A two-domain elevator mechanism for sodium/proton antiport

Chiara Lee¹, Hao Joo Kang¹, Christoph von Ballmoos², Simon Newstead¹, Povilas Uzdavinys², David L. Dotson³, So Ikawa¹,4,5, Oliver Beckstein¹, Alexander D. Cameron¹,4,5,6 & David Drew¹,4

Sodium/proton (Na⁺/H⁺) antiporters, located at the plasma membrane in every cell, are vital for cell homeostasis. In humans, their dysfunction has been linked to diseases, such as hypertension, heart failure and epilepsy, and they are well-established drug targets. The best understood model system for Na⁺/H⁺ antiport is NhaA from Escherichia coli, for which both electron microscopy and crystal structures are available. NhaA is made up of two distinct domains: a core domain and a dimerization domain. In the NhaA crystal structure a cavity is located between the two domains, providing access to the ion-binding site from the inward-facing surface of the protein. To alternate access to this ion-binding site, however, requires a cavity is located between the two domains, providing access to the ion-binding site from the inward-facing surface of the protein.1,4

Like many Na⁺/H⁺ antiporters, the activity of NhaA is regulated by pH, only becoming active above pH 6.5, at which point a conformational change is thought to occur. The only reported NhaA crystal structure so far is of the low pH inactivated form. Here we describe the active-state structure of a Na⁺/H⁺ antiporter, NapA from Thermus thermophilus, at 3 Å resolution, solved from crystals grown at pH 7.8. In the NapA structure, the core and dimerization domains are in different positions to those seen in NhaA, and a negatively charged cavity has now opened to the outside. The extracellular cavity allows access to a strictly conserved aspartate residue thought to coordinate ion binding, directly, a role supported here by molecular dynamics simulations. To alternate access to this ion-binding site, however, requires a surprisingly large rotation of the core domain, some 20° against the dimerization interface. We conclude that despite their fast transport, Na⁺/H⁺ antiporters operate by a two-domain rocking bundle model, revealing themes relevant to secondary-active transporters in general.

Na⁺/H⁺ antiporters are secondary active transporters that are conserved across all biological kingdoms to maintain the internal pH, cell volume and sodium concentration of the cell, a mechanism first proposed by West and Mitchell⁻°. Na⁺/H⁺ antiporters are members of the large monovalent cation proton antiporter (CPA) superfamily that includes, among others, the CPA1 and CPA2 clades. It is generally thought that Na⁺/H⁺ antiporters from the CPA1 clade utilise electro-neutral sodium-proton exchange (SLC9A1–9 in mammals (also known as NHE1–9)), whereas CPA2 members are thought to be electrogenic (SLC9B1–2 in mammals (also known as NHA1–2)), with stoichiometries of 2H⁺:1Na⁺ and 3H⁺:2Na⁺ ions reported.¹⁻². Like all secondary-active transporters, Na⁺/H⁺ antiporters are thought to operate by an alternating access mechanism; however, the different conformational states of the transport cycle have yet to be determined. The inward-facing structure of the well-characterized bacterial CPA2 protein E. coli NhaA is the only representative crystal structure.

Using fluorescent-based methods, we screened members of the CPA2 clade for their suitability for structural studies. NapA from T. thermophilus, which has 21% sequence identity to human NHA2, was thus identified (see Methods and Supplementary Figs 1a and 2). Although the overall sequence homology to the better characterized E. coli NhaA is low, <15% identity, residues identified to be important for transport in NhaA and mammalian homologues are nonetheless well conserved (Supplementary Fig. 2). Using isolated inside-out membrane vesicles it was previously shown that T. thermophilus NapA is active above pH 6, with maximum activity for sodium at pH 8 (ref. 15), similar to E. coli NhaA. To measure activity in an isolated system, we co-reconstituted purified NapA and E. coli F1F0 ATP synthase into liposomes (see Methods). After establishment of a pH gradient by the addition of ATP, proton efflux was monitored in response to Na⁺ or Li⁺ addition (Fig. 1a, b). In this experimental set-up, the apparent Michaelis constant (Kₘ) values for Na⁺ or Li⁺ were determined to be 4.0 ± 0.3 (mean ± s.d.) or 0.41 ± 0.04 mM, respectively, similar to the affinities from inside-out vesicles (Fig. 1c). In experiments with NapA proteoliposomes trapped with a water-soluble pH sensitive dye, dissipation of the membrane potential (ΔΨ) stimulated exchange activity in the presence of a Na⁺ gradient, confirming the electrochemical nature of NapA (Fig. 1d, bottom). Furthermore, in the absence of a H⁺ or Na⁺ gradient, NapA transport activity was solely driven by ΔΨ (Fig. 1d, top). Taken together, T. thermophilus NapA has a similar antiport profile to E. coli NhaA, and consequently functional and structural studies of the two proteins can complement one another.

