Crystal structure of Ca\(^{2+}/H^+\) antiporter protein YfkE reveals the mechanisms of Ca\(^{2+}\) efflux and its pH regulation

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Ca\(^{2+}\) efflux by Ca\(^{2+}\) cation antiporter (CaCA) proteins is important for maintenance of Ca\(^{2+}\) homeostasis across the cell membrane. Recently, the monomeric structure of the prokaryotic Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) antiporter NCX_Mj protein from *Methanococcus jan- naschii* shows an outward-facing conformation suggesting a hypothesis of alternating substrate access for Ca\(^{2+}\) efflux. To demonstrate conformational changes essential for the CaCA mechanism, we present the crystal structure of the Ca\(^{2+}/H^+\) antiporter protein YfkE from *Bacillus subtilis* at 3.1-Å resolution. YfkE forms a homotrimer, confirmed by disulfide crosslinking. The protonated state of YfkE exhibits an inward-facing conformation with a large hydrophilic cavity opening to the cytoplasm in each protomer and ending in the middle of the membrane at the Ca\(^{2+}\)-binding site. A hydrophobic “seal” closes its periplasmic exit. Four conserved α-repeat helices assemble in an X-like conformation to form a Ca\(^{2+}/H^+\) exchange pathway. In the Ca\(^{2+}\)-binding site, two essential glutamate residues exhibit different conformations compared with their counterparts in NCX_Mj, whereas several amino acid substitutions obscure the Na\(^+\)-binding sites. The structural differences between the inward-facing YfKE and the outward-facing NCX_Mj suggest that the conformational transition is triggered by the rotation of the kink angles of transmembrane helices 2 and 7 and is mediated by large conformational changes in their adjacent transmembrane helices 1 and 6. Our structural and mutational analyses not only establish structural bases for mechanisms of Ca\(^{2+}/H^+\) exchange and its pH regulation but also shed light on the evolutionary adaptation to different energy modes in the CaCA protein family.

**Ca\(^{2+}\) transport**

The Ca\(^{2+}\) cation antiporter proteins (CaCAs) comprise a large transporter superfamily existing ubiquitously throughout all biological kingdoms, including animals, insects, plants, fungi, and bacteria (1). They promote Ca\(^{2+}\) extrusion by using an electrochemical gradient of Na\(^+\) or H\(^+\), regulating Ca\(^{2+}\)-mediated signaling processes (2). In animals, all characterized CaCA proteins exclusively use an inward Na\(^+\) gradient for Ca\(^{2+}\) efflux, Na\(^+\)/Ca\(^{2+}\) exchanger proteins (NCXs) are important for maintaining cardiac contractility in cardiac muscle and for neuronal transmission in brain (3). Dysfunction of Na\(^+\)/Ca\(^{2+}\)-K\(^+\) exchangers (NCKXs) impairs skin and retinal pigmentation, as well as synaptic plasticity (4).

To address these important questions, here, we report the crystal structure of an inward-facing conformation of the YfKE protein. This structure, together with protein kinetic analysis, not only provides an important structural characterization of the mechanism of Ca\(^{2+}\) efflux across the cell membrane but also sheds light on the different modes of energy coupling used by members of the CaCA protein superfamily.

**Results**

**Ca\(^{2+}\)-Transport Specificity and Structural Determination of YfKE.** YfKE catalyzes a H\(^+\)-coupled Ca\(^{2+}\) flux in inverted vesicles (SI Appendix, Fig. S2A). Na\(^+\), Li\(^+\), or Mg\(^{2+}\) do not inhibit its transport activity. Modest inhibition was only observed at higher concentrations of Mn\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), or Sr\(^{2+}\), confirming the Ca\(^{2+}\) selectivity of YfKE (SI Appendix, Fig. S3). YfKE is strongly regulated by intracellular pH. Michaelis–Menten kinetic analysis revealed two distinct pH dependencies for its Ca\(^{2+}\)-transport activity or substrate binding (SI Appendix, Fig. S2B).

The *v*\(_{\text{max}}\) values of YfKE vs. pH exhibit a Gaussian distribution,

\[ v_{\text{max}} = \frac{K_v}{K_v + [H^+]^2} \]

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The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 4KJR and 4KJS).

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with an optimal pH of 7. In contrast, its substrate binding appears to be alkaline-dependent [i.e., the apparent Km value at pH 8.5 (51 μM) is dramatically increased to 320 μM at pH 7.5 and becomes undetectable below pH 6].

The YfKE protein was purified from cell membranes using the detergent n-dodecyl-β-maltoside. The purified protein exists in an oligomeric conformation estimated by its gel-filtration elution profile (SI Appendix, Fig. S10B). To facilitate protein expression and crystallization, the mutation K116A was introduced within the loop between TMs 3 and 4. This mutant exhibits a similar transport activity to that of wild type (see Fig. 4C). An additional mutation L77M was introduced for crystallization of the selenomethionine-substituted (Se-Met) protein. Crystals of both native and Se-Met proteins were obtained at pH 4 and diffracted to a resolution of 3.1 Å (SI Appendix, Table S1). The YfKE structure was determined using the multiple-wavelength anomalous-dispersion method combined with the phases of five additional methionine mutants (SI Appendix, Fig. S4 and Table S2). The refined models comprise amino acid residues 4–351, with the missing residues occurring in two cytoplasmic loops between TMs 1–2a (54–57) and between TMs 5–6 (179–201) (SI Appendix, Fig. S5).

