Mechanism of extracellular ion exchange and binding-site occlusion in a sodium/calcium exchanger

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Na+/Ca2+ exchangers use the Na+ electrochemical gradient across the plasma membrane to extrude intracellular Ca2+ and play a central role in Ca2+ homeostasis1–4. NCX catalyzes the uphill extrusion of intracellular Ca2+ across the cell membrane by coupling this process to the downhill permeation of Na+ into the cell5,6, with a 3:1 Na+/Ca2+ stoichiometry6–11. This reaction is, however, inherently reversible; its direction is dictated by only the transmembrane electrochemical ion gradients1. The mechanism of NCX proteins is therefore highly likely to be consistent with the alternating-access model of secondary active transport12. The basic functional unit for ion transport in NCX consists of ten membrane-spanning segments13,14 comprising two homologous halves. Each of these halves contains a highly conserved region, referred to as α-repeat, which is known to be important for ion binding and translocation15,16; in eukaryotic NCX, the two halves are connected by a large intracellular regulatory domain17,18, which is absent in microbial NCX15,19 (Supplementary Fig. 1).

Despite a long history of physiological and functional studies, the molecular mechanism of NCX has been elusive, owing to the lack of structural information. Our recent atomic-resolution structure of NCX_Mj from Methanococcus jannaschii (NCX_Mj) bound to Na+, Ca2+ or Sr2+ in various occupancies and in an apo state. This analysis defines the binding mode and relative affinity of these ions, establishes the structural basis for the anticipated 3:1 Na+/Ca2+-exchange stoichiometry and reveals the conformational changes at the onset of the alternating-access transport mechanism. An independent analysis of the dynamics and conformational free-energy landscape of NCX_Mj in different ion-occupancy states, based on enhanced-sampling molecular dynamics simulations, demonstrates that the crystal structures reflect mechanistically relevant, interconverting conformations. These calculations also reveal the mechanism by which the outward-to-inward transition is controlled by the ion occupancy, thereby explaining the emergence of strictly coupled Na+/Ca2+ antiport.

RESULTS
Extracellular Na+ binding

The assignment of the four central binding sites identified in the previously reported NCX_Mj structure19 was hampered by the presence of both Na+ and Ca2+ in the protein crystals. To conclusively clarify this assignment, we first set out to examine the Na+ occupancy state of these sites without Ca2+. We grew crystals in 150 mM NaCl with the lipidic cubic phase (LCP) technique20, then slowly replaced the crystals in different concentrations of NaCl and EGTA (Online Methods). X-ray diffraction of these soaked crystals revealed a Na+-dependent variation in the electron density distribution at sites S_{ext}, S_{Ca} and S_{int}, thus indicating a Na+-occupancy change (Fig. 1c). Occupancy

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refinement indicated that two Na+ ions bind to S_{mid} and S_{Ca} at low Na+ concentrations (Fig. 1c), with a slight preference for S_{int} (Table 1). Binding of a third Na+ to S_{ext} occurs at higher concentrations, because there was no density at that site at Na+ concentrations of 10 mM Na+ or lower (Fig. 1c); S_{ext} is however partially occupied at 20 mM Na+ and fully occupied at 150 mM Na+ (Fig. 1c). The Na+ occupation at S_{Ca}, together with the expected 3:1 Na+/Ca2+ stoichiometry, suggests that our previous assignment of the S_{mid} site must be reevaluated. Indeed, two observations indicate that a water molecule rather than a Na+ ion occupies S_{mid}, as has been predicted in a recent simulation study. First, the electron density at S_{mid} does not substantially depend on the Na+ concentration. Second, the protein coordination geometry at S_{mid} is clearly suboptimal for Na+ (Supplementary Fig. 1d). The water molecule at S_{mid} forms hydrogen bonds with the highly conserved E54 and E213 (Supplementary Fig. 1d), thereby stabilizing their orientation and allowing proper coordination of multiple Na+ ions at S_{ext}, S_{Ca}, and S_{int}. It can be inferred from this assignment that E54 and E213 are ionized, whereas D240, which flanks S_{mid} (and is replaced by asparagine in eukaryotic NCX) would be protonated, as indicated by the abovementioned simulation study.

Na+-dependent conformational change

The NCX_Mj structures in various Na+ concentrations (Table 2) also reveal that Na+ binding to S_{ext} is coupled to a subtle but important conformational change (Fig. 2). When Na+ at high concentrations binds to S_{ext}, the N-terminal half of TM7 is bent into two short helices, TM7a and TM7b (Fig. 2a, PDB 5HXE). TM7b occludes the four central binding sites from the external solution, and the backbone carboxyl of A206 coordinates the Na+ ion (Fig. 2b-d). However, when S_{ext} becomes empty at low Na+ concentrations, TM7a and TM7b become a continuous straight helix (Fig. 2a, PDB 5HWY), and the carboxyl group of A206 retracts (Fig. 2b-d). TM7a also forms hydrophobic contacts with the C-terminal half of TM6. These contacts are absent in the structure with Na+ at S_{Ca}, in which there is an open gap between the two helices (Fig. 2b). This difference is notable because TM6 and TM1 are believed to undergo a sliding motion relative to the rest of the protein when the transporter switches to the inward-facing conformation. The straightening of TM7ab also opens a passageway from the external solution to S_{ext} and S_{mid}, while S_{Ca} and S_{int} remain occluded (Fig. 2d). Thus, the structures at high and low Na+ concentrations (PDB 5HXC) represent the outward-facing occluded and partially open states, respectively. This conformational change is dependent on the Na+ occupancy of S_{ext} and occurs when Na+ already occupies S_{int} and S_{Ca}. Our crystallographic titration experiment indicated that the K_{1/2} of this Na+-driven conformational transition is ~20 mM. At this concentration, S_{ext} is partially occupied, and the NCX_Mj crystal is a mixture of both the occluded and partially open conformations PDB 5HXC. This structurally derived Na+ affinity agrees well with the external Na+ concentration required for NCX activation in eukaryotes. The finding that the Na+-occupancy change from two to three ions coincides with a conformational change of the transporter also provides a rationale to the Hill coefficient of the Na+-dependent activation process in eukaryotic NCX.

Extracellular Ca2+ and Sr2+ binding and competition with Na+

To determine how Ca2+ binds to NCX_Mj and competes with Na+, we first titrated the crystals with Sr2+ (Online Methods and Table 3). Sr2+ is transported by NCX similarly to Ca2+ (refs. 23,24) and is distinguishable from Na+ by its higher electron density intensity. Protein crystals soaked with 10 mM Sr2+ and 2.5 mM Na+ revealed a strong electron density peak at site S_{Ca}, thus indicating binding of a single Sr2+ ion (Fig. 3a, PDB 5HXS). The Sr2+-loaded NCX_Mj structure adopts the partially open conformation observed at low Na+ concentrations. Binding of Sr2+, however, excludes Na+ entirely. Crystal titrations with decreasing Sr2+ or increasing Na+ demonstrated that Sr2+ binds to the outward-facing NCX_Mj with low affinity and that it can be outcompeted by Na+ even at low concentrations (Supplementary Note 1 and Supplementary Fig. 2a,b). Thus, in 100 mM Na+ and 10 mM Sr2+, Na+ completely replaced Sr2+ (Fig. 3a) and reverted NCX_Mj to the Na+-loaded, fully occluded state (PDB 5DQ).