The structure of NapA at pH 7.8 was solved by multiple isomorphous replacement with anomalous scattering in combination with multi-crystal averaging. The highest resolution data correspond to a triple mutant of NapA, in which three cysteine residues, which have no apparent effect on functional activity, were introduced to facilitate phasing (see Methods, Supplementary Tables 1 and 2 and Supplementary Fig. 1b). The structure was refined at a resolution of 3 Å, R_factor value of 22.3% and R_free value of 24.8% (Supplementary Table 1, Supplementary Fig. 3 and Methods). Electron density maps at 3.7 Å of wild-type NapA at pH 9.0 show no clear structural differences between the wild-type and the triple cysteine mutant.

NapA is built from 13 transmembrane (TM) helices with an N_out-C_in topology (Fig. 2 and Supplementary Fig. 4). Relative to NhaA, it has an additional helix at the amino terminus. To facilitate comparison to NhaA, we refer to the first helix as TM-1 (Fig. 2a and Supplementary Fig. 4). TM-1 to TM5 and TM7 to TM12 are topologically similar but oppositely orientated in the plane of the membrane (Supplementary Fig. 4). These six-transmembrane-helice topology inverted repeats intertwine to form a core (ion-translocation) and dimerization (interface) domain, and are linked together by TM6 (Fig. 2b and Supplementary Fig. 4). The NapA and NhaA structures are very similar (Fig. 2b). As there is a change in the position of the core relative to the dimerization domain, however, the similarity is best seen when the two domains are superposed separately; root mean squared deviation (r.m.s.d.) of 1.8 Å for 134 out of 148 pairs of Cz atoms of the core domain transmembrane helices, and 1.9 Å for 62 out of 88 of the dimerization domain transmembranes (Supplementary Fig. 5a, b). The change in position of

¹Division of Molecular Biosciences, Imperial College London, London SW7 2AZ, UK. ²Centre for Biomembrane Research, Department of Biochemistry and Biophysics, Stockholm University, SE-106 91 Stockholm, Sweden. ³Center for Biological Physics, Department of Physics, Arizona State University, Tempe, Arizona 85287-1504, USA. ⁴Membrane Protein Laboratory, Diamond Light Source, Harwell Science and Innovation Campus, Didcot, Oxfordshire OX11 0DE, UK. ⁵Research Complex at Harwell Rutherford, Appleton Laboratory, Harwell, Oxford, Didcot, Oxfordshire OX11 0FA, UK. ⁶School of Life Sciences, University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK. ⁷Present address: Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK.

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the core relative to the dimerization domain, which may reflect the difference in the pH at which the transporters were crystallized, gives rise to a large negatively charged cavity that is now open to the outside in contrast to the inward-facing funnel seen in NhaA (Figs 2b and 3b). The interaction between TM2 and TM4 and TM5 tightly closes the cytoplasmic side of the cavity.

NapA, like other bacterial and mammalian Na\(^{+}/H^{+}\) antiporters\textsuperscript{6,17,18}, purifies as a dimer and is clearly dimeric in the structure with an extensive interface burying a surface area of 1,800 Å\(^2\) (Fig. 3 and Supplementary Fig. 1c). In molecular dynamics simulations, the dimer sits entirely within a model membrane bilayer (Fig. 3c). The backbone r.m.s.d. from the starting conformation of each monomer increased to about 2.0 Å over the time course of the simulation (Supplementary Fig. 6), indicating a slow relaxation of the crystal structure in the native-like membrane environment. The dimer has a crystallographic two-fold operator approximately parallel to the transmembrane helices, and it is largely made up of tight hydrophobic helix–helix packing between TM1–1 on one monomer and TM7 on the other. There are also contacts between the ends of TM2 and TM9 (Fig. 3a, b). NapA lacks the β-hairpin domain that makes most of the contact between protoners at the extracellular membrane surface in NhaA\textsuperscript{41,42}. The dimer interface in NapA more closely resembles the dimer interface modelled in the 7 Å electron crystallography structure of NhaP1 (ref. 20), a CPA1 member from Methanocaldococcus jannaschii (formerly Methanococcus jannaschii). NhaP1 also has 13 transmembrane helices built up from two six-transmembrane topology repeat units.