**Overall Structure of YfKE Trimer.** In sharp contrast to the monomeric NCX_Mj structure (6), YfKE crystallizes as a threefold symmetric trimer, reminiscent of an equilateral triangle viewed from the periplasmic side of the membrane (Fig. 1 A and B). Two anti-parallel–stacked trimers determine crystal packing in addition to limited contact between adjacent trimers (SI Appendix, Fig. S6). The “triangle” extends 50 Å from the central threefold axis to its triangular tips with each edge spanning 93 Å on the plane of the membrane. Each YfKE protomer contains 11 TMs (TMs 0–10) (SI Appendix, Fig. S5), in contrast to the 10 TMs of NCX_Mj. They can be characterized as two contiguous helical bundles, TMs 1–5 and TMs 6–10, and an N-terminal TM0 located at the distal tip of the triangle. The two helical bundles exhibit an antiparallel orientation with a pseudo twofold symmetry perpendicular to the membrane plane (Fig. L1). Whereas its C terminus resides at the periplasmic surface, its N terminus is embedded in the membrane near the cytoplasm owing to the shortest helix TM0 (Fig. 1B). The sequence of TM0 is the least-conserved (SI Appendix, Fig. S1). In NCXs and NCKXs, the analogous fragment is thought to be a cleaved signal peptide, probably generating 10 TMs (3). In plant CAXs, a larger N-terminal segment serves an autoinhibitory function to regulate its Ca2+-transport activity (11). Despite the poor protein sequence homology (18% identity) between YfKE and NCX_Mj, a YfKE protomer can be superimposed on the NCX_Mj structure with a root mean square deviation (rmsd) value of 1.8 Å, demonstrating the structural conservation of the CaCA protein superfamily (Fig. 2 A–C).

The YfKE trimeric conformation is stabilized by extensive hydrophobic interactions and shape complementation between TMs 3–4 of one protomer and TMs 9–10 of the adjacent protomer with a large interface area of more than 1,200 Å² (Fig. 1B). Along the trimeric axis, three copies of TMs 4 and 9 assemble a vase-like cavity of 4 Å in diameter, which is opened on the periplasmic side of the membrane (SI Appendix, Fig. S7). To further examine the trimeric conformation of YfKE, we generated two double-cysteine mutants, I133C/A322C and I11368|www.pnas.org/cgi/doi/10.1073/pnas.1302515110 Wu et al. with an optimal pH of 7. In contrast, its substrate binding appears to be alkaline-dependent [i.e., the apparent Km value at pH 8.5 (51 μM) is dramatically increased to 320 μM at pH 7.5 and becomes undetectable below pH 6].

The YfKE protein was purified from cell membranes using the detergent n-dodecyl-β-maltoside. The purified protein exists in an oligomeric conformation estimated by its gel-filtration elution profile (SI Appendix, Fig. S10B). To facilitate protein expression and crystallization, the mutation K116A was introduced within the loop between TMs 3 and 4. This mutant exhibits a similar transport activity to that of wild type (see Fig. 4C). An additional mutation L77M was introduced for crystallization of the selenomethionine-substituted (Se-Met) protein. Crystals of both native and Se-Met proteins were obtained at pH 4 and diffracted to a resolution of 3.1 Å (SI Appendix, Table S1). The YfKE structure was determined using the multiple-wavelength anomalous-dispersion method combined with the phases of five additional methionine mutants (SI Appendix, Fig. S4 and Table S2). The refined models comprise amino acid residues 4–351, with the missing residues occurring in two cytoplasmic loops between TMs 1–2a (54–57) and between TMs 5–6 (179–201) (SI Appendix, Fig. S5).

**Inward-Facing Conformation.** One of the most remarkable features of the YfKE structure is the symmetric conformation of its two α-repeat motifs. TMs 2–3 and TMs 7–8 form two anti-parallel “hairpins” that ride each other from each side of the membrane (Fig. 2D). Whereas TMs 3 and 8 are tilted in parallel, kinked TMs 2 and 7 form an X-shaped conformation. Such conformation was also observed in the NCX_Mj structure (6) but not in other transporter proteins, indicating the unique scaffold of the α-repeat regions within the CaCA proteins. Whereas TMs 3 and 8 are well superimposable between NCX_Mj and YfKE structures, TMs 2 and 7 exhibit different conformations at their kink angles (Fig. 2F). Compared with the NCX_Mj structure, TM2a of YfKE rotates clockwise around its kink angle by 40°, generating a larger split toward the cytoplasmic side of the membrane. Meanwhile, its TM7a undergoes an anticlockwise rotation by 24° to approach toward TM2b on the periplasmic side of the molecule. The residue E255 located at the TM7 kink apparently stabilizes this inside-opening conformation. The detailed interaction of this highly conserved residue cannot be resolved at this resolution. Based on the electron density map, the carboxylate side chain of E255 extends toward the kink angle of TM2 to interact with four residues (F67, G68, N69 and A70), stabilizing the TM2 kink conformation (Fig. 2E).

