Sodium/proton exchangers of the SLC9 family mediate the transport of protons in exchange for sodium to help regulate intracellular pH, sodium levels, and cell volume. In electronegative Na⁺/H⁺ antiporters, it has been assumed that two ion-binding aspartate residues transport the two protons that are later exchanged for one sodium ion. However, here we show that we can switch the antipporter activity of the bacterial Na⁺/H⁺ antiporter NapA from being electronegative to electroneutral by the mutation of a single lysine residue (K305). Electroneutral lysine mutants show similar ion affinities when driven by ΔpH, but no longer respond to either an electrochemical potential (Ψ) or could generate one when driven by ion gradients. We further show that the exchange activity of the human Na⁺/H⁺ exchanger NHA2 (SLC9B2) is electroneutral, despite harboring the two conserved aspartic acid residues found in NapA and other bacterial homologues. Consistently, the equivalent residue to K305 in human NHA2 has been replaced with arginine, which is a mutation that makes NapA electroneutral. We conclude that a transmembrane embedded lysine residue is essential for electronegative transport in Na⁺/H⁺ antiporters.

secondary active transporters  |  proton transport  |  membrane protein  |  Na⁺/H⁺ exchangers  |  energetics

Sodium/proton antiporters are found in all cells, where they help to regulate intracellular pH, sodium levels, and cell volume. (1) Na⁺/H⁺ antiporters are members of the large monovalent cation:proton antiporter (CPA) superfamily that includes, among others, the CPA1 and CPA2 clades (2). It is generally thought that Na⁺/H⁺ antiporters from the CPA1 clade catalyze electroneutral sodium–proton exchange (SLC9A1-9/NHE1-9, in mammals), whereas CPA2 members are thought to be electronegic (SLC9B1-2/NHA1-2, in mammals) (2), with stoichiometries of 2H⁺:1Na⁺ and 3H⁺:2Na⁺ ions reported (3, 4). Their dysfunction is associated with a number of different diseases, and they are well-established drug targets (1, 2). In bacteria, Na⁺/H⁺ antiporters use the proton-motive force to extrude sodium out of the cell and, for this reason, are important classes of secondary active transporters for both bacterial homeostasis and pathogenesis (5).

Bacterial Na⁺/H⁺ antiporters harbor the “NhaA fold” (6, 7), which was first observed in Escherichia coli NhaA and has since been observed in an unrelated sodium-coupled bile acid symporter (8). The NhaA fold consists of a dimer domain and an ion translocation (core) domain, which is characterized by a six-helical bundle harboring two opposite-facing discontinuous helices that crossover near the center of the membrane. Although bidirectional proton (H⁺) and sodium (Na⁺) translocation is strictly coupled in antiporters, the underlying molecular mechanism is still not fully understood. It has been assumed that, for electronegative Na⁺/H⁺ antiporters, two protons are carried across the membrane by two strictly conserved aspartate residues (2), which release their protons on the other side of the membrane in exchange for binding one sodium ion. Previous studies have shown that, for electronegative transporters, both carboxyl-containing residues are essential (2, 9, 10), but, for electroneutral transporters, only one of the two aspartate residues is conserved (2). Despite this prevailing view, there is no direct measurement for proton transport by these aspartate residues per se, and this is not the only plausible mechanism. In the recent crystal structure of NapA, an electronegative Na⁺/H⁺ antiporter from Thermus thermophilus, the two strictly conserved aspartate residues D157 and D156 are well positioned to bind protons; however, D156 was also found salt-bridged to a neighboring lysine residue, K305 (11). Likewise, in a new crystal form of dimeric E. coli NhaA at inactive pH, a salt bridge between the equivalent charged residues was also evident (12). The formation of the salt bridge between one of the conserved ion-binding aspartates suggests a different mechanism than direct protonation of the carboxyl residues, that is, one in which the lysine residue itself could be a proton carrier (12).

Previous studies have shown that the mutation of K305 in NapA to alanine (11, 13) or the equivalent lysine in E. coli NhaA to alanine, arginine, or histidine retains some antipporter activity for Li⁺ and the latter two also for Na⁺ (10), but this activity has not yet been characterized in detail (10). In this study, we have analyzed the effect of pH to Na⁺ and Li⁺ catalyzed transport of NapA wild type (WT), mutants of K305, and other residues in the vicinity of the proposed ion binding site. Our data support a transport model in which protons and Na⁺ (Li⁺) compete...
for the same ion binding site. Although most K305 mutations in NapA are functional, only the substitution with histidine can generate a membrane potential, revealing the essential role of K305 as a proton carrier and for conferring electrogenicity. We further show that these findings are consistent with the electroneutral antiport activity measurements of the purified human Na⁺/H⁺ exchanger NHA2, a protein that harbors the same strictly conserved aspartate residues, but where the residue equivalent to K305 has been replaced by arginine.

**Results**

**pH-Dependent Activity Is an Intrinsic Property of the Ion Binding Site.** Using solid-supported membrane electrophysiology, it was shown that the strongly pH-dependent activity for the homologous antiporter *E. coli* NhaA can be fitted by a simple kinetic model in which Na⁺ and protons compete for the same ion binding site (14). At acidic pH values, the *K*ₐ for Na⁺ is strongly affected by competition of the elevated proton concentration to the same binding site, whereas *V*ₘₐₓ of the transporter is unaltered. At alkaline pH, however, where affinity for Na⁺ is high because of the low proton concentration, activity is dictated by an altered *V*ₘₐₓ. Together with other biochemical data (14), these results strongly suggested that pH regulation is an intrinsic property of the ion binding site and not a separate step in the transport cycle, as was originally proposed (1, 15).