Similar titration experiments showed that Ca2+ binding and Sr2+ binding to NCX_Mj are not exactly alike. The electron density distribution of native outward-facing NCX_Mj from crystals grown in 150 mM Na+.

Figure 1 Na+ binding to outward-facing NCX_Mj. (a) Overall structure of native outward-facing NCX_Mj from crystals grown in 150 mM Na+. N- and C-terminal halves are colored yellow and cyan, respectively. Colored spheres represent the bound Na+ (green) and water (W, red). (b) Structural details and definition of the four central binding sites. Only residues flanking these sites are shown for clarity (in this and subsequent figures). The electron density (gray mesh, 1.9 Å F_{o}- F_{c} ion omit map contoured at 4σ) at S_{mid} was modeled as water (red sphere), and that at S_{ext}, S_{Ca} and S_{int} was modeled as Na+ ions (green spheres). (c) Concentration-dependent change in Na+ occupancy. All F_{o}- F_{c} ion omit maps are calculated to 2.4 Å and contoured at 3σ. The displacement of A206 reflects the Na+-dependent conformational change from the partially open to the occluded state (observed at low and high Na+ concentrations, respectively). At 20 mM Na+, both conformations coexist. No significant changes were observed in the side chains involved in ion or water coordination at the S_{Ca}, S_{int} and S_{mid} sites.

Table 1 Concentration-dependent Na+ occupancy of outward-facing NCX_Mj

| Sites      | 2.5 mM Na+ | 10 mM Na+ | 20 mM Na+ | 100 mM Na+ | 150 mM Na+ |
|------------|------------|-----------|-----------|------------|------------|
| S_{int}    | 0.84       | 0.92      | 0.99      | 0.96       | 1.00       |
| S_{Ca}     | 0.75       | 0.87      | 0.97      | 1.00       | 1.00       |
| S_{ext}    | –          | –         | 0.59      | 0.79       | 0.93       |
| Total      | 1.58       | 1.79      | 2.55      | 2.75       | 2.93       |

Conformation: Partially open, Partially open, Mixed, Occluded, Occluded.

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from crystals soaked in high Ca\(^{2+}\) and low Na\(^+\) (Table 4) indicates that Ca\(^{2+}\) binds to Smid as well as Sc, with a preference for Sc (Fig. 3b, PDB 5HXR). Binding of Ca\(^{2+}\) to both sites simultaneously is highly improbable because of their proximity, and at least one water molecule appears to coordinate the ion (Fig. 3b). The partial Ca\(^{2+}\) occupancy at Smid is probably caused by D240, which flanks this site and can in principle coordinate Ca\(^{2+}\). Previous functional and computational studies, however, have indicated that D240 becomes protonated during transport\(^2\)\(^2\). Indeed, in most NCX proteins D240 is substituted by asparagine, thus probably weakening or abrogating Ca\(^{2+}\) binding to Smid. Sc is therefore the functional Ca\(^{2+}\) site. Similarly to Sr\(^{2+}\), Ca\(^{2+}\) binds with low affinity to outward-facing NCX_Mj and is readily displaced by Na\(^+\) (Supplementary Note 1 and Supplementary Fig. 2c). This finding is consistent with physiological and biochemical data for both eukaryotic NCX and NCX_Mj indicating that the apparent Ca\(^{2+}\) affinity is much lower on the extracellular than the cytoplasmic side\(^2\)\(^1\),\(^2\)\(^3\),\(^2\)\(^5\)–\(^2\)\(^7\). Specifically, our crystallographic titration assay indicated that Ca\(^{2+}\) binds with submillimolar affinity, a result in good agreement with the external apparent Ca\(^{2+}\) affinities deduced functionally for cardiac NCX (K\(_m\) of ~0.32 mM)\(^2\)\(^6\) and NCX_Mj (K\(_m\) of ~0.175 mM)\(^2\)\(^1\).

Together, these crystal titration experiments demonstrated that the four binding
Figure 4 Spontaneous changes in the structure of outward-occluded, fully Na+-occupied NCX_Mj. (a) Representative simulation snapshots of NCX_Mj with Na+ bound at S_{ext}, S_{Ca} and S_{int} (orange cartoons, green spheres) and with Na+ bound only at S_{Ca} and S_{int} (marine cartoons, yellow spheres) (b) Close-up of the backbone of the N-terminal half of TM7 (TM7ab), in the same Na+-occupancy states depicted in a. Black lines indicate (i, i + 4) carbonyl-amide pairs along the helix; specific O-N distances are indicated in angstroms (magenta). (c) Representative simulation snapshots (same as above) with Na+ bound at S_{Ca} and S_{int} (marine cartoons, yellow spheres) and without any Na+ bound (gray cartoons). (d) Close-up of the ion-binding region in the fully Na+-occupied state. Approximate distances between TM2, TM3 and TM7 are indicated in angstroms. (e) Close-up view of the ion-binding region in the partially Na+-occupied state. (f) Close-up view of the ion-binding region in the Na+-free state. (g–i) Mathematical descriptors of the changes described above, calculated from the simulations of each Na+-occupancy state depicted in a–f. (g) Probability distributions of an analytical descriptor of the backbone hydrogen-bonding pattern in TM7ab (Online Methods, equation (2)). (h) Mean value ± s.d. from 250-ns time-series of a quantitative descriptor of the solvent accessibility of the S_{Ca} site (Online Methods, equation (1)). (i) Mean value ± s.d. (from 250-ns time-series) of a quantitative descriptor of the solvent accessibility of the S_{Ca} site (Online Methods, equation (1)).
We initially performed a series of exploratory MD simulations to examine which features of the NCX_Mj structure might depend on the ion-binding-site occupancy. Specifically, we first simulated the outward-occluded form, in the ion configuration that we previously predicted and now confirmed by the high-Na\(^+\) crystal structure described above (Fig. 1b). That is, Na\(^+\) ions occupy S_{ext}, S_{Ca} and S_{int}, whereas D240 is protonated, and a water molecule occupies S_{mid}. We then relocated the Na\(^+\) ion at S_{ext} from the site to the bulk solution (Online Methods) and then allowed this system to evolve freely over time. We subsequently displaced the Na\(^+\) ions at S_{Ca} and S_{int} and carried out an analogous simulation. These initial simulations revealed noticeable changes in the transporter, which were consistent with those observed in the new crystal structures. The most notable change upon displacement of Na\(^+\) from S_{ext} was the straightening of TM7ab (Fig. 4a). When three Na\(^+\) ions are bound, TM7ab primarily folds as two distinct, noncollinear α-helical fragments, owing to the loss of the backbone carbonyl-amide hydrogen bonds between F202 and A206, and T203 and F207 (Fig. 4b). This distortion occludes S_{ext} from the exterior (Fig. 4d, h, i) and appears to be induced by the Na\(^+\) ion itself, which pulls...
the carbonyl group of A206 into its coordination sphere (Fig. 4g). However, with $S_{\text{ext}}$ empty, TM7ab forms a canonical $\alpha$-helix (Fig. 4a,b,g), thereby creating an opening between TM3 and TM7, which in turn allows water molecules from the external solution to reach into $S_{\text{ext}}$ (Fig. 4c,h,i); i.e., the transporter is no longer occluded. Displacement of Na$^+$ from $S_{\text{Ca}}$ and $S_{\text{int}}$ induces further changes (Fig. 4c). The most noticeable change is an increased separation between TM7 and TM2 (Fig. 4f), which had previously been brought together by concurrent backbone interactions with the Na$^+$ ion at $S_{\text{Ca}}$ (Fig. 4d,e). TM1 and TM6 also slide further toward the membrane center relative to the outward-occluded state (Fig. 4c). Together, these changes open a second aqueous channel leading directly into $S_{\text{Ca}}$ and $S_{\text{int}}$ (Fig. 4f,h,i). The transporter thus becomes fully outward open.