The substrate-binding cavity is open to the extracellular side. The cavity begins above the dimerization interface and funnels between the dimerization and core domains, ending in the middle of the membrane (Fig. 3). It is considerably more open than the inward-facing cavity of NhaA\textsuperscript{4} and is negatively charged, being lined with glutamate residues. Although there is a similar distribution of charged residues in NhaA and NapA, few, including those that have been predicted to be involved in pH sensing\textsuperscript{4}, are conserved between the two proteins (Supplementary Fig. 7). Near the base of the cavity are two highly conserved aspartate residues, Asp 156 and Asp 157, located on TM5 (Supplementary Figs 2 and 7), probably coordinate ion binding on their position, conservation with mammalian Na\(^{+}/H^{+}\) antiporters\textsuperscript{6}, phenotypes of mutants\textsuperscript{1,21}, isothermal titration calorimetry (ITC) experiments\textsuperscript{8} and molecular dynamics simulations\textsuperscript{8}. In NapA, mutation of either residue to alanine\textsuperscript{15} or asparagine abolishes transport activity completely (Supplementary Fig. 1d and Supplementary Table 2). With NapA crystals grown at pH 7.8, the aspartate residues are likely to be deprotonated. Consistent with this, Asp 157 is orientated towards the base of the cavity rather than hydrogen bonding with the backbone of TM4, as seen for Asp 164 in NhaA, an interaction that requires Asp 164 to be protonated (Supplementary Fig. 5c). Using solid-state membrane electrophysiology it was previously shown that NhaA transports cations equally well in either direction and the transport activity profile fits a simple H\(^{+}\) versus Na\(^{+}\) kinetic binding model to a single common site\textsuperscript{25}. To investigate whether Na\(^{+}\) ions would bind as expected, we carried out equilibrium molecular dynamics simulations of outward-facing NapA in a model membrane bilayer. Simulations were carried out with both Asp 157 and Asp 156 deprotonated, as they are likely to be in the crystal structure, and also as combinations of their neutral and charged forms. With both aspartates charged, Na\(^{+}\) ions spontaneously entered the negatively charged extracellular cavity to bind to Asp 157 (Fig. 3c and Supplementary Fig. 8). Na\(^{+}\) ions were concentrated at Asp 157 (Supplementary Fig. 8) and multiple distinct binding and unbinding events could be observed, which is qualitatively consistent with weak binding. By contrast, Na\(^{+}\) ion binding was not observed when Asp 157 was protonated and was markedly reduced when Asp 156 was neutral (Supplementary Fig. 8). In molecular dynamics simulations of
It results in the positive dipole ends of TM4a and the antiparallel crossing over of transmembrane helices is a unique feature. Discontinuous helices are a common feature for ion-binding in transporters,

The two antiparallel discontinuous helices, TM4a–b and TM11a–b, cross which is positioned at the base of the cytoplasmic cavity.

Asp 156 and Asp 157 on TM5 are located next to the point at which

the two antiparallel discontinuous helices, TM4a–b and TM11a–b, cross over in the core domain (Supplementary Fig. 5c). Although discontinuous helices are a common feature for ion-binding in transporters,

the antiparallel crossing over of transmembrane helices is a unique feature of the NhaA fold.

It results in the positive dipole ends of TM4a and TM11a facing one another and likewise the negative dipole ends of TM4b and TM11b. In NhaA, the dipoles are proposed to be neutralized by the side chains of an aspartate (Asp 133; TM4a–b) and a lysine (Lys 300; TM10), respectively (Supplementary Fig. 5c). The lysine (Lys 305) is retained in NapA but the aspartate is replaced by a serine, as it is in human NHA2 (Supplementary Figs 5c and 7). The positions of these two residues are pseudosymmetrically related; the functional significance of this swap is unclear.