Although, in NhaA, both Na⁺ and Li⁺-driven activity show a clear pH dependence profile, the activity for NapA was reported to be atypical in that its activity was shown to be pH-dependent for Na⁺, but not for Li⁺ over the physiological pH ranges tested (13). The simplest interpretation is that the different pH activity profiles reflect the 10-fold lower apparent affinities for Na⁺ compared with Li⁺ (11, 13, 16). As such, in NapA, protons could be more effective at competing for the binding of Na⁺ than for Li⁺. To test this hypothesis, purified NapA was coreconstituted together with *E. coli* FₐF₁ ATP synthase into liposomes (*Materials and Methods* and Fig. 1A). In these liposomes, FₐF₁ ATP synthase has an inside-out orientation, whereas, based on maleimide-PEG-5k labeling efficiency to a cytoplasmic located cysteine mutant V31C, NapA has a ~60 to 70% right-side-out orientation (Fig. S1A), i.e., preference is toward the physiological orientation. In these experiments, the ATP synthase acidifies the inner volume of the liposomes by ATP-driven proton pumping, which is monitored by a decrease in the fluorescence of the ∆H⁺-sensitive dye 9-amino-6-chloro-2-methoxyacridine (ACMA). Addition of Na⁺ or Li⁺ ions at various concentrations drives proton efflux by NapA, which is seen as an increase in ACMA fluorescence (dequenching) (Fig. 1B).

Based on the above reasoning, weakening the ion binding site by point mutations should result in Li⁺-driven activity that is now pH-dependent over the physiologically relevant pH ranges tested. To explore this rationale, potential ion binding
site residues T126 and S127 (Fig. 1D and Fig. 2A) were substituted with alanine; mutants of the conserved aspartates D156 and D157 cannot be directly assessed, as they are completely nonfunctional (11, 13). A weakening of the binding affinity was indeed observed, as the T126A mutation caused a sixfold increase in the apparent $K_m$ for Li$^+$ from 0.5 to 3.7 mM at pH 8 (Table 1). As predicted, activity for T126A was now also strongly pH-dependent for Li$^+$, with no detectable activity at pH 6 (Fig. 1D). The same pattern, but less pronounced, is also observed for S127A (Fig. 1D). This weaker effect could be because the side-chain of S127 is more distal to the predicted ion binding site than the side-chain of T126 (Fig. 2A). Finally, a third mutation, K305A, was investigated and displayed similar behavior, supporting our idea that K305 affects the ion binding properties in NapA (Table 1). Taken together, the results show that the pH-dependent activity of NapA is an intrinsic property of the ion binding site.

**Converting Electrogenic NapA into an Electroneutral Transporter.** Recently, an outward-facing crystal structure of NapA was determined at active pH 6.5 and refined at 2.3 Å resolution (16).
However, it was still not possible to unambiguously identify a bound Na\(^+\) ion in the electron density maps at the proposed ion binding site (16). Molecular dynamics (MD) simulations of the NapA crystal structure were therefore performed to investigate potential Na\(^+\) ion coordination (16). Similar to the binding mode seen in MD simulations of *E. coli* NhaA (11, 16), conserved aspartic acids D157 and D156 as well as T126 were seen to coordinate Na\(^+\) in the middle of the discontinuous helix crossover (Fig. 2A, Lower). However, unlike NhaA, where Na\(^+\) binding in MD simulations was also seen to favor breakage of the salt bridge between D163 and K300 residues (12), in NapA, the equivalent salt bridge between K305 and D156 residues always remained intact, even upon Na\(^+\) binding (16). This discrepancy may reflect the parameters used to model ion binding in the MD simulations or structural differences between the two proteins. Regardless, it highlights that experimental data are essential before any firm conclusions can be made regarding the potential role of K305 as a proton carrier. Hence, we forthwith substituted K305 to neutral and negatively and positively charged residues and determined the *Kₐ* of the purified mutants in our proteoliposome-based transport setup.

Only the substitution of K305 to histidine was able to support WT Na\(^+\) -driven transport activity (Table 1). The K305H variant also showed a pH-dependent activity indistinguishable from that of WT NapA (Fig. S1B). Overall, apart from the K305Q mutant that showed a fivefold higher *Kₐ* for Na\(^+\), all other lysine variants were almost completely inactive for Na\(^+\) up to ∼300 mM. All K305 variants do, however, retain some Li\(^+\) -driven transport activity (Fig. 2B), where K305H and K305Q were the least perturbing variants, with *Kₐ* values for Li\(^+\) equal to and threefold higher than WT, respectively (Table 1). Surprisingly, the variant with the poorest Li\(^+\) -driven transport activity was the substitution of K305 to arginine with a *Kₐ* greater than 50 mM at pH 8. The apparent *Kₐ* dropped slightly to ∼35 mM at pH 8, but was again higher at pH 9 (>80 mM). At these higher pH values, Na\(^+\) -driven activity for the K305R variant was likewise immeasurable up to ∼300 mM (Table 1). Thus, a positively charged residue is, in of itself, insufficient to retain robust transport activity in this position. Other lysine variants K305A, K305E, and K305C had *Kₐ* values that were between 10- and 60-fold higher than WT (Fig. 2B and Table 1).