To more rigorously characterize the influence of the ion-occupancy state on the conformational dynamics of the exchanger, we carried out a series of enhanced-sampling MD calculations designed to reversibly simulate the transition between the outward-occluded and fully outward-open states and thus quantify the free-energy landscape encompassing these states (Online Methods). As above, we initially examined three occupancy states: with Na$^+$ in $S_{\text{ext}}$, $S_{\text{Ca}}$ and $S_{\text{int}}$ with Na$^+$ in only $S_{\text{Ca}}$ and $S_{\text{int}}$ and without Na$^+$.

These calculations demonstrate that the Na$^+$-occupancy state of the transporter has a profound effect on its conformational free-energy landscape. When all Na$^+$ sites are occupied, the global free-energy minimum corresponds to a conformation in which the ions are maximally coordinated by the protein (Fig. 5a,c); TM7ab is bent and packs closely with TM2 and TM3, and so the binding sites are occluded from the solvent (Fig. 5b). At a small energetic cost, however, the transporter can adopt a metastable ‘half-open’ conformation in which TM7ab is completely straight, and $S_{\text{ext}}$ is open to the exterior (Fig. 5a,b). The Na$^+$ ion at $S_{\text{ext}}$ remains fully coordinated, but an ordered water molecule now mediates its interaction with the carbonyl group of A206, thus relieving the strain on the backbone hydrogen bond between TM2 and TM3 (Fig. 5c). This semiopen conformation is nearly identical to that found to be the most probable when Na$^+$ occupies only $S_{\text{Ca}}$ and $S_{\text{int}}$ (two Na$^+$ ions; Fig. 5a), thus demonstrating that binding (or release) of Na$^+$ to $S_{\text{ext}}$ occurs in this metastable conformation. Interestingly, this doubly occupied state can also access conformations in which the second aqueous channel leading to $S_{\text{Ca}}$ between TM7 and TM2 and over the gating helices TM1 and TM6, as mentioned above, also opens (Fig. 5b,c). Crucially, however, the free-energy landscape for this partially occupied state demonstrates that the occluded conformation is no longer energetically feasible (Fig. 5a). Displacement of the two remaining Na$^+$ ions from $S_{\text{Ca}}$ and $S_{\text{int}}$ further reshapes the free-energy landscape of the transporter (no ions; Fig. 5a), which now can adopt only a fully open state featuring the two aqueous channels (Fig. 5b,c). The transition to the occluded state in this apo state is again energetically unavailable.

From a mechanistic standpoint, the open and semiopen states are each compatible with two different Na$^+$ occupancies, thus explaining how sequential Na$^+$ binding to energetically accessible conformations (before those binding events) progressively reshapes the free-energy landscape of the transporter; by contrast, the occluded conformation is forbidden unless the Na$^+$ occupancy is complete. This processivity is logical because three Na$^+$ ions are involved but also implies that in the Ca$^{2+}$-bound state, which includes a single ion, the transporter ought to be able to access all three conformations: the outward-open state, to allow release (or rebinding) of Ca$^{2+}$; the occluded conformation, in transition to or from the inward-facing state; and therefore the semiopen intermediate between the outward-open and occluded forms. By contrast, occupancy by H$^+$, which, as previously mentioned, is not transported, might be compatible with a semiopen state as well as with the fully open conformation but should not be conducive to occlusion.

To assess this hypothesis, we carried out enhanced-sampling simulations for the Ca$^{2+}$- and H$^+$-bound states of outward-facing NCX_Mj, which were analogous to those described above for Na$^+$ (details on how the structures of the Ca$^{2+}$-bound state were predicted in Supplementary Note 2 and Supplementary Figs. 3 and 4). The calculated free-energy landscape for Ca$^{2+}$-bound NCX_Mj confirms the hypothesis outlined above (one Ca$^{2+}$ ion, Fig. 6a) and is consistent with NCX_Mj transporting a single Ca$^{2+}$. The occluded, dehydrated conformation is one of the major energetic minima, but clearly the exchanger can also adopt the semiopen and open states required for Ca$^{2+}$ release and Na$^+$ entry, via either of the aqueous access channels that lead to $S_{\text{ext}}$ and $S_{\text{Ca}}$ (Fig. 6b,c). By contrast, protonation of E54 and E213 makes the occluded conformation energetically unfavorable, consistently with the fact that NCX_Mj does not transport protons; in this H$^+$-bound state, however, the exchanger can adopt the semiopen conformation captured in the low pH, apo crystal structure (two H$^+$ ions, Fig. 6a–c).
This systematic computational analysis of outward-facing NCX_Mj clearly demonstrates that the alternating-access and ion-recognition mechanisms in this Na+/Ca2+ exchanger are coupled through the influence of the bound ions on the free-energy landscape of the protein, which in turn determines whether the occluded conformation is energetically feasible. This occluded conformation, which is a necessary intermediate between the outward- and inward-open states, and which entails the internal dehydration of the protein, is attainable only upon complete occupancy of the binding sites.