Although the mutation of Glu 333 to alanine affected the apparent affinity for Li$^+$ only slightly (<3-fold), the affinity for Na$^+$ was severely decreased (>15-fold), which is in agreement with the results obtained from an Asp 133 to alanine mutation in the NhaA protein (Supplementary Table 2). By contrast, the mutation of the highly conserved Lys 305 to alanine severely decreased both Na$^+$ and Li$^+$ affinity (>20-fold; Supplementary Table 2). It was recently speculated that Lys 300 in NhaA could have more than a stabilizing role and that it could also form part of the pH activation mechanism, as mutation to arginine changed the pH at which NhaA becomes active.

In the NapA structure, Lys 305 forms a salt bridge with Asp 156 (Supplementary Figs 5c and 8a). Notably, we have recently observed a similar interaction in a different crystal form of NhaA at low pH (O.B. et al., manuscript in preparation). We would expect, however, that after cation binding to Asp 156 and Asp 157 the salt bridge is disrupted. As such, the interaction observed here supports the role of lysine in pH activation.

Previously, it was proposed that the exceptionally fast transport of Na$^+$/H$^+$ antiporters would primarily involve local rearrangements of the finely electrostatically balanced discontinuous helices in the core domain. However, the major structural difference between NapA and NhaA is in the position of the core domain in relation to the dimerization domain (Supplementary Fig. 5a, b). With reference to the dimerization...
interface, which seems likely to remain stable during transport across the membrane\(^{19,26}\), the core domain in the NhaA structure is rotated by 21° relative to the core of NapA (Fig. 4a, b). This large rotation of the core domain closes the cavity seen on the outside of NapA and opens the cytoplasmic funnel on the inside, as observed in NhaA (Supplementary Videos 1 and 2). During this process, the two cation binding aspartates, which are in line with the tip of TM8 in the dimerization domain of the outward-facing NapA structure, are shifted 10 Å towards the cytoplasmic surface of the transporter (Fig. 4a). This elevation movement of a substrate-binding domain, in this case to carry Na\(^+\) (Li\(^+\)) ions from one side of the membrane to the other in exchange for protons, resembles that of the transport mechanism seen in the glutamate transporter GltPh (ref. 27). A two-domain transport mechanism was also recently predicted in NhaA, on the basis of a consideration of the two symmetry-related inverted repeats as well as elastic network models and biochemical cross-linking\(^{28}\). Such a mechanism was also proposed for the bile acid sodium symporter ASBT\(_{NM}\) (ref. 24), which is a structural homologue of NhaA and NapA.

In summary, the structure of NapA is consistent with a single ion-translocation site mechanism, of which the strictly conserved Asp 157 (Asp 164 in NhaA) is ideally positioned for binding ions in outward-facing states (Fig. 4b). To provide alternating access to the substrate-binding domain, in this case to carry Na\(^+\) (Li\(^+\)) ions from one side of the membrane to the other in exchange for protons, resembles that of the transport mechanism seen in the glutamate transporter GltPh (ref. 27). A two-domain transport mechanism was also recently predicted in NhaA, on the basis of a consideration of the two symmetry-related inverted repeats as well as elastic network models and biochemical cross-linking\(^{28}\). Such a mechanism was also proposed for the bile acid sodium symporter ASBT\(_{NM}\) (ref. 24), which is a structural homologue of NhaA and NapA.

**METHODS SUMMARY**

NapA was cloned into a cleavable green fluorescent protein (GFP)–His\(_{6}\) fusion vector pWaldGFPe\(^{18}\). The fusion protein was expressed in *E. coli*, solubilized in 1% dodecyl-β-D-maltopyranoside (DDM), and purified to homogeneity in either DDM or 1% nonyl-β-D-maltopyranoside. The NapA proteoliposome uptake assay was modified as previously described\(^{17}\). Crystals were grown at either pH 7.8 or 9.0 by the vapour diffusion method. Data were collected on beamlines I02 and I03 at the Diamond Light Source or ID 23-1 and 23-2 beamlines at the European Synchrotron Radiation Facility. The protein was derivatized before crystallization by incubation with 2.5 mM mercury acetate. The NapA structure was solved by multiple isomorphous replacement with anomalous scattering in combination with molecular dynamics simulation and crystal averaging and refined at a resolution of 3 Å.