Because NapA is electricrogenic (two protons are exchanged for one Na\(^+\) (Li\(^+\)) ion), every ion gradient-driven turnover builds up a membrane potential that opposes, and eventually inhibits, proton transport. If the membrane potential is dissipated by the addition of valinomycin (in the presence of potassium), ion-driven transport is no longer hindered, and a new steady-state level can be reached (11). In contrast, the addition of valinomycin should not increase the transport activity in electroneutral Na\(^+\)/H\(^+\) antiporters, as there is no membrane potential generated to oppose proton transport. We exploited this behavior to analyze the electrogenicity of the measurable Li\(^+\) transport activity in the K305 variants. To this end, WT NapA WT and ion-binding variants were reconstituted into proteoliposomes in the presence of the water-soluble pH ratiometric dye pyranine, but in the absence of Li\(^+\) (Fig. 2C). As shown for WT NapA, after the addition of Li\(^+\), the fluorescence signal rapidly reached a first steady-state level (Fig. 2D). Upon the subsequent addition of valinomycin, the membrane potential was dissipated, and a second, final steady-state level was reached. Transport activity before and after valinomycin addition showed an approximately twofold to threefold ratio increase for WT NapA (Fig. 2D and E), which is similar to that previously observed for *E. coli* NhaA (18). For the ion-binding variants S127A and T126A, the relative ratio increase in activity after the addition of valinomycin was also similar to that observed for WT (Fig. 2E and Fig. S1C). For the lysine variants K305A and K305Q, however, no obvious increase in exchange activity was observed with a ratio close to 1 (Fig. 2E). The difference is unlikely due to the poor activity of the K305A and K305Q variants, because they showed similar *Kₐ* values for Li\(^+\) as the valinomycin-sensitive variants T126A and S127A (Table 1). Indeed, with the exception of K305H, all lysine variants including K305R no longer responded to valinomycin (Fig. 2E and Fig. S1C).

As WT NapA is electrogenic, we have recently shown that Na\(^+\)/H\(^+\) exchange can be efficiently driven solely by a membrane potential (ΔΨ) in the absence of proton and sodium gradients (11). If all of the K305 variants, except for histidine, are indeed electroneutral, it should not be possible to drive their activity under these conditions. To test this hypothesis, WT NapA and all of the ion binding site variants were reconstituted into pyranine-encapsulated liposomes containing 100 mM KCl and 100 mM Li\(^+\) at pH 7.8 and suspended into the same buffer composition, but with only 1 mM KCl (Fig. 3A). With no ion gradients present, the only driving force applied was an electrical potential of ∼116 mV (inside negative), which is generated when valinomycin was added to the proteoliposomes (Fig. 3B). As shown in Fig. 3B, WT NapA, T126A, S127A, and K305H variants all showed strong ΔΨ-driven activity. In contrast, all of the K305 variants, which were previously shown to be valinomycin-insensitive, showed little or no ΔΨ-driven transport activity (Fig. 3B). We further assessed the dependence of antiporter activity on the electrical potential in the range from ∼5 to ∼116 mV, as shown in Fig. S1D. Again, only WT NapA and the K305H, T126A, and

### Table 1. *Kₐ* values in mM of WT NapA and human NHA2 and studied mutants from pH 6.0 to 8.0

<table>
<thead>
<tr>
<th>NapA</th>
<th>Na(^+), mM</th>
<th>Li(^+), mM</th>
<th>Na(^+), mM</th>
<th>Li(^+), mM</th>
<th>Na(^+), mM</th>
<th>Li(^+), mM</th>
<th>Na(^+), mM</th>
<th>Li(^+), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>38.8 ± 5.4</td>
<td>3.5 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>0.55 ± 0.03</td>
<td>3.7 ± 0.2</td>
<td>0.61 ± 0.04</td>
<td>4.0 ± 0.3</td>
<td>0.41 ± 0.04</td>
</tr>
<tr>
<td>K305A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>K305H</td>
<td>34.1 ± 9.3</td>
<td>3.5 ± 0.2</td>
<td>5.2 ± 0.3</td>
<td>0.51 ± 0.02</td>
<td>3.5 ± 0.2</td>
<td>0.52 ± 0.04</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>K305Q</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>K305R</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>S127A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3.2 ± 0.4</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>T126A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>7.2 ± 0.3</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D156N K305Q</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>3.2 ± 0.4</td>
<td>—</td>
</tr>
<tr>
<td>hsNHA2 WT</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>7.2 ± 0.3</td>
<td>—</td>
</tr>
<tr>
<td>hsNHA2 R362K</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

The apparent *Kₐ* for K305R mutant are 34.7 ± 4.7 mM and 84.7 ± 17.2 mM for Li\(^+\), at pH 8.5 and pH 9.0, respectively.
S127A variants showed a linear increase in activity with increasing voltages (Fig. 3 C and D and Fig. S1 E and F), while the electoneutral variants showed no voltage-dependent increase in activity (Fig. 3D and Fig. S1 E and F).