DISCUSSION

The alternating-access hypothesis implies that the switch between outward- and inward-open conformations of a given secondary active transporter must not occur unless the appropriate type and number of substrates are recognized. This control mechanism is functionally crucial because it precludes the backflow of the species that is transported uphill and also prevents the dissipation of the driving electrochemical gradients. It is, however, also nontrivial: antiporters, for example, do not undergo the alternating-access transition without a cargo, which is precisely what symporters must do to reset their transport cycles. Similarly puzzling is that a given antiporter undergoes this transition after recognition of substrates of different charge, size and number. Yet, when multiple species are cotranslocated, partial occupancies must not be conducive to the alternating-access switch. Here, we provided new insights into this intriguing mechanism of conformational control through structural studies and quantitative molecular simulations of a Na+/Ca2+ exchanger.

Specifically, our studies of NCX_Mj reveal the mechanism of forward ion exchange (Fig. 7). The internal symmetry of outward-facing NCX_Mj and the inward-facing crystal structures of several Ca2+/H+ exchangers20,28–30 have indicated that the alternating-access mechanism of NCX proteins entails a sliding motion of TM1 and TM6 relative to the rest of the transporter. Here, we demonstrate that conformational changes in the extracellular region of the TM2–TM3 and TM7–TM8 bundle precede and are necessary for the transition and are associated with ion recognition and/or release. The most apparent of these changes involves the N-terminal half of TM7 with TM6 and thus enables TM6 and TM1 to freely slide to the extracellular solution. Interestingly, the bending of TM7 associated with the occlusion of the ion-binding sites also unlocks its interaction with TM6 and thus enables TM6 and TM1 to freely slide to the inward-facing conformation. We anticipate that the intracellular ion-exchange process involves analogous conformational changes.

The crystal structures of NCX_Mj reported here, with Na+, Ca2+, Se2+ or H+ bound, capture the exchanger in different conformational states. These states represent only a subset of all possible states, but they ought to reflect inherent preferences of the transporter, modulated by the experimental conditions. For example, in the crystal of NCX_Mj in LCP, the extracellular half of the gating helices (TM6 and TM1) form a lattice contact, which might ultimately restrict the degree of opening of the ion-binding sites in some cases (for example, in the apo, low pH structure). Nonetheless, the calculated free-energy landscapes, derived without knowledge of the

| Table 2 Data collection and refinement statistics for NCX_Mj structures obtained from crystals soaked with varying amounts of Na+ and no Ca2+, and at low pH and no Na+ or Ca2+ |
|---|---|---|---|---|---|---|
| Data collection | Space group | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | P2₁2₁2₁ | C2 |
| Cell dimensions | a, b, c (Å) | 90, 72, 96.78 | 90, 72, 96.64 | 90, 72, 96.78 | 90, 72, 96.36 | 90, 72, 96.21 | 90, 72, 96.21 |
| Resolution (Å) | 2.0 (2.1–2.0) | 2.10 (2.14–2.10) | 2.28 (2.28–2.32) | 1.90 (1.93–1.90) | 1.90 (1.93–1.90) | 2.80 (2.85–2.80) |
| Refinement | R_work / R_free | 0.21 / 0.25 | 0.19 / 0.22 | 0.19 / 0.23 | 0.19 / 0.24 | 0.179 / 0.207 | 0.20 / 0.26 |
| R_work / R_free | 0.21 / 0.25 | 0.19 / 0.22 | 0.19 / 0.23 | 0.19 / 0.24 | 0.179 / 0.207 | 0.20 / 0.26 |
| Protein | 2,206 | 2,274 | 2,366 | 2,229 | 2,229 | 4,410 |
| Ligand / ion | 154 / 2 | 161 / 5 | 162 / 6 | 257 / 4 | 257 / 4 | 121 / 2 |
| Water | 36 | 67 | 67 | 67 | 67 | 39 |
| R.m.s. deviations | Protein | 34.28 | 34.91 | 39.75 | 39.75 | 39.75 | 42.98 |
| Ligand / ion | 55.86 / 31.75 | 55.25 / 36.47 | 58.24 / 38.93 | 46.11 / 22.13 | 46.11 / 22.13 | 54.29 / 63.47 |
| Water | 41.16 | 46.97 | 49.74 | 37.80 | 37.80 | 33.17 |
| Bond lengths (Å) | 0.004 | 0.006 | 0.008 | 0.003 | 0.006 | 0.003 |
| Bond angles (°) | 0.819 | 0.915 | 1.269 | 1.024 | 1.024 | 0.966 |

Values in parentheses are for highest-resolution shell. 5% of the data were used in the R_free calculation. Ligand atoms are from lipids, PEG 400 and acetates.
Table 3 Data collection and refinement statistics for NCX_Mj structures obtained from crystals soaked with varying amounts of Na\(^+\) and Sr\(^{2+}\)

| Data collection | 10 mM Sr\(^{2+}\), 2.5 mM Na\(^+\) | 1 mM Sr\(^{2+}\), 2.5 mM Na\(^+\) | 0.1 mM Sr\(^{2+}\), 2.5 mM Na\(^+\) | 10 mM Sr\(^{2+}\), 10 mM Na\(^+\) | 10 mM Sr\(^{2+}\), 100 mM Na\(^+\) |
|----------------|----------------------------------|----------------------------------|----------------------------------|--------------------------------|----------------------------------|
| PDB | 5HXS | 5JDL | 5JDM | 5JDN | 5JDQ |
| **Space group** | \(P2_1 \times 2_1\) | \(P2_1 \times 2_1\) | \(P2_1 \times 2_1\) | \(P2_1 \times 2_1\) | \(P2_1 \times 2_1\) |
| **Cell dimensions (Å)** | \(a, b, c\) | 49.52, 72.35, 96.00 | 49.76, 72.62, 95.62 | 49.80, 72.27, 94.84 | 49.67, 72.46, 96.43 | 49.88, 72.43, 95.91 |
| **Resolution (Å)** | \( \alpha, \beta, \gamma \)** | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 | 90, 90, 90 |
| **Completeness (%)** | 98.8 (90.4) | 94.9 (96.1) | 99.9 (100.0) | 99.8 (99.9) | 99.6 (99.7) |
| **Redundancy** | 6.5 (5.4) | 6.7 (6.9) | 7.1 (6.8) | 6.2 (4.5) | 5.9 (6.0) |
| **Reefinement** | | | | | | |
| **Resolution (Å)** | 50–2.80 | 50–2.90 | 50–2.54 | 50–2.30 | 50–2.50 |
| **No. reflections** | 8,927 | 7,611 | 11,483 | 16,488 | 12,665 |
| **\(R_{free}\)** | 0.22 / 0.27 | 0.22 / 0.27 | 0.23 / 0.26 | 0.20 / 0.25 | 0.21 / 0.24 |
| **No. atoms** | Protein | 2,227 | 2,249 | 2,217 | 2,271 | 2,223 |
| **Ligand / ion** | 110 / 2 | 133 / 3 | 155 / 3 | 101 / 3 | 104 / 6 |
| **Water** | 10 | 2 | 5 | 34 | 17 |
| **B factors** | Protein | 61.55 | 67.82 | 60.83 | 46.42 | 56.75 |
| **Ligand / ion** | 74.56 / 63.32 | 85.56 / 70.47 | 80.33 / 90.41 | 64.79 / 38.25 | 71.27 / 53.50 |
| **Water** | 59.22 | 61.31 | 68.58 | 51.39 | 55.07 |
| **R.m.s. deviations** | Bond lengths (Å) | 0.004 | 0.005 | 0.005 | 0.007 | 0.003 |
| **Bond angles (º)** | 0.887 | 1.076 | 0.963 | 1.057 | 0.733 |

Values in parentheses are for highest-resolution shell. 5% of the data were used in the \(R_{free}\) calculation. Ligand atoms are from lipids, PEG 400 and acetates.