Adjacent to the X-conformation of TMs 2 and 7, TMs 1 and 6 exhibit the major conformational difference between YfKE and NCX_Mj. The entire TM1 helix of YfKE is moved ∼15 Å from the TM6 position of NCX_Mj. TM6, the longest helix (45 Å), traverses the V-shaped groove formed by TMs 1 and 2b to bend toward TM7a (Fig. 1 A and B). One-third of TM6 extends above the projected membrane boundary. It may lie on the periplasmic membrane surface, giving the opposing polarity of its two helical
surfaces. The conformational differences of TMs 1 and 6 appear to be associated with changes in the kink angle of their adjacent TMs 2a and 7a occurring on each side of the membrane. In YfkE, TMs 6–7a lie toward TM3, resulting in closure of the large periplasmic cavity that is observed in the NCX_Mj structure (6). Meanwhile, its TMs 1–2a swing away from the X-like conformation by widening the TM2 kink angle, generating a large funnel-like cavity opened at the cytoplasmic side of the YfkE molecule (Figs. 2C and 3A; see below).

Intracellular Substrate Entry. Of major interest regarding the structure of YfkE is the possibility of identifying the Ca\(^{2+}\)-translocation pathway and the Ca\(^{2+}\)-binding site. The native YfkE crystal was obtained in the presence of 2 mM Ca\(^{2+}\). There was no significant change in the overall protein conformation of the structures with or without substrate. Efforts to obtain anomalous signals by collecting the native crystal data at a wavelength of 1.6 Å did not localize any metal ion in the structure. Therefore, the YfkE structure obtained at pH 4 may represent a protonated inactive conformation.

To transport Ca\(^{2+}\), a hydrophilic ion-translocation pathway is predicted. In YfkE, the trimeric central cavity would readily exclude any ion because of its hydrophobicity (SI Appendix, Fig. S7). A Ca\(^{2+}\)/Na\(^{+}\)-translocation pathway is formed by the four \(\alpha\)-repeat helices in the NCX_Mj structure (6). We hypothesize that Ca\(^{2+}\) efflux occurs through a similar pathway in each protomer of YfkE based on the following. (i) The residues along the pathway are highly conserved among Ca\(^{2+}\)/H\(^{+}\) antiporter proteins and the CaCA superfamily. Many of them contribute their side-chain oxygen atoms to form the sole hydrophilic tunnel across the membrane in YfkE (Fig. 3 and SI Appendix, Fig. S1). Several polar residues including E72, E255, two CaCA fingerprint residues each from the \(\alpha\)-1 or \(\alpha\)-2 motif, reside in the middle of the pathway. (ii) Single alanine substitution of these two residues completely abolishes Ca\(^{2+}\)-transport activity in everted vesicles (Fig. 4C). To exclude any effect of protein expression and folding, all YfkE mutant proteins were confirmed using Western blot analysis of the vesicles (SI Appendix, Fig. S10A) and further purified by size-exclusion chromatography (SEC). The SEC profiles and CD spectroscopic analyses suggest that all mutants exist in an overall protein conformation similar to that of wild type (SI Appendix, Fig. S10 B and C).