The absence of a stimulating effect after dissipation of $\Delta \Psi$ in all of the K305 variants except for histidine, together with their inability to be driven solely by an electrical potential, suggests that the K305 residue is essential for electrogenic transport. Consequently, these mutants should not generate a membrane potential when driven by ion gradients. To confirm this, we used the fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide [DiSC$_3$(5)], because it is sensitive to an electrical membrane potential (negative inside) and has been previously used to show electrogenic transport of E. coli NhaA (19). In these experiments, either NapA WT or K305 variants were reconstituted into liposomes and the DiSC$_3$(5) dye added (Fig. 4A). Upon addition of Na$^+$ or Li$^+$, an uneven Na$^+$(Li$^+$)/H$^+$ stoichiometry, as found in WT NapA, quickly generated a membrane potential, which was observed by a decrease in DiSC$_3$(5) fluorescence (Fig. 4B, black trace). In the presence of potassium, the membrane potential was abolished by valinomycin and the signal returned close to the starting level (Fig. 4B); to establish a background signal, proteoliposomes containing either no protein or the inactive variant D157N were used (Fig. 4B, green and orange traces). Next, we tested the variants S127A and K305H, which showed electrogenic activity in the previous experiments. As shown in Fig. 4C, upon the addition of lithium, these variants generated a membrane potential, wherein S127A showed a weaker response than either K305H or WT. In contrast, variants K305A, K305Q, and K305R showed no membrane potential generation, as their responses were indistinguishable from the background levels of the negative controls (Fig. 4D). These data are thus in complete agreement with the other two assays, reinforcing the view that the pumping stoichiometry in K305 mutants has been altered and is now close, or equal, to 1:1. The only exception, again, was K305H, which, remarkably, appeared to generate an electrical potential similar to that observed for NapA WT (Fig. 4C).

Low Internal pH Facilitates Ion Exchange Activity in NapA. In NapA WT proteoliposomes encapsulated with pyranine, a very strong change in fluorescence was observed upon the addition of Li$^+$ at pH 8 (Fig. 2D). In contrast, the addition of Li$^+$ to the electoneutral K305 variants showed a much smaller response (Fig. S1C). The difference in the Li$^+$-driven response could not be simply explained by a difference in activities, because, in the $\Delta \mathrm{pH}$-driven ACMA experiments, the apparent ion affinities of a number of the electoneutral and electrogenic mutants were determined to be similar, for example, S127A and K305Q (Table 1). The main difference between the two proteoliposome setups is that, in the $\Delta \mathrm{pH}$-driven experiments, ATP synthase pumps protons into liposomes until the internal pH becomes quite acidic (<pH 6), whereas, in the pyranine containing liposomes, the pH is ~7 on both sides of the membrane, i.e., in absence of the ATP synthase, a proton gradient is not present during the pyranine experiments, and transport is driven solely by the applied Li$^+$ gradient. We reasoned that perhaps a low internal pH might facilitate exchange activity by increasing the likelihood of protonating key ion-binding residues, which would promote intracellular Li$^+$ release in a simple competition mechanism. To test this hypothesis, NapA WT and K305 variants in pyranine-encapsulated proteoliposomes were prepared, and the pH was adjusted to 6.6, 7.3, or 8.0 (Fig. 5A). Subsequently, proteoliposomes were diluted into buffer at each of the respective pHs, and Li$^+$-driven proton efflux was followed as described above. At pH 8, transport in the K305A and K305Q variants is quickly exhausted and does not even reach the first steady-state level seen in WT NapA (Fig. 5A). At pH 7.3, however, K305A and K305Q variants still perform similarly, but somewhat better compared with WT. At this pH, it is also seen that the transport process is now clearly slower in the NapA variants. Most interestingly, at pH 6.6, K305Q clearly performs better than K305A and almost reaches the first steady-state level of WT. Variant T126A also showed a pH-dependent decrease of the initial rate; however, the sensitivity toward $\Delta \psi$ is seen at all pH values. The observed effects reinforce that K305 is at the core of the transport mechanism.
An Interaction Between D156 and K305 Residues Is Central for Transport. In addition to the role of K305 as a proton carrier, MD simulations have also indicated that the salt bridge formed between corresponding residues in NhaA is sensitive to ion binding (12, 20). We therefore speculated that perhaps the reason why mutations of the equivalent D156 aspartate in NapA homologues are always inactive (1, 10, 11, 13) may not necessarily be because the variants are incapable of Na\(^+\) (Li\(^+\)) binding but because such a mutation generates an “unpaired” lysine. To probe the importance of this interaction, we combined the nonfunctional D156N mutant with a K305Q variant, which was the least perturbing of the neutral lysine variants tested (Table 1). Strikingly, the D156N-K305Q mutant now showed robust ΔpH-driven antiport activity with ~40% ACMA dequenching for both Li\(^+\) and Na\(^+\) ions at high ion concentrations (Fig. 5B). Although the apparent \(K_m\) for Li\(^+\) and Na\(^+\) are higher than WT (Table 1), this nonetheless demonstrated that, mechanistically, the second aspartate is not essential for ion-coupled transport, which is consistent with its replacement to asparagine in electroneutral Na\(^+\)/H\(^+\) exchangers (2). As expected, the activity for the D156N-K305Q double mutant is also electroneutral, as no increase in antiport activity after valinomycin addition was observed (Fig. 5C).