Experimental data, confirmed that the crystallized structures correspond to mechanistically relevant, interconverting states. The simulations also demonstrated how this landscape is drastically reshaped after each ion-binding event. Indeed, we show that the presence or absence of the occluded state in this landscape explains the antiport function of NCX_Mj and its 3:1 Na\(^+\)/Ca\(^{2+}\) stoichiometry. We posit that a similar principle might govern the alternating-access mechanism in other transporters; that is, we anticipate that for both symporters and antiporters, the feasibility of the occluded state, and its dependence on substrate binding, ultimately explains their specific coupling mechanisms.

In multiple ways, our findings provide an explanation for existing functional, biochemical and biophysical data for both NCX_Mj and its eukaryotic homologs. The striking quantitative agreement between the protein conformational free-energy landscape and the ion-binding affinities inferred from our crystallographic titrations also demonstrated how this landscape is drastically reshaped in response to mechanistically relevant, interconverting states. The simulations also demonstrated how this landscape is drastically reshaped after each ion-binding event. Indeed, we show that the presence or absence of the occluded state in this landscape explains the antiport function of NCX_Mj and its 3:1 Na\(^+\)/Ca\(^{2+}\) stoichiometry. We posit that a similar principle might govern the alternating-access mechanism in other transporters; that is, we anticipate that for both symporters and antiporters, the feasibility of the occluded state, and its dependence on substrate binding, ultimately explains their specific coupling mechanisms.

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Table 4  Data collection and refinement statistics for NCX_Mj structures obtained from crystals soaked with varying amounts of Na\(^+\) and Ca\(^{2+}\)

|                  | 10 mM Ca\(^{2+}\), 2.5 mM Na\(^+\) PDB 5HXR | 1 mM Ca\(^{2+}\), 2.5 mM Na\(^+\) PDB 5JDF | 0.1 mM Ca\(^{2+}\), 2.5 mM Na\(^+\) PDB 5JDG | 10 mM Ca\(^{2+}\), 10 mM Na\(^+\) PDB 5JDH |
|------------------|---------------------------------------------|-------------------------------------------|---------------------------------------------|---------------------------------------------|
| **Data collection**                      |                                             |                                           |                                             |                                             |
| Space group      | P\(^2\)\(_1\)\(_2\)\(_1\)                     | P\(^2\)\(_1\)\(_2\)\(_1\)                  | P\(^2\)\(_1\)\(_2\)\(_1\)                   | P\(^2\)\(_1\)\(_2\)\(_1\)                   |
| **Cell dimensions**                   |                                             |                                           |                                             |                                             |
| a, b, c (Å)      | 49.70, 72.52, 96.94                         | 49.80, 72.26, 95.80                      | 49.48, 72.47, 96.30                        | 49.88, 72.22, 96.10                        |
| α, β, γ (°)      | 90, 90, 90                                   | 90, 90, 90                               | 90, 90, 90                                 | 90, 90, 90                                 |
| Resolution (Å)   | 2.45 (2.49–2.45)                             | 2.65 (2.70–2.65)                         | 2.40 (2.44–2.40)                           | 2.20 (2.24–2.20)                           |
| R\(_{free}\) (%) | 11.8 (94.2)                                  | 11.1 (92.1)                              | 11.2 (91.3)                                | 10.3 (99.1)                                |
| I(σ(I))          | 24.4 (1.6)                                   | 20.9 (1.6)                               | 20.4 (1.6)                                 | 22.4 (1.8)                                 |
| CC\(_{1/2}\)     | (0.611)                                      | (0.696)                                   | (0.632)                                    | (0.549)                                    |
| Completeness (%) | 99.7 (100.0)                                 | 99.9 (100.0)                             | 99.2 (100.0)                               | 99.7 (100.0)                               |
| Redundancy (°)   | 7.8 (7.8)                                    | 7.0 (6.5)                                 | 6.9 (7.0)                                  | 7.1 (7.1)                                  |
| **Refinement**   |                                             |                                           |                                             |                                             |
| Resolution (Å)   | 50–2.45                                      | 50–2.65                                   | 50–2.40                                    | 50–2.2                                     |
| No. reflections  | 12996                                       | 10548                                     | 13736                                      | 18080                                      |
| R\(_{free}\) / R\(_{work}\) | 0.22 / 0.26                               | 0.22 / 0.26                              | 0.20 / 0.26                                | 0.19 / 0.24                                |
| No. atoms        |                                             |                                           |                                             |                                             |
| Protein          | 2211                                        | 2225                                      | 2228                                       | 2284                                       |
| Ligand / ion     | 114 / 3                                     | 130 / 3                                   | 162 / 3                                    | 164 / 4                                    |
| Water            | 16                                          | 20                                        | 42                                         | 57                                         |
| B factors        |                                             |                                           |                                             |                                             |
| Protein          | 64.97                                       | 71.22                                     | 53.50                                      | 45.16                                      |
| Ligand / ion     | 78.98 / 60.53                               | 90.03 / 72.90                            | 70.67 / 45.53                              | 69.09 / 42.92                              |
| Water            | 68.57                                       | 81.02                                     | 64.12                                      | 55.45                                      |
| R.m.s. deviations|                                             |                                           |                                             |                                             |
| Bond lengths (Å) | 0.002                                       | 0.004                                     | 0.002                                      | 0.004                                      |
| Bond angles (°)  | 0.630                                       | 1.143                                     | 0.686                                      | 0.650                                      |

Values in parentheses are for highest-resolution shell. 5% of the data were used in the R\(_{work}\) calculation. Ligand atoms are from lipids, PEG 400 and acetates.