Along the pathway, the intracellular cavity is formed by TMs 1, 2a, 7b, 8, and 10 (Fig. 3C). The cavity has a water-accessible entry 5 Å in diameter calculated by MOLE (12) and gradually narrows to the intersection of TMs 2a and 7b in the middle of the membrane (Fig. 3B and SI Appendix, Fig. S11). Several polar residues including E49, N64, S258, E273, S278, and Q281 contribute to the net electronegativity of the cavity (Fig. 3A). Mutation of Q281 to alanine nearly abrogated Ca\(^{2+}\)-transport activity, whereas the N64A or S258A mutants remained largely unaffected, suggesting that the residues deeply embedded in the membrane are more important for Ca\(^{2+}\)-transport activity than those near the cytoplasmic entry (Fig. 4C). Therefore, one would expect Ca\(^{2+}\) diffusion in the cavity to reach a binding position near residue Q281.
**Ca\(^{2+}\)**-Binding Site and Closed Periplasmic Exit. At the end of the cytoplasmic cavity, six conserved residues from the four \(\alpha\)-repeat helices, G68 and N69 from TM2, N99 from TM3, N252 and E255 from TM7, and S278 from TM8, assemble an oxygen “umbrella” conformation in the middle of the membrane (Fig. 4A). Alanine scanning of the umbrella suggests that each asparagine residue, particularly N69, is important in maintaining Ca\(^{2+}\)-transport activity (Fig. 4C), supporting the hypothesis that Ca\(^{2+}\) binding takes place at a position near the oxygen umbrella. In the NCX\(_{Mj}\) structure, these conserved residues or their counterparts form an ion binding region to coordinate 3 Na\(^{+}\) and 1 Ca\(^{2+}\) (6). However, in contrast to NCX\(_{Mj}\), these ion-binding sites undergo significant conformational changes in the YfkE structure: (i) two glutamate residues (E72 and E255) coordinating Ca\(^{2+}\) in NCX\(_{Mj}\) exhibit different conformations in the YfkE structure (Fig. 5A); and (ii) the Na\(^{+}\)-binding sites are replaced by amino acid substitutions to remove available ligands and occlude the binding sites (Fig. 5B). E72 rotates its carboxylate side chain away from the Ca\(^{2+}\)-binding site toward the periplasmic side, whereas H256 moves along the helix to occupy the position of E213 (NCX\(_{Mj}\) numbering), the counterpart residue of E255 in YfkE, and places its imidazole ring on the Na1 position. This residual translocation leads to a helical distortion at the TM7 kink region (Fig. 5A). S95, N99, and S278 are well superimposed with their counterpart residues involved in the Na\(^{+}\)-binding sites of NCX\(_{Mj}\), whereas N69 replaces S51 that coordinates Na-1. I282 substitutes D240 in the Na-2 binding site; N252 (S210 in NCX\(_{Mj}\)) inserts its side chain onto the Na-2 site. T209 involved in the Na-3 binding is also replaced by G251 in the YfkE structure (Fig. 5B).

Right above the umbrella, there is no obvious path opening to the periplasmic surface, because of the tightly packed helices (Fig. 4B). Direct Ca\(^{2+}\) passage along TMs 3 and 8 is prevented because of the tilting of TMs 2b–3 on TM8 and their hydrophobic interface (SI Appendix, Fig. S12). TM5 and the membrane-buried loop between TMs 8 and 9 also restrict any large movement of TM8. Instead, the tilted TMs 2b–3 generate another funnel-like cavity together with TMs 1 and 7a that is connected to the Ca\(^{2+}\)-binding site (Fig. 4B). The location of the essential residue E72 suggests Ca\(^{2+}\) passage through this cavity. Compared with the open cytoplasmic entry, the periplasmic tunnel is completely blocked by TM6 lying at the exit. Furthermore, unlike the overall polarity of the cytoplasmic entry, the periplasmic tunnel is more hydrophobic. Eight residues aligned along the closed tunnel or located at the periplasmic exit (including I75 and A79 from TM2b; L84, I87, and V88 from TM3; V225 and F228 from TM6; and V247 from TM7a) form a hydrophobic “seal,” eliminating water accessibility to the Ca\(^{2+}\)-translocation pathway from the periplasmic side of the membrane.

**Discussion**

In this study, we have determined the crystal structure of the H\(^{+}\)-coupled Ca\(^{2+}\) transporter YfkE. The crystal structure supplemented by crosslinking analysis demonstrates that the CaCA protein exists as a trimer. A trimeric structure has been observed in other transporter structures, including AmtB and Gltph (13, 14), but is unexpected in the CaCA family because both mammalian NCXs and NCKXs are predicted to form a homodimer (15, 16). The YfkE structure provides structural evidence that varied oligomeric conformations may exist in the CaCA protein family. It is noteworthy that several aliphatic residues on the trimer interface, including I142, L158, L319, and I343, are conserved among the Ca\(^{2+}\)/H\(^{+}\) exchanger proteins (SI Appendix, Figs. S1 and S2). Whether such a trimeric conformation also exists in
other CAX proteins may require further investigation. Additionally, whether the trimeric conformation plays a specific role in the Ca\(^{2+}\)/H\(^{+}\) exchange mechanism remains an interesting question. As shown in Fig. 2C, the trimer-forming helices in the inward-facing YfkE structure are well superimposable with their counterparts in the outward-facing NCX_Mj structure, arguing that the trimer conformation is not directly involved in Ca\(^{2+}\) transport or responsible for their difference in energy coupling. Instead, the trimer may support Ca\(^{2+}\) transport by stabilizing the protein in cell membranes given that large conformational changes are expected to occur in other parts of the protein. Studying this aspect in the future could provide insights into the working mechanism of CaCA proteins in general.