Human NHA2 Antiport Activity Is Likely Electroneutral. In recent years, human Na\(^+\)/H\(^+\) exchangers NHA1 and NHA2 have been described, with the latter proposed to have a role in hypertension and insulin secretion (21–23). Compared with the human cation exchangers NHE1-9, NHA1 and NHA2 have higher sequence similarity to the bacterial homologues, especially NapA (21% protein sequence identity). NHA2 (SLC9B2) also appears to be the only mammalian Na\(^+\)/H\(^+\) exchanger where both aspartic acid residues equivalent to D156 and D157 are conserved (Fig. S2). However, unlike NapA, the residue equivalent to K305 is an arginine (Fig. S2). Furthermore, in contrast to the NHEs that use the inwardly directed Na\(^+\) gradient to extrude protons, NHA2 is thought to be similar to (most) bacteria that use the proton gradient/membrane potential to extrude sodium (24). Consistent with this idea, NHA2 is found to localize to late endosomes, where it further colocalizes with the vacuolar ATPase (V-ATPase) (25), which establishes a low pH on the inside of endosomes. Because of the two conserved aspartates and the similarity to the bacterial homologues, NHA2 was thought to be electronegic, but experiments in whole cells have not supported this assumption (2, 22–24, 26). We therefore aimed to clarify the energetics of human NHA2 in an isolated system using purified components.

First, an N-terminal tail Δ71 amino acid truncation mutant of human NHA2 was produced using fluorescent-based screening methods in Saccharomyces cerevisiae (27, 28) and purified in the detergent dodecyl-β-D-maltopyranoside (DDM) (Materials and Methods and Fig. S3A). Next, together with ATP synthase from E. coli, hNHA2 was coreconstituted into liposomes made from a mixture of brain and soybean lipids, which consistently gave the highest activity out of the various combinations of lipids tested (Materials and Methods). Using the same antiport assay as for NapA, we were able to obtain robust transport activity for hNHA2 after the addition of either Li\(^+\) or Na\(^+\) ions (Fig. 6A). Similar to E. coli NhaA (29), steep pH-dependent sensitivity for both Na\(^+\) and Li\(^+\) ions was observed between pH 6.5 and 8.5, with a maximal activity at pH 8.5 (Fig. 6B). The apparent affinities (\(K_m\)) for Li\(^+\) and Na\(^+\) were determined at pH 8.0 to be in a physiological range, ~33 and 67 mM, respectively (Fig. S3B).
To test if Li\(^+\) catalyzed activity of hNHA2 is electrogenic, it was forthwith reconstituted into proteoliposomes in the presence of pyranine, as shown previously for NapA. Although the signal for Li\(^+\) catalyzed proton efflux was small, a consistent difference compared with the nonfunctional ion-binding aspartate variant D279N was observed (Fig. 6C and Fig. S3C). Under these conditions, the addition of valinomycin, however, showed either a small or no clear increase in hNHA2 antiport activity at either pH 7.3 or 6.6, respectively (Fig. 6C and Fig. S3D). To clarify this ambiguous response to valinomycin, we assessed the dependence of NHA2 antiporter activity in the presence of a ~116-mV potassium diffusion potential (Fig. 6D). However, no \(\Delta\Psi\)-driven proton influx could be observed for hNHA2 with traces indistinguishable from that observed for empty liposomes.

**Discussion**

Recently, we reported crystal structures of NapA in both outward- and inward-facing conformations (16). By comparing these two structures, we could show that alternating access to the ion binding site is likely achieved through large, elevator-like rearrangements of the core ion translocation domain. To begin to establish how ion binding and transport is coupled to these structural rearrangements, it is essential to dissect the residues required to drive electrogenic activity. Although a thallium-bound structure of the homologous protein NhaP from *Pyrococcus abyssi* has provided some insight into how Na\(^+\) may bind (30), because NhaP is electroneutral, it provides little insight into the mechanism required to establish proton transport in electrogenic Na\(^+\)/H\(^+\) antiporters.

Here, we have probed the role of the highly conserved transmembrane embedded lysine residue K305, which was implicated recently as a potential proton carrier, because of its salt-bridge formation, to one of the strictly conserved ion-binding aspartates in both NapA and NhaA crystal structures (11, 12, 16).

Poignantly, the K305 residue is also positioned in the same location as the Na\(^+\)/H\(^+\) ion in sodium-driven bile acid symporters, which are structural homologues of Na\(^+\)/H\(^+\) exchangers (8, 31). Our results clearly show that, apart from a K305 to histidine variant, which retains WT behavior, all other variants tested lost their ability for activity (i) to be stimulated by the dissipation of the membrane potential, (ii) to be driven with an electrical gradient alone, and (iii) to generate an electrical potential when driven by ion gradients. Thus, we were able to switch the activity of NapA from being electrogenic to electroneutral by the mutation of lysine K305 to any of the amino acids tested, except for histidine. The exceptional ability of histidine to retain WT activity supports these conclusions, as it is the only amino acid replacement that can still change its protonation state within a physiological pH range of ~4 to 9. In contrast, a K305R variant shows no clear activity even at pH 9, presumably because the pK\(_A\) of arginine is too high and will predominantly be protonated over this pH range (Table 1). Interestingly, the apparent K\(_{in}\) values and the kinetic traces for the K305H are indistinguishable from WT, and, in this regard, it is somewhat surprising that this mutation, as far as we can ascertain, has never been acquired during evolution.