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

Accession codes. Atomic coordinates and structural factors for the structures specified in Tables 2–4 have been deposited in the Protein Data Bank under accession codes 5HWX, 5HWY, 5HXC, 5HXE, 5HYA, 5HXH, 5HXS, 5JDL, 5JDM, 5JDN, 5JDQ, 5XHR, 5JDF, 5JDG and 5JDH.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.L. and Y.J. designed the experimental studies and analyzed the resulting data. J.L., C.L. and Y.H. performed the experimental research. F.M. and J.D.F.-G. designed the computational research and analyzed the corresponding data. F.M. performed the computational work. J.L., Y.J., F.M. and J.D.F.-G. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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**ONLINE METHODS**

**Protein expression, purification and crystallization.** NCX₉₋₅j was expressed, purified and crystallized as previously described[23]. Briefly, the NCX₉₋₅j gene with a C-terminal hexahistidine tag was cloned into the pQE60 vector and expressed in *Escherichia coli* BL21(DE3)pLysS. Harvested cells were homogenized and incubated in buffer containing 50 mM HEPES pH 7.2, 50 mM NaCl, 12 mM KCl, 10 mM CaCl₂ and 40 mM DDM. After incubation at room temperature (RT) for 3.5 h, the supernatant was collected by centrifugation and loaded onto a TALON Co²⁺ affinity column (Clontech). The nonspecifically bound contaminants on the column were washed with buffer containing 50 mM HEPES, pH 7.2, 50 mM NaCl, 12 mM KCl, 10 mM CaCl₂ and 1 mM DDM. The bound NCX₉₋₅j was eluted by increasing the imidazole concentration to 300 mM. The eluate was treated with thrombin to remove the hexahistidine tag and was dialyzed against 20 mM HEPES, pH 7.2, 50 mM NaCl, 12 mM KCl, 10 mM CaCl₂ and 1 mM DDM at RT overnight. After overnight digestion, the sample was loaded onto a second Co²⁺ affinity column to remove any free hexahistidine tag and contaminant proteins. NCX₉₋₅j in the flow-through was collected and further purified by gel filtration with a Superdex 200 (10/300) column (GE Healthcare) in 20 mM HEPES, pH 7.2, 50 mM NaCl, 12 mM KCl, 10 mM CaCl₂ and 0.5 mM DDM. The purified protein was then concentrated to 40 mg/ml for crystallization.

Native NCX₉₋₅j was crystallized with the LCP technique, as previously described[23]. Concentrated NCX₉₋₅j was first reconstituted into 1-oleoyl-rac-glycerol (Sigma) in a protein/lipid weight ratio of 1:1.5, with the two-syringe method[24]. Protein-laden LCP droplets of 35 nm were dispensed onto Corning 96-well protein-crystallization plates and overlaid with 5 µL of precipitant solution containing 40–42% PEG 400, 100 mM MES, pH 6.5, and 100 mM NaAc. Crystals were observed after 48 h and grew to full size after 2 weeks. The native crystals belonged to space group P2₁2₁2₁ with a cell dimension of a = 49.5 Å, b = 72.9 Å and c = 96.2 Å, and contained one subunit per asymmetric unit. Because the LCP droplet accounted for less than 1% of the total crystallization volume, the salt composition in the crystallization condition was determined mainly by the overlying solution and was estimated to have 150 mM Na⁺ (from the MES buffer and NaAc) and 30 µM Ca²⁺ (from the LCP droplet). In these concentration conditions (high Na⁺ and low Ca²⁺), Ca²⁺ does not bind to NCX₉₋₅j (as shown in our crystallographic titration experiments), and thus this native crystal structure represents NCX₉₋₅j in 150 mM Na⁺. The native crystals were used in all subsequent titration experiments to define low-Na⁺, Ca²⁺ and Sr²⁺-loaded structures.

To obtain the apo crystal form, the protein was first purified in a solution containing 20 mM HEPES-Tris, pH 7.2, 100 mM NMDG, 10 mM CaCl₂ and 0.5 mM DDM. The crystals were obtained in LCP with crystallization solution containing 200 mM LiCl, pH 4.0, and 35% PEG 400. The apo NCX₉₋₅j crystals belonged to space group C2 with a cell dimension of a = 164.2 Å, b = 46.8 Å, c = 97.0 Å and β = 106.2°, and contained two protein subunits per asymmetric unit.

**Crystal titrations.** Once the native crystals had reached their full size, the crystallization solutions overlaying lipid/protein droplets were gradually replaced by titration solutions through multiple steps of solution exchange. In general, 2–3 µL of existing crystallization solution (normally in 5 µL) was replaced by the same volume of titration solution, and this was followed by overnight equilibration. The same procedures were repeated 6–10 times until the ion components in the crystal drops reached the targeted concentrations. For titration experiments to define concentration-dependent Na⁺ binding, the titration solutions contained 100 mM MES-Tris, pH 6.5, 44% PEG 400, 10 mM CaCl₂ and 40 mM NaCl, in the following proportions: 100 mM CsCl; 90 mM CsAc and 10 mM NaAc; 80 mM NaCl and 20 mM NaAc; and 100 mM NaCl. Notably, Ca²⁺ does not bind NCX proteins and is commonly used as a Na⁺ substituent to maintain the ionic strength of the solutions. Because complete removal of Na⁺ would deteriorate the crystals, we had to maintain a minimum Na⁺ concentration of approximately 2.5 mM in the crystal drops. The final Na⁺ concentrations in this set of titration experiments were approximately 2.5, 10, 20 and 100 mM, respectively. Notably, the observed Na⁺-dependent conformational change occurred while the proteins were in crystal form and embedded in lipid.

In the titration experiments carried out to define the mode of divalent cation binding and competition with Na⁺, the soaking solutions contained 100 mM MES-Tris, pH 6.5, 44% PEG 400, 100 mM mixture of CaCl₂ and NaAc and various concentrations of XCl₂, where X = Ca²⁺ or Sr²⁺, in the following proportions: 100 mM CaCl₂ and 10 mM XCl₂; 100 mM CaAc and 1 mM XCl₂; 100 mM CaAc and 0.1 mM XCl₂; 90 mM CaAc, 10 mM NaAc and 10 mM XCl₂; and 100 mM NaAc and 10 mM XCl₂. After multiple steps of solution exchanges, the final soaking conditions contained 0.1, 1, or 10 mM of XCl₂ together with 2.5 mM Na⁺; or 10 mM XCl₂ together with 2.5, 10 or 100 mM Na⁺.