**The Mechanism of pH Regulation.** Ca\(^{2+}\) efflux via YfkE is tightly regulated by intracellular pH. Our structure obtained at pH 4 demonstrates an acid-locked conformation. Interestingly, the structure of the Na\(^{+}\)/H\(^{+}\) antiporter NhaA from *Escherichia coli* crystallized at the same pH also exhibited an acid-inactive conformation (17). Despite the lack of sequence homology and structural similarity between these two H\(^{+}\)-coupled ion-exporter proteins, their inactive states were both trapped at their inward-facing conformations, raising the hypothesis that the inward-facing conformation may be energetically favorable for acidic inactivation of H\(^{+}\)-coupled transporter proteins. However, YfkE apparently uses a different mechanism for pH regulation than that of NhaA. In contrast to the acid-inactive conformation of NhaA, which is triggered by a structural element outside its ion-translocation pathway (17), the inactivation of YfkE likely takes place within its Ca\(^{2+}\)-binding site. At acidic pH, the protonated E255 stabilizes the highly kinked TM2a to seize the protein in a widely open inward-facing conformation (Fig. 2D and E). This stable interaction stabilizes TM7a and TM6 to attach to TM3, consequently blocking the periplasmic pathway (Fig. 4B). At alkaline pH, the deprotonated E255 may move away from the TM2 kink, liberating the X structure of TMs 2 and 7 for any conformational movement. Owing to their high sequence homology and similar pH-regulatory properties (18), this unique pH-regulatory mechanism may be shared by all members of the CAX protein family.

**Ca\(^{2+}\)/H\(^{+}\) Exchange Mechanism.** The inward-facing conformation of the YfkE structure and the outward-facing conformation of the NCX_Mj structure provide important structural information about the Ca\(^{2+}\)/cation exchanging mechanism. The fact that protonated YfkE exists in an inward-facing conformation suggests that H\(^{+}\) influx resets the protein to a state ready for cytoplasmic Ca\(^{2+}\) access. Based on the Ca\(^{2+}\)-bound NCX_Mj structure, Ca\(^{2+}\) binding in the cytoplasmic cavity would induce a closure rotation of TM2a around the TM2 kink to narrow the cytoplasmic cavity (Fig. 6A). Constraint of the kink rotation by mutating G68 to alanine causes a 50% reduction in the V\(_{\text{max}}\) value, supporting this view (Fig. 4C). The deprotonated E255 liberated from the TM2 kink would then move toward the oxygen umbrella to join the Ca\(^{2+}\)-binding site, as shown in the NCX_Mj structure (6) (Fig. S4).

In the YfkE inward-facing conformation, the residue H256 blocks the Ca\(^{2+}\)-binding site under the oxygen umbrella (Fig. 4A). Attempts to remove this blocking residue resulted in 90% loss of the transport activity (V\(_{\text{max}}\)) and threefold reduction in the K\(_{\text{m}}\) value, suggesting its critical role for transport activity rather than substrate binding (Fig. 4C). Historically, the imidazole ring of H256 is in the protonated state at crystallization pH 4, it is stabilized as a plug under the oxygen umbrella (Fig. 4A). Upon its deprotonation, H256 would unlock its imidazole ring from the plug position, providing a specific position for Ca\(^{2+}\) binding.

The movement of H256 and E255 may take place simultaneously with the helical distortion, because of their adjacent Cys residue. As a result, the TM7 kink hinge may induce an outward rotation of the tilted TM7a together with the adjacent TM6 toward the exterior of the lipid bilayer (Fig. 6B). These motions would subsequently open the Ca\(^{2+}\)-translocation pathway by sliding the hydrophobic seal on the periplasmic surface, resulting in an outward-opening conformation providing access to the Ca\(^{2+}\)-binding site.

Opening of the periplasmic exit may allow water access to the periplasmic tunnel to facilitate inward H\(^{+}\) flux. To accomplish H\(^{+}\)/Ca\(^{2+}\) exchange at the Ca\(^{2+}\)-binding site, H\(^{+}\) has to travel a long distance of 20 Å from the periplasmic surface to residues E255 and H256 deeply buried in the middle of the membrane.
(Fig. 4B). Passive diffusion may not afford a rapid H\(^+\) uptake given the hydrophobic nature of the periplasmic tunnel. Instead, residue E72 residing along the tunnel may more efficiently facilitate H\(^+\) influx given its remarkable mobility illustrated in the two structures. Shifting E72 upward of the carbohydrate group of E72 shortens its distance to the periplasmic surface to 12 Å, making it an ideal candidate to import periplasmic H\(^+\). The protonated carbohydrate group of E72 may flip toward the Ca\(^{2+}\)-binding site to transfer the H\(^+\) to the cytoplasmic side. As a consequence, the TM7a and 6 move back to close the periplasmic entry may facilitate H\(^+\) delivery in the cavity (Fig. 3C). However, conformational changes in these residues would be necessary for direct H\(^+\) relay from the Ca\(^{2+}\)-binding site given their distances of more than 10 Å from E255 and H256.

In the YfkE structure, both Na\(^+\) and Ca\(^{2+}\) are bound to central binding sites (6). In YfkE, we predict that E72 and E255 are involved in binding an alternate substrate because a protonated glutamate residue may not be able to coordinate with Ca\(^{2+}\). Although we cannot exclude partial substrate occupancy of the binding site, Ca\(^{2+}\) release can be immediately triggered by protonating the Ca\(^{2+}\)-binding site. E72 may also assist Ca\(^{2+}\) escape toward the periplasmic surface by flipping its side chain back to the upright conformation. The protonated H256 residue may return to the plug position under the umbrella. As a consequence, TM7a and 6 move back to close the periplasmic entry may facilitate H\(^+\) delivery in the cavity (Fig. 3C). However, conformational changes in these residues would be necessary for direct H\(^+\) relay from the Ca\(^{2+}\)-binding site given their distances of more than 10 Å from E255 and H256.