In light of our data, this means that, almost certainly, the residues equivalent to D157 and K305 are the two dominant proton carriers in all homologous electrogenic Na\(^+\)/H\(^+\) antiporters. However, if D156 is not a proton carrier, why is this residue so essential for activity in electroneutral antiporters (1, 2)? Because D156 is not conserved in electroneutral antiporters, we hypothesized that its role in electrogenic antiporters, rather than strictly for ion coordination, is also to interact with K305, providing the required molecular environment for electrogenic antiport. To evaluate the importance of the D156 and K305 interaction, we simultaneously replaced D156 and K305 residues with uncharged polar residues and showed, for the first time, that...
antiporter activity of a nonfunctional aspartate mutant is recoverable (Fig. 5B). Moreover, although D156N-K305Q was active, the D156N-K305N double mutant showed no activity. We speculate that the still polar, but slightly shorter, asparagine residue was not capable of restoring transport activity because the interaction between residues D156N and K305N is lost. It seems likely that D156 mutations might, therefore, be lethal because an unpaired lysine K305 results in highly unfavorable interactions, although more data are required to corroborate this.

Why is an interaction between D156 and K305 residues so important? As can be seen in NapA structures at physiological pH, the salt bridge between D156 and K305 is intact in the absence of a bound Na" ion (11, 16). This observation is in agreement with MD simulations of NhaA, in which the salt-bridge interaction needed to be broken to fully coordinate Na" (12). Our transport data show that, similarly to NhaA (14), the pH-dependent activity of NapA fits a kinetic model in which Na" and protons compete for the same ion binding site. As such, Na" (Li") binding and subsequent salt-bridge breakage should also catalyze proton release from K305. Consistent with this conventional antiport model (32), a recent MD study calculated that the pK_{a} of the equivalent lysine to K305 in NhaA (K300) is only lowered to a deprotonatable value once Na" binds (20). If, however, an uncharged residue replaces K305, the unpaired aspartate D156 will likely influence the efficiency of proton binding to D157. Our experiments using Li"-driven antiport at different pH values support this hypothesis as, compared to WT, the polar electroneutral K305 variants became more active at lower pH values (Fig. 5A).

To explore the general requirement of a transmembrane embedded lysine for conferring electrogenicity in Na"/H" antiporters, we analyzed the energetics of human NHA2 (hNHA2) antiport activity. (A) Representative ACMA fluorescence traces for hNHA2 antiport activity using the setup described for NapA in the legend of Fig. 1A. (B) The pH-dependent sodium (150 mM) and lithium (60 mM) activity for hNHA2. (C) The hNHA2 activity was monitored with liposome-entrapped pH-sensitive fluorophore dye pyranine recorded at 510 nm (excitation 406 and 460 nm). In hNHA2, addition of LiCl (60 mM) at pH 7.3 does not seem to build up a membrane potential, as no additional increase in H" efflux was observed following addition of valinomycin at 4 min (black trace). As a comparison, the inactive aspartate mutant D279N is shown (green trace). (D) The dependency of hNHA2 antiporter activity in the presence of a -116 mV potassium diffusion potential, displaying an absence of ∆Ψ-driven proton influx. The pyranine traces depicting the relative activities are shown for WT NapA (black) and the electroneutral NapA mutant K305A (red), in comparison with hNHA2 (green) and empty liposomes (blue).
prerequisite for facilitating the larger, elevator-like structural transitions seen in NapA crystal structures (11, 16), somewhat analogous to the core domain compaction intermediates seen upon substrate binding in other elevator proteins (33). Certainly, such an Na\textsuperscript{+} (Li\textsuperscript{+})-sensitive salt bridge would be an elegant way to rapidly catalyze ion exchange with the conformational changes required for transport (34–36) (Fig. 7).

Taken together, our data challenges the prevailing assumption that the two ion-binding aspartates are the proton carriers in electrogenic Na\textsuperscript{+}/H\textsuperscript{+} antiporters. This assumption was based on the fact that mutations of these residues results in an inactive transporter and that, in the electroneutral homologues, only one out of the two aspartates is conserved. Here, however, we have shown that (i) the lysine residue K305 is essential for electrogenic transport, (ii) activity of a nonfunctional D156N mutant can be rescued by the further mutation of lysine K305 to glutamine, and (iii) the activity of human NH2A2, which harbors two aspartates, is nonetheless electroneutral, a suggestion that has previously been proposed from in vivo functional experiments (2, 37). We thus conclude that a transmembrane embedded lysine residue is a proton carrier and is essential for electrogenic Na\textsuperscript{+}/H\textsuperscript{+} antiport.

**Materials and Methods**

Expression and Purification of Recombinant NapA and Human NH2A2 Protein. NapA was cloned into pWaldo-GFPα with a C-terminal TEV-cleavable GPF-His\_tag and overexpressed in the E. coli strain Lemo21 (DE3) as described previously (12). NapA and mutants were purified in the detergent DDM (Generon) by Ni-nitrilotriacetic acid (NTA) chromatography and size-exclusion chromatography as described previously. Human NH2A2 (residues 70 to 480) was cloned into the GAL1 inducible TEV-cleavable GFP-His\_tag vector pDGGFP2, transformed into the S. cerevisiae strain FY217 (MATα, ura3–52, lys2Δ201, and pep4Δ), and overexpressed as described previously (27, 28). Human NH2A2 was purified in DDM and cholesteryl hemisuccinimide Tris salt (CHS) by Ni-NTA chromatography and size-exclusion chromatography, as described previously (27).