**Figure 4 | Alternating access model of sodium-proton antiporter.** a, Surface representation showing a section through the outward-facing NapA structure (left) and inward-facing NapA model (right) (see Methods and Supplementary Videos 1 and 2). The position of Asp 157 is denoted by an asterisk, and the helices have been coloured as in Fig. 3. For the sake of clarity, only one molecule is shown. b, Schematic of the proposed transport mechanism that illustrates the conformational changes with the core moving against the dimerization domain. Protons (shown in blue) bind to the core domain in the outward-facing state (left) causing it to switch to the inward-facing state (right). On the inside, protons are exchanged for sodium (green) and the core domain moves back to the outside. Asp 157 (shown in red), which is crucial for binding both one of the protons and the sodium ion, moves approximately 10 Å during this process. Other residues involved in ion binding are not shown.

Full Methods and any associated references are available in the online version of the paper.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** A.D.C. and D.D. designed the project. Cloning, expression screening, protein purification and crystallographic data were collected by C.L. and D.D. with assistance from H.J.K., S.N., S.I. and A.D.C. Data collection and structural determination were carried out by C.L., D.D. and A.D.C. Experiments for functional analysis were designed by C.v.B. and D.D. and carried out by C.v.B., C.L., P.U. and D.D. Molecular dynamics simulations were carried out by D.D. and O.B. A.D.C. and D.D. wrote the manuscript with contributions from C.L., H.J.K., C.v.B. and O.B.

**Author Information** The coordinates and the structure factors for NapA have been deposited in the Protein Data Bank under accession code 4BWZ. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.D.C. (acameron@warwick.ac.uk) or D.D. (ddrew@imperial.ac.uk).
METHODS

Thermus thermophilus NapA sequence (Uniport accession number Q7ZIM4); residues progressively substituted to cysteine are underlined, and additional C-terminal residues retained after TFE cleavage are shown in italics (see next section for cloning details).

MHGAEHELIFLYLLAQLAMIFKRLNQPVVVGLVLGVLGVGLYW
HGEIEILFALGAVFFMVGETLGKLIDKAVEFLAVLWALCYG
LYELELGTFALPFLGLTAVSVGITARVLQELGILPSYRLGAADAVDV
GILVAVVGNTGEQTGVQAIRTVLVSGVFLAVLFSTLALRPLERPV
GSPGLFALGAVGLMVLMASIALGPIAVGFGLMLSEVEYKRYELPIFIASES
FLAPPFAMVFVRLSALPSVLVLAIGTVTIALGVKSLGALTQGV
RSALTWVGMAPREGVGLVALGLKAGAVNEEYAIYLMVFVFTFLAPF
AKLPILFAATERAARKEGNSLQFYYCGT

Expression screening, mutagenesis, protein purification and characterization.

NhaA homologues were cloned as green fluorescent protein (GFP)–His6 fusions into the vector pWaldGFP (ref. 13), as fluorescence from the C-terminal GFP fusion is a reliable reporter of membrane-integrated expression.29 The monodispersity of expressed fusions were screened in a number of different detergents by fluorescence-detection size-exclusion chromatography.30 NapA from T. thermophilus was selected as a suitable candidate showing stability in a wide range of detergents including the harsh detergent N-dodecyl-N,N-dimethylamine-N-oxide.31 Expression levels of the protein were initially low in standard culture conditions, but improved significantly using MemStar, which is a new strategy for boosting expression of membrane proteins in E. coli (C.L. et al., manuscript in preparation). In brief, Lemo21(DE3) cells32 were grown at 37 °C in PASM-5025 media33, with and without selenomethionine incorporation, and induced with 4 μM isopro- pyl-β-D-thiogalactoside (IPTG) at an absorbance (A600nm) of 0.5 for overnight incubation at 25 °C.