This substrate-shifting alternate model is consistent with the outward-facing conformation seen in the NCX_Mj structure (6), suggesting that the CaCA proteins share a conserved mechanism for Ca\(^{2+}\)/cation exchange. The structures of YfkE and NCX_Mj provide two examples of the inward-facing and outward-facing conformations. Whether any other conformational state(s), such as the Ca\(^{2+}\)/bound conformation or the partially protonated state that Liao et al. proposed for NCX_Mj (6), is/are available during one Ca\(^{2+}\)/H\(^+\) exchange is unknown. Based on the two structures, transition from the inward-facing to the outward-facing conformation is triggered by alternative substrate binding at the kinks of the α-repeat helices to alter the kink angles of TM2 and 7 (Fig. 2B). These helical rotations occurring on each side of the membrane may be coupled via their adjacent TMs 1 and 6 (Fig. 2A and C). These two interactive helices move together on the exterior of the trimer, resulting in alternative opening and closing of the Ca\(^{2+}\)/H\(^+\) exchanger pathway at each side of the molecule.

One of the most intriguing questions regarding the YfkE structure is how the different CaCA proteins adapt different driving forces while maintaining their Ca\(^{2+}\) specificity. Despite the possibility that Ca\(^{2+}\) binding may change the conformation of the binding site, comparison of the apo form of YfkE with the Ca\(^{2+}\)/Na\(^+\) binding site of NCX_Mj provides insight into this fundamental question (Fig. 5). In addition to the two conserved glutamate residues, other residues coordinating the three Na\(^+\) ions in NCX_Mj undergo significant modifications, which apparently abolish the Na\(^{+}\) binding sites. Strikingly, these residues reassemble to form a cluster of side-chain oxygen atoms to generate a more compact binding site that would only accommodate Ca\(^{2+}\). These sequence differences conserved in either the Na\(^{+}\)-coupled NCXs and NCXKs or the H\(^{+}\)-driven CAX family (SI Appendix, Fig. S1) may reflect evolutionary adaptation to different energy driving forces while maintaining substrate specificity.

**Methods and Materials**

Both native and SeMet-YfkE were expressed in E. coli with a polyhistidine tag inserted within the cytoplasmic loop between TMs 5 and 6, extracted by dodecylmaltoside, and purified by a metal-affinity column. The proteins were further purified by SEC and concentrated to 8 mg mL\(^{-1}\) for crystallization. Crystals were obtained using the sitting-drop vapor-diffusion method. Both native and Se-Met crystals were diffracted to 3.1 Å resolution after dehydration treatment. The crystal diffraction data were processed using XDS (19). The structures were determined using the multiple-wavelength anomalous dispersion method using AutoSHARP (20). The structures were built using Coot (21) and refined to the final R/Revs values (%) of 22/26 for the native structure and 24/27 for the Se-Met structure using Refmac (22). Transport analyses were carried out by measuring 46Ca\(^{2+}\) influx into everted vesicles prepared as described previously (23). Disulfide crosslinking was performed using an iodine-catalyzed approach (24).

The details of methods and materials are provided in SI Appendix.

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Supporting Information

Crystal structure of Ca\(^{2+}\)/H\(^{+}\) antiporter protein YfkE reveals the mechanisms of Ca\(^{2+}\) efflux and its pH regulation

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SI Appendix, Methods and Materials

**Protein preparation**  YfkE was cloned into the pET22b vector from *Bacillus subtilis* genome. A hexa-His tag was inserted at the residue 188 within the cytoplasmic loop between TM5 and 6 to facilitate protein purification and the generated protein is referred to as YfkE wild type. To improve protein expression and crystallization, the mutation K116A was introduced within the cytoplasmic loop between TMs 3 and 4. The native YfkE protein was expressed in *E.coli* strain BL21 (DE3) in autoinduction medium (1) at 37 °C for 3 h followed by overnight incubation at 25 °C. Cells were harvested and homogenized in phosphate buffer containing 20 mM sodium phosphate, pH 7.4, 500 mM NaCl and 20 mM imidazole. Cell rupture was carried out by three passages through a C3 homogenizer (Avestin) at 15,000 p.s.i. Cell debris was removed by centrifugation. The supernatant was pelleted by ultracentrifugation for 1 h. Membrane fractions suspended in the same phosphate buffer as described above were solubilized by addition of 1% (w/v) n-dodecyl-β-maltoside (DDM, Anatrace) at 4 °C. After 1 h incubation, the solubilized solution was centrifuged at 40,000 rpm for 25 min. The supernatant was loaded on to a Ni-NTA affinity resin (GE healthcare). The resin was washed with phosphate buffer containing 60 mM imidazole and 0.05% DDM (w/v). The YfkE protein was eluted with phosphate buffer containing 400 mM imidazole and 0.05% DDM. The eluted protein was further purified by SEC using a Superdex-200 10/300 GL column (GE Healthcare) equilibrated in buffer containing 20 mM sodium phosphate, 500 mM NaCl, 0.05% DDM (w/v), pH 7.4. The final protein solution was concentrated to 8 mg mL\(^{-1}\) and stored at -80 °C before crystallization. An additional mutation L77M was introduced to crystallize Se-Met protein. Se-Met protein of YfkE was expressed in the BL21(DE3) C43 *E.coli* strain using a published protocol (2), and purified using similar
approach to that for the native protein. After protein eluted from Ni-NTA resin, 5mM DTT was added to the eluted protein solution and maintained in all subsequent buffers.