Na\textsuperscript{+}/H\textsuperscript{+} Cysteine Accessibility (NapA V31C Mutant). Accessibility of cysteine residue was assessed by electrophoresis gel analysis following conjugation of methoxypolyethylene glycol maleimide (mPEG-Sk; Sigma-Aldrich) to solvent-exposed cysteine thiol groups of the NapA mutant V31C. Five micrograms of purified NapA protein and 300 µL of liposomes, reconstituted with only NapA protein (see reconstitution protocol in Coupled Proton Transport Assay Using ATP Synthase and NapA, reconstitution without ATP synthase), were incubated with 5 mM mPEG-Sk for 40 min at room temperature (RT). Liposomes were spun down for 30 min at 215,000 × g. Pellets were resuspended in the sample buffer. The resulting liposome samples with and without purified protein were analyzed by SDS/PAGE (NuPAGE Novex 4 to 12% Bis-Tris Protein Gels; Life Technologies).

**Coupled Proton Transport Assay Using ATP Synthase and NapA.** L-\(\alpha\)-Phosphatidycholine lipids from soybean (soybean lipids, type II, Sigma-Aldrich) were mixed with buffer consisting of 10 mM 3-(N-morpholino)propane sulfonic acid (Mops), pH 6.5, 5 mM MgCl\_2, 100 mM KCl (MMK pH 6.5) to a final concentration of 10 mg/mL (w/v) and vortexed until homogenized. The lipids were flash-frozen in liquid nitrogen and then thawed, in a total of eight freeze-thaw cycles. Liposomes were subsequently extruded using polycarbonate filters (Whatman) with a pore size of 200 nm. For reconstitution experiments, 250 µL of liposomes were destablized by addition of sodium cholate (0.65% final concentration) and mixed with 100 µg of NapA and E. coli F\_GFP, ATP synthase with an ~2:1 molar ratio (NapA:ATP synthase), and the suspension was incubated for 30 min at RT. Detergent was removed using a PD-10 desalting column, and the sample was collected in a final volume of 2.3 mL. Fifty microliters of NapA and ATPase containing proteoliposomes were diluted into 1.5 mL of reaction buffer (MMK buffer in the appropriate pH from 6.3 and 6.9 to 9) containing 2.5 mM ACMA and nigericin. An outward-directed pH gradient (acidic inside) was established by the addition of 130 µM ATP, as detected by a change in ACMA fluorescence at 480 nm using an excitation wavelength of 410 nm in a fluorescence spectrophotometer (Cary Eclipse; Agilent Technologies). After ~3 min equilibration, the activity of NapA WT and mutants thereof was assessed, after addition of the indicated concentrations of NaCl or LiCl, by a change in ACMA fluorescence. The addition of Na\textsuperscript{+}Cl\_2 to a final concentration of 20 mM, dissipates the proton motive force and results in an almost complete dequenching using this experimental setup.

