a negative residue (Glu) replaced Lys-300 (K300E) presented an anomalous behavior compared with the other investigated variants. Although there were two small inflection points (around 50 and 70 °C, Fig. 4, A and B), the F350/330 values were higher than those of the other variants, indicating the presence of a considerable population of unfolded protein already at starting temperatures.

Even at room temperature all variants display elevated F350/330 values compared with the WT. Because high F350/330 for this particular protein corresponds to the unfolded state (Fig. 4), this observation points to a reduced structural stability of all variants also at the temperature where the functional assays were performed.

**Functional and structural classification of the investigated Lys-300 variants**

Summarizing the structural and functional properties of the investigated Lys-300 variants, we can classify them into three groups, as follows.

**WT-like variants (K300H and K300R)**—Compared with the WT they have lower but substantial currents, perform well in the growth assay, and display WT-like activity in the biochemical assay. In the DSF assessment, they show a step function characteristic of a well defined protein melting transition and melting temperatures 4 to 6 °C lower than the WT.

**Variants with low activity (K300C and K300A)**—These variants show zero to very small effects in the growth assay, reduced activity in the biochemical assay, and small currents. In the DSF measurement they have no well defined melting transition and display shallow slopes or double peaks in the first derivative.

**Inactive variants (K300L and K300E)**—No activity was detected irrespective of the applied assay. The DSF traces showed no defined melting transition but are characterized by shallow slopes with no peak or inverted and multiple peaks in the first derivative.

**Discussion**

Lys-300 plays a central role in the NhaA Na\(^+\)/H\(^+\) exchanger. Mutation in this position resulted in a certain loss of function in all investigated variants so far. In general, a reduced transport activity may result from impaired functionality but also from structural instability of the protein. Because our electrophysiological analysis revealed indications of protein instability, it was absolutely essential to use an independent method for assessment of protein stability, like the DSF technique, in parallel with the functional characterization.

**Lys-300 stabilizes the NhaA structure**

We found a reduced thermal stability of the variants that depended on the character of the amino acid residue in position 300. Only a conservative replacement of Lys-300 by an amino acid with a basic side chain, arginine and histidine, yielded protein with a well defined step function characteristic for a protein melting transition (Fig. 4). The transition temperatures of the variants are 4 – 6 °C lower than that of the WT, indicating a reduced structural stability. In line with the lower stability of K300H and K300R, a lower activity was detected in the growth, the biochemical, and the electrophysiological assays as compared with the WT.

Low thermal stability and very small or no activity were detected in the other variants, K300C, K300A, K300L, and K300E. In fact, thermal stability and activity seem to be correlated. This indicates that the effect of low activity is at least in part a consequence of the reduced thermal stability rather than directly affecting transport activity. Note that this would not be obvious in an ordinary biochemical assay where the sample is only used once, and a reduced activity would be interpreted as low activity of the particular variant unless special care is taken to detect temporal inactivation of the sample.

The observed structural role of Lys-300 is in agreement with its location in the middle of a most evolutionary-conserved segment (100%) in the middle of TM X (Gly–299, Lys–300, Gly–303) on one side of the helix facing the active site (Fig. 1). Both the original crystal structure of EcNhaA (20) and the more recent one (24) showed that Lys-300 is located between or near the C termini of the short helices IVp and X1c in the middle of the membrane so that it compensates their partial negative dipoles (Fig. 1B). The positive charge of Lys-300 has, therefore, been proposed to be important for structural integrity and activity of NhaA (20). Indeed, thermal stability decreases as the nature of the amino acid in position 300 changes from a positively charged (Lys, Arg, and His) to a hydrophobic (Leu, Ala) or negatively charged side chain (Glu).

Alternatively, Lys-300 may be engaged in a salt bridge as proposed by the structure of Lee et al. (24). In this case the positive charge of Lys-300 is effectively neutralized by Asp-163 and is unable to compensate the partial negative opposing dipole of the small helices (IVp and X1c). Nevertheless, the salt bridge itself may confer stability to the fold and function as a so-called “ion-lock” as described for the melibiose permease (45).