**Protein crystallization** Both native and selenomethionine-substituted YfkE proteins were crystallized in buffer containing 23-25% PEG 400, 200 mM ammonium sulfate, 20mM NaCl, 0.1M sodium acetate, pH 4.0 and 3% hexanediol using the sitting-drop vapor diffusion method at room temperature. 2 mM Ca$^{2+}$ was supplemented to the native protein crystallization buffer. Both crystals diffract to a maximum 8 Å resolution, estimated by in house Rigaku X-ray source at room temperature. The crystals were further treated using the crystal dehydration and salvage kit (Jena Biosciences) for 30 min at room temperature. This dehydration treatment is critical to improve the crystal quality to 4 Å resolution. The dehydrated crystals were cryo-protected in Paraffin oil and flash frozen quickly under liquid Nitrogen.

**Data collection, data processing and structure determination** The native YfkE dataset was collected at the Swiss Light Source (SLS, Switzerland) and the MAD datasets were collected at the Advanced Light Source beamline 4.2.2 (Berkeley, CA, USA). Both the native and the Se-Met crystals diffract to the highest resolution of 3.1 Å. Data were processed using the program *XDS* (3). Statistics of data collection and data processing are given in *SI Appendix*, Table S1. The data were initially processed to the space group of $R3_2$ with one molecule in the asymmetric unit. Both exhibit nearly perfect twining (~45%), estimated by the *phenix.xtriage* program (4). The initial phase was calculated using the MAD method and density modification by the *AutoSHARP* program (5). Each YfkE peptide has 12 native methionine residues. Ten strong Se sites were unambiguously localized. A partial YfkE model containing the main chains of 11 α helices was built using *Coot* (6) and refined in the $R3_2$ space group using *Refmac* (7) till $R / R_{free}$ stabilized at 38% / 42%. The electron density map was gradually improved after several rounds of the MR-SAD (Molecular Replacement combined with SAD phasing) approach using *Phaser* (8) and model building with the peak data. To help model building and to verify the YfkE model, five additional methionine mutants on different TM helices were also crystallized. Their peak datasets were collected at ALS or SLS (*SI Appendix*, Table S2). New heavy atom sites were localized by calculating their anomalous difference maps using *AutoSHARP* (5) in the $R3_2$ space group. The native YfkE structure was determined by the molecular replacement method in the $R3$ space group using *Phaser* (8). Twin refinement was performed in the $R3$ space group by
Refmac (7) with twin law (k, h, -l). The final models were refined to \( R / R_{\text{free}} \) values of 22\% / 26\% for the native structure and 24\% / 27\% for the Se-Met L77M structure. All residues fall into the allowable region of Ramachandran plot. Statistics of structure refinements are given in SI Appendix Table S1. The structures of YfkE native and Se-Met proteins have been deposited to Protein Data Bank (PDB accession codes: 4KJR & 4KJS). All structural figures were prepared using PyMOL (9).

**Disulfide crosslinking** Disulfide crosslinking experiments were performed using an iodine-catalyzed approach described by Kaback for LacY (10). *E.coli* strain BL21(DE3) cells expressing YfkE wild type or cysteine mutants were suspended in lysis buffer (20 mM sodium phosphate, 500 mM NaCl, 10\( \mu \)g mL\(^{-1} \) lysozyme, pH7.4) and then ruptured by sonication. Crosslinking was triggered by adding aqueous iodine (final concentration of 0.5 mM) into the whole cell lysate at room temperature. After 30 min incubation, reactions were stopped by adding 10 mM N-ethylmaleimide and then were separated by SDS-PAGE. Crosslinking was visualized by western blot using anti-peta-His antibody horseradish peroxidase-conjugate.