Wild-type NapA and mutants generated by Quikchange protocol (Agilent Technologies) were purified essentially as previously described.33 Membranes were isolated from E. coli cultures and solubilized in 1% dodecyl-β-D-maltosylpyanoside (DDM; Generon) for 2 h in buffer containing 1× PBS, 150 mM NaCl and 10 mM imidazole. The suspension was cleared by ultracentrifugation at 120,000g for 1 h. The sample was mixed with 1 ml of Ni-NTA Superflow resin (Qiagen) per 1 mg of GFP–His6, and incubated for 2 h at 4 °C. Slurry was loaded onto a glass Econo-Column (Bio-Rad) and washed in 1× PBS buffer containing 0.1% DDM, 150 mM NaCl and 20 mM imidazole for 20 column volumes. Bound material was washed for a further 20 column volumes in the same buffer containing 50 mM imidazole. The NapA–GFP–His6 fusion was eluted in two column volumes of 1× PBS buffer containing 6% nonyl-β-D-maltopyanoside (NM; Generon), 150 mM NaCl and 250 mM imidazole. The eluted protein was dialysed overnight in the presence of stoichiometric amounts of His6-tagged tobacco etch virus protease in 1.5 l of buffer 250 mM imidazole. The eluted protein was dialysed overnight in the presence of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.5% NM. Dialysed sample containing NapA transport activity. In this set-up, we followed ΔNa or Δv driven H+ transport as a consequence of electrogenic Na+/H+ exchange activity. In this sensitive assay, liposomes containing the highly soluble and membrane impermeable pH sensitive dye pyranine were used to follow H+ influx or efflux. A Na+ gradient was established by the addition of NaCl, whereas an electrical membrane potential was established with a K+/valinomycin diffusion potential.

Reconstitution of NapA into liposomes containing pyranine was essentially performed as described.35 In brief, to a 500 μl liposome (40–80 nm) suspension (soy bean lipids, type II, SIGMA, 20 mg ml−1) in buffer A (10 mM MOPS-PO4, pH 7.5), 45 μl cholate (20% stock solution) and 17 μl NapA (50 μM, purification buffer) was added and incubated for 30 min at room temperature with occasional mild mixing. The cholate was removed via a PD-10 gel filtration column (GE Healthcare) equilibrated with buffer A and the proteoliposomes in the void volume were collected (~1.2 ml). They were diluted to 8 ml with buffer A, collected via ultra-centrifugation (200,000g; 4 °C, 30 min) and resuspended in 250 μl buffer A. Then, 125 μl of proteoliposomes was mixed with 1 mM pyranine (0.1 M stock solution) and the desired NaPO4 and K2SO4 concentrations, frozen in liquid nitrogen, thawed in water and briefly sonicated in a bath type sonicator (2 × 5 s). The freeze/sonication procedure was repeated once. The external pyranine was subsequently removed via a precapped G25 gel filtration column (GE Healthcare) and the proteoliposomes, equilibrated in buffer A, were collected from the void volume of the column.

Pyranine fluorescence measurements monitoring pH changes on the inside of the proteoliposomes were performed as described.35 Typically, an amount of 20 μl liposomes containing the desired Na+ and K+ concentrations was mixed with 2.5 ml buffer containing the same buffer with the appropriate salt concentrations. After 30 s, exchange activity was either initiated by the addition of 50 mM Na+ to the outside (0.5 mM Na+ on the inside), establishing an inwardly-directed Na+ gradient (~120 mV), and leading to H+ efflux. Accordingly, in a system in the absence of Na+ gradient (50 mM Na+ on both sides), but in the presence of a K+ gradient (100 mM K+ inside, 1 mM K+ outside liposomes), addition of valinomycin (10 mM) established a membrane potential of ~120 mV (inside negative) driving H+ influx (and Na+ efflux).36

Crystallization and preliminary screening. Crystals were grown at 20 °C using the hanging drop vapour diffusion method. A 1μl aliquot of pure protein was mixed 1:1 with reservoir solution containing 0.001 M zinc sulphate, 0.05 M HEPES, pH 7.8 and 22–36% PEG 600 (monants) or 0.05 M glycine, pH 9.0, 0.5 M magnesium acetate, 26% PEG 400 (wild type). Crystals appeared overnight and reached maximum size after 3–4 days. For specified crystals, coverslips were transferred for overnight incubation with reservoir solution containing 2% increments of PEG 400. Dehydrated crystals in 30–36% PEG 400 were finally soaked with 1 μl reservoir solution containing 1% NaCl and 40% PEG 400 followed by flash freezing in liquid nitrogen before data collection.

Data were collected at the European Synchrotron Radiation Facility and Diamond Light Source. Most of the crystals were triclinic, however, very occasionally crystals were collected from an orthorhombic crystal of the triple mutant that had been reannealed on the beamline. This crystal was grown with 0.025% dichloroethane (Hampton Research) as an additive.