In conclusion, whether the underlying mechanism be charge compensation or salt-bridge formation, a positive charge in position 300 is indispensable for the structural integrity of NhaA. This has to be taken into account when mechanistic conclusions are drawn based on a comparison of the activities of Lys-300 variants. On the other hand, the mutation clearly affects also the transport mechanism directly as evidenced by the cation affinity drop of most variants. The functional role of Lys-300 will be addressed in the following.

**Lys-300 is not essential for electrogenic Na\(^+\)/H\(^+\) exchange**

NhaA, like most (and possibly all) members of the CPA2 evolutionary branch (CPA = cation proton antiporter) of cation proton antiporters, is electrogenic; that is, it functions with a H\(^+\)/Na\(^+\), Li\(^+\) stoichiometry > 1 (for NhaA a 2H\(^+\)/1Na\(^+\) stoichiometry was determined in Refs. 9 and 33). If Lys-300 is an essential proton donor/acceptor in the NhaA reaction mecha-
Figure 5. Mechanistic models for Na\(^+\)/H\(^+\) exchange in NhaA. The NhaA transport mechanism consists of two Na\(^+\)/H\(^+\) exchange reactions separated by conformational transitions changing accessibility of the substrate binding site from periplasmic to cytoplasmic and back. The figure shows these exchange reactions for the two different transport mechanisms proposed for NhaA. A, in the two-aspartates model (20), the two transported protons bind to the two aspartic acids. B, in the salt-bridge model (24) one proton resides on Asp-164, and the second proton resides in the Asp163–Lys-300 salt bridge. For further discussion of the models see “Discussion.”

As characteristic for a secondary active transporter, the NhaA transport mechanism consists of at least two Na\(^+\)/H\(^+\) exchange reactions separated by conformational transitions changing accessibility of the substrate binding site from periplasmic to cytoplasmic and back (32, 34). In the conventional two-aspartates model (20) the two transported protons bind to the two aspartic acid residues and are released to the cytoplasm by Na\(^+\) binding to the aspartates (Fig. 5B). In the “salt-bridge model” one proton resides on Asp-164 and the second proton in the Asp163–Lys-300 salt bridge (Fig. 5B). As suggested previously (34), when intracellular pH becomes more alkaline, intracellular Na\(^+\) ions successfully compete with protons for binding to the proton acceptors in the binding site. In the two-aspartates model this leads to the displacement of the two protons from the two aspartates. In the salt-bridge model upon Na\(^+\) binding, the first Asp-164 releases its proton. Molecular dynamics simulations (24) showed that subsequently Asp163 switches from interacting with Lys-300 to contributing to binding the Na\(^+\) ion, and a second H\(^+\) is released from Lys-300. The Na\(^+\)-bound form of the transporter would then switch to the outward-facing conformation where the sodium ion can be released, the salt bridge can reform by binding of H\(^+\) to Lys-300 and to Asp-164, and finally the transporter switches back to the inward-facing conformation.
In summary, in the salt bridge mechanism proposed by Lee et al. (24), Lys-300 plays a role of an essential proton donor/acceptor in the NhaA reaction cycle. This implies that replacing Lys-300 of NhaA by a residue that cannot protonate will yield either an inactive mutant or a variant, which is electroneutral, a conclusion that is in agreement with our experiments.

The arguments given above would obviously be in favor of the two-aspartates model. However, there is a third possibility, namely that the transporter, after replacement of Lys-300 by an uncharged residue like alanine, switches from the salt-bridge model to the “two aspartates” model. The considerable lower currents recorded in this case may be explained by the fact that the transporter is optimized for the salt-bridge model and works with lower turnover in the two-aspartates model. In both cases transport is electrogenic, as experimentally demonstrated in the electrophysiological experiments. An electroneutral mechanism where only one H⁺ is bound and exchanged for one Na⁺ ion can be ruled out.