**Data collection and structure determination.** After soaking, crystals were mounted on 100-µm Mitegen Microloops and frozen in liquid nitrogen. All diffractometer data were collected at the Advanced Photon Source (APS) GM/CA-CAT beamlines 23ID-B or 23ID-D with a beam size of 35 µm × 50 µm. Data were processed and scaled with HKL2000[37], and the structures were determined by molecular replacement in PHASER[38] with our previously published NCX₉₋₅j structure (PDB 3V5U[39]) as a search model. Model building was completed with COOT[40], and structure refinement was performed with PHENIX[41]. The data sets from crystals soaked in solutions containing 2.5 to 100 mM Na⁺ were collected with an X-ray wavelength of 1.033 Å; the crystal grown with 150 mM Na⁺, and those soaked with Ca²⁺ and Sr²⁺ solutions, were obtained with a wavelength of 0.9793 Å. Finally, the data from the crystal grown at low pH with no Na⁺ or Ca²⁺ were collected with a 2.0-Å wavelength beam. The resulting statistics for data collection and refinement are shown in Tables 2–4. All structure figures were prepared in PyMOL (http://www.pymol.org/). The ion passageways in low- and high-Na⁺ structures as well as the apo state were analyzed with CYAV[42]. Because of the variation in diffraction resolution and intensity among crystals, ion-occupancy comparisons were made on the basis of the diffraction data obtained in the titration experiments scaled against common reference data before map calculation. The NCX₉₋₅j crystal obtained with 2.5 mM Na⁺ was used as the reference.

**Conventional molecular dynamics (MD) simulations.** Conventional (i.e., not enhanced) MD simulations were carried out with NAMD 2.7–2.9 (ref. 42) at constant temperature (298 K), pressure (1 atm), and membrane surface area (~69 Å² per lipid), and with periodic boundaries in all directions. All calculations used the standard CHARMM27/CMAP force field[43,44], except for NBFIX corrections for the interaction between carboxyloxygens and Na⁺[45] and Ca²⁺ [Supplementary Note 3 and Supplementary Fig. 5]. Electrostatic interactions were calculated with PME with a real-space cutoff of 12 Å; the same cutoff distance was used for all van der Waals interactions.

Five ion-occupancy states of the transporter were considered, namely with three Na⁺, two Na⁺, two H⁺ or one Ca²⁺, and with no ions bound; in all cases D240 was protonated[26]. For the three-Na⁺ state, we reanalyzed a 200-ps trajectory of NCX₉₋₅j reported previously[22]. NCX₉₋₅j had been embedded in a POPC lipid membrane with GRIFEN[45]. The initial configuration of the two-Na⁺ state was generated from an equilibrated configuration of a three-Na⁺ state, from which the Na⁺ ion at S452 was displaced by means of a slow alchemical transformation that annihilates the bound ion and reorients it in the bulk solution (in the same simulation box). The resulting two-Na⁺ state was simulated for 250 ns. Similarly, the state with no Na⁺ bound was generated from an equilibrated configuration of the two-Na⁺ state, from which the remaining Na⁺ ions were displaced; this state was again simulated for 250 ns. For the two-H⁺ state, an initial configuration was generated from an equilibrated configuration of the three-Na⁺ state, by gradually annihilating the Na⁺ ions from the binding sites and creating protonated E54 and E213 side chains; concurrently, acetic acid molecules in the bulk solution (in the same simulation box) were deprotonated, and Na⁺ ions were introduced. A second initial configuration of the two-H⁺ state was obtained from an equilibrated configuration of the simulation with no ions bound, by slowly transforming deprotonated E54 and E213 into their protonated form, while doing the opposite to acetic acid molecules in the bulk solution. These two initial configurations of the two-H⁺ state were then equilibrated for 800 ns. All annihilation/creation simulations were carried out with the FEP module of NAMD and comprised 32–50 intermediate simulations of 400 ps each for each transformation. A soft-core van der Waals potential with a radius-shifting coefficient of 2 Å² was used. The annihilated Na⁺ ions were confined within their corresponding binding sites with flat-bottom distance restraints. Specifically, the Na⁺ in S452 was concurrently maintained within 4 Å of the E54 Cβ, A206 Cβ, S77 Cβ, T209 Cβ and S210 Cβ. The Na⁺ ions in S452 and S551 were concurrently kept within 4 Å of the E213 Cβ and A47 C, respectively. The Na⁺ ions and acetic acid molecules in the bulk...
solution were kept at a distance greater than 37 Å from the membrane center. Finally, the initial configuration of the Ca$^{2+}$ state was generated on the basis of the published NCX-Mj X-ray structure by placing Ca$^{2+}$ in the ScA site and two water molecules coordinating Ca$^{2+}$ at and near the Smid site, to satisfy the expected coordination geometry (Supplementary Note 2 and Supplementary Figs. 3 and 4). This configuration was initially equilibrated through a series of simulations in which r.m.s. deviation–based restraints of gradually diminishing strength were applied to the protein Cα atoms as well the side chains involved in Ca$^{2+}$ coordination. A 250-ns equilibration was then carried without any restraints.

**Enhanced-sampling MD simulations.** Free-energy landscapes were calculated with Bias-Exchange Well-Tempered Metadynamics (BE-WT-MetaD)46,47, with GROMACS4.5.5/PLUMED48,49. The force-field and simulation conditions were equivalent to those used in the unbiased MD simulations. The accumulated simulation time for each of the ion-occupancy states studied was 1.6 μs. Each of these calculations consisted of 16 concurrent, interdependent simulations (or replicas); in 15 of these replicas, a WT-MetaD biasing potential was applied on different subsets of collective variables, as specified below, and the remaining replica was unbiased. Attempts to exchange coordinate configurations among replicas were made every 2–5 ps, with the Metropolis criterion46. The inputs for each calculation were equilibrated configurations extracted from the unbiased MD simulations.

The choice of collective variables to be biased in the BE-WT-MetaD simulations was also based on analysis of the unbiased MD trajectories (Fig. 4g–i). Specifically, to enhance the reversible opening and closing of the water channels reaching from the extracellular bulk solution into either the Sext or the ScA binding sites, we used the following time-dependent collective variable (Fig. 4h–i):

$$V_{1,2}(t) = \frac{\beta}{\ln \sum_i \exp[\beta r_i(t)]}$$

where $r_i$ denotes the distance between the oxygen atom of each water molecule in the system $i$ and the center of the binding site considered ($V_{1,S} c_{1,S}$ for $S_{ScA}$, $V_{2,S} c_{2,S}$ for $S_{ScB}$), and $\beta$ is 10–100 nm. When the binding site was occupied, the ion was used to define its center. If the site was empty, its center was defined as the center of mass of the oxygen atoms coordinating the ion if bound. Bound water molecules at or near $S_{mid}$ (coordinating the Na$^+$ or Ca$^{2+}$ ions) were not considered.