**Functional Assays of NapA in Proteoliposomes Using Pyrane.** ΔP or ΔΨ-driven proton H\textsuperscript{+} transport, resulting from electrogenic Na\textsuperscript{+}/H\textsuperscript{+} exchange activity, was monitored in liposomes using the highly soluble and membrane-impermeable pH-sensitive dye pyranine. An Na\textsuperscript{+} or Li\textsuperscript{+}-sensitive dye pyranine. An Na\textsuperscript{+} or Li\textsuperscript{+}-sensitive dye pyranine. An Na\textsuperscript{+} or Li\textsuperscript{+} gradient was established by the addition of NaCl or LiCl, whereas an electrical membrane potential was established with a K\textsuperscript{+}/valinomycin diffusion potential. Reconstitution of NapA into liposomes containing pyranine was essentially performed as described (11). In brief, a 500 µL liposome suspension (soybean lipids, type II, 20 mg/mL; Sigma-Aldrich) in buffer A (10 mM Mops-Tris, pH 8.0), 20 µL cholate [20% (w/v) stock solution] and 100 µg NapA purified as described previously was added, and the resulting samples were incubated for 30 min at RT. Cholate was removed via a PD-10 gel filtration column (GE Healthcare) equilibrated with buffer, with the reconstituted proteoliposomes collected in ~2.3 mL. The liposome sample was dialyzed overnight against buffer A at 4 °C for 16 h. The resulting dialyseate was diluted to 8 mL by addition of buffer A, and the protoliposomes were collected by ultracentrifugation (200,000 × g, 4 °C, 45 min) and resuspended in ~250 µL 2 mM Mops-Tris, pH 8.0. Subsequently, the proteoliposomes were mixed with 1 mM pyranine (0.1 M stock solution) and the desired Na\textsubscript{2}SO\_4 and K\textsubscript{2}SO\_4 concentrations (300 µM total volume of liposomes mixture). The resulting sample was twice frozen in liquid nitrogen, thawed in water, and briefly sonicated for 30 s. The nonincorporated pyranine was (subsequently) removed by size-exclusion chromatography using a G25 column (GE Healthcare) preequilibrated in buffer containing 2 mM Mops-Tris pH 8.0, 50 mM K\textsubscript{2}SO\_4 and either 0.5 or 50 mM Li\textsubscript{2}SO\_4. The proteoliposomes were collected from the first 1 mL of the eluate. Samples were diluted in 8 mL by addition of buffer A, and the proteoliposomes were collected by ultracentrifugation (200,000 × g, 4 °C, 45 min) and resuspended in ~250 µL 2 mM Mops-Tris, pH 8.0. Subsequently, the proteoliposomes were mixed with 1 mM pyranine (0.1 M stock solution) and the desired Na\textsubscript{2}SO\_4 and K\textsubscript{2}SO\_4 concentrations (300 µM total volume of liposomes mixture). The resulting sample was twice frozen in liquid nitrogen, thawed in water, and briefly sonicated for 30 s. The nonincorporated pyranine was (subsequently) removed by size-exclusion chromatography using a G25 column (GE Healthcare) preequilibrated in buffer containing 2 mM Mops-Tris pH 8.0, 50 mM K\textsubscript{2}SO\_4 and either 0.5 or 50 mM Li\textsubscript{2}SO\_4. The proteoliposomes were collected from the first 1 mL of the eluate. Samples were diluted in 8 mL by addition of the equilibration buffer, concentrated by ultracentrifugation (200,000 × g, 4 °C, 45 min), and resuspended in ~250 µL 2 mM Mops-Tris, pH 8.0. Subsequently, the proteoliposomes were mixed with 1 mM pyranine (0.1 M stock solution) and the desired Na\textsubscript{2}SO\_4 and K\textsubscript{2}SO\_4 concentrations (300 µM total volume of liposomes mixture). The resulting sample was twice frozen in liquid nitrogen, thawed in water, and briefly sonicated for 30 s. The nonincorporated pyranine was (subsequently) removed by size-exclusion chromatography using a G25 column (GE Healthcare) preequilibrated in buffer containing 2 mM Mops-Tris pH 8.0, 50 mM K\textsubscript{2}SO\_4 and either 0.5 or 50 mM Li\textsubscript{2}SO\_4. The proteoliposomes were collected from the first 1 mL of the eluate. Samples were diluted in 8 mL by addition of the equilibration buffer, concentrated by ultracentrifugation (200,000 × g, 4 °C, 45 min), and resuspended in ~250 µL 2 mM Mops-Tris, pH 8.0. Subsequently, the proteoliposomes were mixed with 1 mM pyranine (0.1 M stock solution) and the desired Na\textsubscript{2}SO\_4 and K\textsubscript{2}SO\_4 concentrations (300 µM total volume of liposomes mixture). The resulting sample was twice frozen in liquid nitrogen, thawed in water, and briefly sonicated for 30 s. The nonincorporated pyranine was (subsequently) removed by size-exclusion chromatography using a G25 column (GE Healthcare) preequilibrated in buffer containing only NapA protein (see reconstitution protocol in Coupled Proton Transport Assay Using ATP Synthase and NapA, reconstitution without ATP synthase), were incubated with 5 mM mPEG-Sk for 40 min at room temperature (RT). Liposomes were spun down for 30 min at 215,000 × g. Pellets were resuspended in the sample buffer. The resulting liposome samples with and without purified protein were analyzed by SDS/PAGE (NuPAGE Novex 4 to 12% Bis-Tris Protein Gels; Life Technologies).

**Fig. 7.** Schematic of the NapA transport cycle. See last paragraph of Discussion for details.
and 0.25 mM K$_2$SO$_4$, i.e., no Li$^+$ gradient is present). Exchange activity was initiated after 20 s by addition of valinomycin (10 nM), establishing a membrane potential of −116 mV (inside negative) driving H$^+$ influx (and Li$^+$ efflux). Further potassium diffusion potentials were used to drive H$^+$ influx from 0 mV to −116 mV by modifying the outside-to-inside ratio of K$_2$SO$_4$ based on the Nernst equation. The ratio of pyranine 406 nm/460 nm (emission 510 nm) was converted into pH using a calibration curve, obtained using empty liposomes incubated at different pH values (see Fig. S1E). Proton influx and efflux were converted to ΔpH by subtraction of the pH (t = 0) from the measured pH upon initiation of transport activity.

**Measurement of Membrane Potential Generation.** The preparation of proteoliposomes for Li$^+$-driven antiport activity is identical to that described in Functional Assays of NAP$_A$ in Proteoliposomes Using Pyranine except that ATP synthase was omitted during reconstitution. For measurements, 50 μL of proteoliposomes were mixed in a cuvette containing 1.5 mL of measuring buffer [50 mM Mops-Tris, pH 8.0, 10 mM K$_2$SO$_4$, 10 mM (NH$_4$)$_2$SO$_4$, 2.5 mM DSC$_5$ (S) was added, and a baseline was recorded (excitation 651 nm; emission 675 nm). After 30 s, 50 mM LiCl was added and mixed rapidly. Finally, 130 mM valinomycin was added to abolish the membrane potential.

**ACKNOWLEDGMENTS.** This work was funded by grants from the Swedish Research Council (to D.D. and C.v.B.) and The Knut and Alice Wallenberg Foundation (to D.D. and C.v.B.). The authors are grateful for support to The Center for Biomembrane Research by the Swedish Foundation for Strategic Research. M.C. was a recipient of a Wenner-Gren post-doctoral fellowship, and D.D. acknowledges support from The European Molecular Biology Organization (EMBO) through the Young Investigator Program.