Conclusions and a hypothetical transport mechanism

In conclusion, the two major contributions of the present study are the experimental proof that Lys-300 is important for the stability of the transporter and, although not essential, may play an important role for an effective transport process. Electrogenic Na⁺/H⁺ exchange can do without it, although with reduced capacity. It is, however, possible that the functional importance attributed to Lys-300 is at least in part a consequence of its structural role.

Given that Lys-300 stabilizes the structure of the NhaA Na⁺/H⁺ exchanger by engaging in a salt bridge with Asp-163, we can also envision that this is important for the dynamics of the transport process. An effective strictly coupled Na⁺/H⁺ exchange process requires that only the fully loaded transporter (2 H⁺ or 1 Na⁺ bound in the case of NhaA) can perform this transition and that it is inhibited in the empty apo transporter (46). We suggest that the formation of the Lys-300–Asp-163 salt bridge may be an inhibitory element by making the structure more rigid so that the energetic barrier for the conformational transition of the apo transporter is increased. Binding of substrates (Na⁺ or H⁺) would then break the salt bridge and allow the transitions of the loaded transporter.

In this concept the transport process proceeds as follows. 1) In the periplasmic open conformation without substrates Asp-164 is unprotonated, and Asp-163 is engaged in a salt bridge with Lys-300, which stabilizes this conformation. 2) When two H⁺ ions bind from the periplasmic side to Asp-163 and Asp-164, the salt bridge is broken, the structure becomes less rigid, and a conformational transition can take place altering the accessibility and releasing the transported H⁺ ions to the cytoplasmic space. 3) Because now Asp-163 is unprotonated, it can again form a salt bridge with Lys-300, effectively inhibiting the reorientation of the unloaded transporter. 4) When Na⁺ binds from the cytoplasm, the salt bridge is broken as demonstrated recently by a molecular dynamics study (24), and a conformational transition allows Na⁺ release at the periplasmic side of the membrane.

The transport mechanism outline above elegantly explains why Lys-300 apparently has a dual role, structural as well as functional, and is consistent with the data provided by Lee et al. (24). If replaced by an uncharged residue like alanine the Na⁺/H⁺ exchanger would still be functional but partly uncoupled, which compromises its function especially when substrate gradients are present. This may explain why K300A NhaA is still functional but with low activity. Experimental efforts to demonstrate the uncoupled function of Lys-300 mutants like K300A NhaA will be required to substantiate this hypothetical mechanism.

Finally, we would like to stress the importance of understanding the transport mechanism of E. coli NhaA. Although the prokaryotic NhaA is evolutionarily remote from the eukaryotic sodium/proton exchangers (NHEs and NHAs), we successfully modeled NHE1 and NHA2 on the basis of the crystal structure of NhaA (47, 48). Therefore, the results presented here can guide experiments that would lead to a better understanding of the functionality also of the human antiporters.

Experimental procedures

Genetic constructs

Mutant variants of NhaA in which Lys-300 was replaced were obtained in plasmid pAXH3, a pET20b derivative (19). The preparation of mutants K300E and K300C was previously described in Kozachkov et al. (36), whereas K300R and K300H were first described in Maes et al. (22). The mutants K300L and K300A were obtained by site-directed mutagenesis using a PCR-based protocol with pAXH3 as a template. The nhaA gene DNA of each construct was sequenced to verify the mutation.

Salt resistance assays

Survival of E. coli EP432 (35) expressing NhaA variants K300L and K300A under conditions of high concentrations of Na⁺ or Li⁺ was assessed as previously described (22).