To enhance the reversible formation and disruption of selected backbone hydrogen bonds in TM7ab (Fig. 4g), we used an analogous collective variable:

$$V_{3}(t) = \frac{\beta}{\ln \sum_{ij} \exp[\beta (r_{ij}(t))]$$

This in case, the index $i$ denotes atoms P202 O and T203 O, whereas the index $j$ denotes atoms A206 N and P207 N. To preclude the artificial unrolling of TM7ab driven by this bias, an upper-bound $V_{max} \alpha = 0.7$ nm was imposed with a boundary potential of the form $k(V_3(t) - V_{3,\max})$ if $V_3(t) > V_{3,\max}$, where $k = 10^5$ nm$^{-4}$ k J/mol. In addition, to control the bending and straightening of TM7ab more globally, we used the following path–collective variables50:

$$V_{4}(t) = \frac{\exp[-\lambda d_1(t)] + 2 \exp[-\lambda d_2(t)]}{\exp[-\lambda d_1(t)] + \exp[-\lambda d_2(t)]}$$

where $d_1$ and $d_2$ denote the mean-square differences between the conformation of TM7ab and either the straight or bent conformations, respectively, and $\lambda = 100$ n m$^{-2}$.

A boundary potential was also applied to confine the ions and water molecule bound to $S_{ScA}, S_{ScB}, S_{mid}$ and $S_{CaA}$ to their corresponding binding sites. Specifically, the variable confined was:

$$V_{c}(t) = \sum_{i} \frac{1 - r_i(t)/r_i^{\delta}}{1 - (1 - r_i(t)/r_i^{\delta})^{\gamma}}$$

where $r_i(t)$ denotes the distance between the ion and each of its coordinating oxygen atoms; $r_i^{\delta}$ was set to 0.24 nm for the Na$^+$ ions and to 0.30 nm for Ca$^{2+}$. Notably, the upper-bound value of $V_c(t)$, by definition, approximately the coordination number in the bound state, whereas $V_c(t)$ becomes 0 as the ion becomes unbound. For the Na$^+$ ion bound to $S_{CaA}$ and $S_{CaB}$, therefore, a lower-bound value of $V_{c,\min}$ was 4.3 imposed with potential of the form $k(V_c(t) - V_{c,\min})$ when $V_c(t) < V_{c,\min}$, where $k = 2,500$ k J/mol. An analogous restraint was used for the Na$^+$ ion at $S_{CaA}$, with $V_{c,\min} = 4.75$. Similarly, for the Ca$^{2+}$ ion at $S_{CaA}$, a lower-bound value $V_{c,\min} = 7.4$ was imposed with a potential of the form $k(V_c(t) - V_{c,\min})$ when $V_c(t) < V_{c,\min}$, with $k = 400$ k J/mol. Notably, these restraints do not perturb the chemical structure of the ion-coordination sphere when the ion is bound, i.e., $V_c(t) > V_{c,\min}$. The displacement of the bound water molecules in the ion-coordination sphere by equivalent water molecules in the solvent was prevented similarly. The specific sets of collective variables biased in each of the replica WT-MetaD simulations, as well as further details on the biasing potentials introduced, are specified in Supplementary Table 1.

**Derivation of conformational free-energy landscapes.** To translate the data gathered in the BE-WT-MetaD simulations into conformational free-energy landscapes, we sought to identify a low-dimensional representation of the data that is nevertheless also intuitive and representative. We ultimately settled on two structure-based descriptors of the degree of opening of the each of the aqueous channels leading to the ion-binding sites (Fig. 4), defined as:

$$S_{1,2} = \frac{\beta}{\ln \sum_{ij} \exp[\beta (r_{ij}(t))]$$

where $r_{ij}$ denotes a set of pairwise distances for specific Cα atoms in the protein, for a given simulation snapshot. For $S_1$, index $i$ refers to the Cα of residues 198 to 209 in TM7, and index $j$ refer to those in residues 68–80 and 290–297, in TM3 and TM10, respectively. For $S_2$, index $i$ refers to the Cα of residues 51–64 in TM2, whereas index $j$ refers to those in residues 177–193 and 198–209, in TM6 and TM7, respectively. Therefore, $S_1$ describes the effective separation between TM7 and TM3/TM10, on the extracellular half of the protein, and thus reports on the accessibility to the Sext site. Analogously, $S_2$ measures the separation between TM2 and TM6–TM7, also on the extracellular side, and thus reports on the accessibility to $S_{CaA}$.

The conformational free energy of NCX-Mj as a function of $S_{1,2}$ and state was then computed for each ion-occupancy state separately (Figs. 5a and 6a). These landscapes were obtained through reweighting of the biased probability distribution from the BE-WT-MetaD sampling, with the WHAM method50; through this approach we combine the statistics gathered in all replicas and can consider alternate free-energy projections50,51.

To correct the landscape calculated for the Ca$^{2+}$ bound state (Fig. 6a) on the account of the excess amount of charge transferred from the ion to the protein (Supplementary Note 4, Supplementary Fig. 6 and Supplementary Table 2), we reprocessed all the sampling obtained during the original BE-WT-MetaD simulations, introducing in the WHAM equations a reweighting factor $w$ for each configuration $X$:

$$w(X) = \exp\left(-\frac{U_c(X) - U(X)}{k_B T}\right)$$

where $U$ denotes the ‘uncorrected’ CHARMM27/NBFIX potential-energy function, and $U_c$ denotes the corrected function. To calculate $U_c(X)$, the charge of Ca$^{2+}$ was reduced to +2.8e from its standard value of +3e, and the difference was distributed among the surrounding protein residues (as specified in Supplementary Note 4). To minimally alter the charge distribution used in the original CHARMM27 force field, the charge added to each protein atom was
proportional to the absolute value of its uncorrected charge. The statistical errors for all free-energy landscapes are provided in Supplementary Figure 7.

**Derivation of representative structures and water-density maps.** Representative structures and water-density isosurfaces (Figs. 5b,c and 6b,c) were derived for each BE-WT-MetaD simulation by clustering all sampling in the multidimensional space of $V_1$, $V_2$ and $V_4$ (equations (1–3)), plus a descriptor $S_3$ of the proximity between TM6–TM7 and TM2–TM3/TM10, on the extracellular side of the protein. More precisely:

$$S_3 = \sum \frac{1 - |r_{ij}(t)/\delta_{ij}|^8}{\sqrt{2}}$$

where $r_0 = 7.5 \text{ Å}$, and $r_{ij}$ denotes a specific set of pairwise Cα distances, for a given simulation snapshot. Similarly to $S_1$ and $S_2$ (equation (6)), index $i$ refers to Cα atoms in the extracellular halves of TM6 and TM7, and index $j$ refers to Cα atoms in the extracellular halves of TM2, TM3 and TM10. We thus obtained $\sim 2,000$ clusters for each of the simulation systems (with r.m.s.-deviation cutoff values of 1.3 Å for $V_1$ and $V_2$, 0.1 Å for $V_p$, and 6.25 Å for $S_3$). With the WHAM equations, we calculated the relative free energy of each of these clusters and then identified the major basins in this space with the MCL method (55,56) (with $P = 1.4$). Water-occupancy maps were calculated for each of these major free-energy basins, only with the sampling gathered by the unbiased replicas.

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