Structure determination. To obtain phases, two mutagenesis mutants were introduced into the protein to enable derivatization with mercury. Three positions were chosen (Met 20, Val 66 and Val 328) and single, double and triple mutants created as described above. Mercury-derivatized protein was prepared by incubation of the protein at 20 °C for 1 h with 2.5 mM mercury acetate. The structure was solved using MIRAS from four triclinic crystals (native, mercury derivatized and seleno-methionine) as shown in Supplementary Table 1. Data were processed using the Xds pipeline37 to DXS, with further processing using the CCP4 suite of programs.38 Heavy atom sites were located from anomalous difference Patterson maps of the double mutant using the program RSPS.39 Phases were calculated and refined
were less than 3.8 Å apart after superposition. Figures were drawn using Pymol and structure restraints were applied and refinement was interspersed with rebuilding. Peaks in anomalous difference maps indicated the presence of two zinc ions bound to the protein on the periplasmic surface to residues at the N terminus of TM1 and the loop between TM1 and TM2. The final refinement statistics are shown in Supplementary Table 1.

Molecular dynamics simulations of the NapA dimer in a mixed POPE/POPG bilayer were carried out with the Gromacs simulation package, either version 4.5.5 or a development version of 4.6. All simulations employed the CHARMM force field including CMAP and the original TIP3P water model and updated CHARMM parameters for POPE and POPG lipids (CHARMM-GUI), as implemented in Gromacs. The ratio of POPE to POPG molecules was about 4:1 to approximate the major components of the E. coli membrane. We used a multi-scale approach to embed the dimer into the membrane and performed the velocity rescaling algorithm for the thermostat (time constant 0.1 ps) and pressure (time constant 323 K and pressure 105 Pa) using the S-LINCS algorithm or SETTLE (for water molecules). The classical equations of motion were integrated with a leap frog integrator and a time step of 2 fs.

Conformations were selected for analysis.

The simulation protocol included an initial energy minimisation of the atomic system and a 1-ns equilibrium simulation during which the protein heavy atoms were restrained with a harmonic force with force constant of 1,000 kJ mol⁻¹ nm⁻². An initial unrestrained simulation of the dimer with Asp 156 and Asp 157 in their default protonation state was run for 100 ns. Eight additional 100-ns simulations were performed in three sets (two simulations in set 1, five in set 2, one in set 3). The starting configuration for each set was generated from the last frame of the initial simulation by exchanging any sodium ion within 3 Å of Asp 157 or Asp 156 with a random bulk water molecule. Repeats in each set always differed by the seed of the random number generator, thus leading to differing initial assignments of velocities and generation of independent trajectories through the stochastic component of the velocity rescaling thermostat, as seen from the different r.m.s.d. time series in Supplementary Fig. 6.

Additional simulations were performed to assess the influence of the protonation state of the conserved residues Asp 156 and Asp 157 on sodium binding as performed previously for NhaA. Two independent 100-ns simulations were performed for each of (1) Asp 156 protonated (negatively charged) and Asp 157 protonated (neutral), (2) Asp 156 protonated and Asp 157 deprotonated, and (3) both Asp 156 and Asp 157 protonated. Charge states were modified with the Gromacs tool protamine. These simulations were started from the 100-ns frame of the initial simulation (default charge states) with any sodium ions near the aspartates exchanged with a bulk water molecule as a well-equilibrated starting conformation. In each case, a 1-ns position restraint simulation (as above) was followed by a 100-ns production equilibrium simulation.

Simulations were analysed with MDAnalysis and Gromacs tools. To calculate the sodium density, data from all nine simulations with the deprotonated aspartates were used at 1-ps intervals. Ion binding and unbinding to each protomer appeared to be independent so that data for both protomers were combined by superpositioning trajectories of both protomer A and protomer B on the coordinates of protomer A from the start of the initial simulation. The density was calculated by histogramming sodium coordinates in cubic volume elements at a resolution of 1 Å in a fixed coordinate system defined by the initial coordinates of protomer A. The thicknesses of bilayer regions were calculated from the distributions of the headgroup phosphate and acyl chain atoms along the membrane normal, using trajectories superpositioned on the dimer.

Images showing simulation data were prepared with VMD and the Bendix plugin for curved helices or UCSF Chimera.
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