Determination of Na⁺, Li⁺/H⁺ antiporter activity in isolated everted membrane vesicles

Everted membrane vesicles from EP432 transformed with the respective plasmids were prepared as previously described (49). Everted membrane vesicles were used to determine Na⁺/H⁺ or Li⁺/H⁺ antiporter activity with an assay based on the measurement of Na⁺- or Li⁺-induced changes in the ΔpH as measured by acridine orange, a fluorescent probe of ΔpH (50). The fluorescence assay was performed in a 2.5-ml reaction mixture containing 100–150 μg of membrane protein, 0.1 μM acridine orange, 150 mM choline chloride, 50 mM Bis-tris propane, and 5 mM MgCl₂, and pH was titrated with HCl. Membrane vesicles were acidified by the addition of 2 mM Tris-D-lactate, inducing fluorescence quenching of the acridine orange dye. Dequenching of fluorescence upon the addition of either Na⁺ or Li⁺ indicates that protons are exiting the vesicles in antiport with either cation. As shown previously (51), the end level of dequenching is a good estimate of antiporter activity, and the ion concentration that gives half-maximal dequenching is a good estimate of the apparent Kᵥ for the antiporter activity. For determination of the apparent Kᵥ, the end level of dequenching for different concentrations of the tested cations
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(0.01–100 mM) at the indicated pH levels was used, and the apparent \( K_m \) values were calculated by linear regression of a Lineweaver-Burk plot.

Overexpression, purification, and reconstitution

C-terminally His-tagged proteins were overexpressed in E. coli BL21(DE3) cells and purified using immobilized metal affinity chromatography as previously described (2). Reconstitution of the purified proteins into proteoliposomes was performed using E. coli polar lipid extract (Avanti Polar Lipids, Alabaster, AL) at a lipid-to-protein ratio of 5, essentially as previously described (34).

SSM-based electrophysiology

Electrophysiological measurements were performed essentially as previously described (34). In brief, 30 \( \mu \)l of proteoliposomes at a lipid-to-protein ratio of 5 were added to the SSM sensor and allowed to adsorb for at least 1 h on top of the preformed octadecanethiol/phospholipid hybrid bilayer. Transient currents were also recorded for Na\(^+\) concentration jumps performed on “empty” liposomes that did not contain transporter proteins.

A single solution exchange protocol (52) was employed, with solutions exchanged over the SSM sensor in the order non-activating–activating–non-activating. All solutions contained 25 mM MES, 25 mM MOPS, 25 mM Tris, 100 mM KCl, 5 mM MgCl\(_2\), and 1 mM dithiothreitol and were titrated to the desired pH with HCl or KOH. In addition, non-activating solutions contained 200 mM extra KCl, whereas activating solutions contained \( x \) mM NaCl and \( (200 - x) \) mM KCl instead. A similar protocol exchanging NaCl for LiCl was employed for measuring Li\(^+\)-dependent transport activity.

The amplitude of the recorded transient currents was used as a measure of steady-state transport activity. Recorded currents were corrected by subtracting the amplitude of transients generated by solution exchange effects recorded on the same sensor in the case where the latter were substantial compared with the transporter-dependent transients.

Differential scanning fluorometry

Protein stability of the purified NhaA variants (WT and K300X, \( X = \text{Ala, Cys, Glu, His, Leu, Arg} \)) was analyzed by DSF. Glass capillaries were loaded with 10 \( \mu \)l of protein at a concentration of 0.5 mg/ml in buffer containing 100 mM KCl, 5 mM MgCl\(_2\), 0.03% DDM, 25 mM potassium acetate (pH 4), and placed in the thermal plate of a Prometheus NT.48 instrument (NanoTemper Technologies, Munich, Germany). Temperature was increased in a range of 20 to 95 °C at a heating rate of 1 °C/min. Thermal protein unfolding was followed by monitoring the tryptophan fluorescence at emission wavelengths of 350 and 330 nm upon excitation at 280 nm with an excitation power setting of 10%.

Melting curves were obtained by plotting the ratio of the two emission intensities (F350/F330) versus the temperature. The thermal unfolding transition midpoint or melting temperature (\( T_{\text{mp}} \) °C) corresponded to the inflection point of the melting curves and was estimated via first derivative analysis.

Author contributions—E. P. and K. F. initiated and directed the project. O. C., M. D., and M. P.-R. performed the experiments described. O. C., M. D., M. P.-R., E. P., and K. F. analyzed the data and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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