**Ca\(^{2+}\) transport assay** Ca\(^{2+}\) efflux assay was performed by measuring Ca\(^{2+}\) uptake into everted membrane vesicles. YfkE everted vesicles were prepared using a method described by Rosen and Tsuchiya with modification (11). Briefly, *E.coli* strain BL21(DE3) cells carrying the YfkE expression vector were grown in Luria broth medium at 37°C. After cell density reached 0.4 at OD600, protein expression was induced by adding 0.2 mM Isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG) for 2 h at 25°C. Cells were then harvested and washed with TKDS buffer (10 mM Tris-HCl, 140 mM KCl, 0.5 mM DTT, 250 mM sucrose, pH 7.3). Everted vesicles were generated by single passage of cells through a C3 homogenizer (Avestin) at 4,000 p.s.i. After the cell debris was removed, the supernatants were centrifuged to pellet the membrane fractions. The vesicles were homogenized in TKDS buffer and quick frozen in liquid Nitrogen for use.

Thawed vesicles were diluted to 0.13 mg mL\(^{-1} \) total protein concentration with TKDS buffer (pH 8.0) containing 5 mM potassium phosphate. Prior to assays, the vesicles were incubated with 5 mM NADH for 10 min. Reaction was triggered by addition of \(^{45}\)CaCl\(_2\) into the vesicles. Samples were taken at various times, filtered through a nitrocellulose membrane (0.22
μm) on a Millipore filtration manifold and immediately washed with 10 ml of TKDS buffer, pH 7.3. The filters were air-dried and counted in a liquid scintillation counter. i) For time-dependent transport assay (SI Appendix, Fig. S2A), 500 μM substrate was used. ii) For cation inhibition assays (SI Appendix, Fig. S3), the vesicles were incubated with 200 μM (10 fold) or 2 mM (100 fold) of CaCl₂, LiCl, NaCl, MgCl₂, MnCl₂, CdCl₂, CoCl₂, NiCl₂ or SrCl₂, before 20 μM ⁴⁵Ca²⁺ was added to the reactions for 10 min. iii) For pH dependent transport measurement (SI Appendix, Fig. S2B), YfkE vesicles were diluted into MES-Tris buffer solutions at appropriate pHs prior to measurements. iv) For Michaelis Menten kinetic analyses (Fig. 4C and SI Appendix, Fig. S2B), the transport activity was measured at seven different [Ca²⁺] ranged from 5 to 500 μM. Apparent $K_m$ and $V_{max}$ values were calculated using Graphpad Prism™ software. All assays were performed in triplicate. Everted vesicles prepared with the cells hosting empty vector were used as control.

**Circular dichroism (CD) spectroscopy** Prior to CD spectroscopic analysis, Ni-NTA purified YfkE wild type or mutant proteins were passed through a P-10 desalting column (GE healthcare) equilibrated with phosphate buffer (20 mM sodium phosphate, 400 mM Na₂SO₄, 0.05% DDM, pH 7.4) to remove Cl⁻. The protein concentration of all samples was adjusted to 0.2 mg mL⁻¹. CD spectra were collected at room temperature over a wavelength range from 190 to 260 nm with a Jasco J-815 CD spectrometer using a 0.02-cm cylindrical cell.
Fig. S1 | Structural-based sequence alignment of CaCA family proteins. The protein sequences are respectively from H⁺/Ca²⁺ exchanger proteins: *Bacillus subtilis* (YfkE_BACSU), *Arabidopsis thaliana* (CAX1_ARATH), *Oryza sativa* subsp. *japonica* (Rice, CAX1A_ORYSJ), *Saccharomyces cerevisiae* (VCX1_YEAST); Na⁺/Ca²⁺ exchanger proteins: *Methanosarcina acetivorans* (MaX1_METAC), *Methanocaldococcus jannaschii* (NCX_Mj), Na⁺/Ca²⁺ exchanger from human Na⁺/Ca²⁺ exchanger 1 (NCX1_HUMAN, truncated from residues 277 to 756) and human K⁺-dependent Na⁺/Ca²⁺ exchanger 2 (NCKX2_HUMAN, truncated from residues 317 to 450). As indicated in parentheses, the large intracellular loops between TMs 5-6 are not subjected to alignment. The sequence numbering is for YfkE. Two conserved α-repeat regions (α-1 and α-2) are highlighted. The sequence alignment was generated using the program ClustalW (12) and the figure was prepared by the program ESPript (13).
Fig. S2 | Ca\textsuperscript{2+} transport activity of YfkE and its pH dependence. (A) Time-dependent Ca\textsuperscript{2+} transport assay in membrane vesicles made from cells expressing YfkE (squares) or the empty vector (triangles). (B) Michaelis-Menten kinetic analysis of the YfkE wild type protein showing pH dependence of the $K_m$ (circle) and $V_{max}$ (square) values.

Fig. S3 | Cation inhibitory assay of YfkE. $^{45}$Ca\textsuperscript{2+} uptake assays of YfkE were measured in the presence of 10 fold (blue bars) or 100 fold (red bars) higher concentration of other cations. The results were normalized with the empty vector control. The $^{45}$Ca\textsuperscript{2+} transport activity of YfkE is inhibited much stronger by additional cold Ca\textsuperscript{2+} than Cd\textsuperscript{2+}, Co\textsuperscript{2+}, Sr\textsuperscript{2+} or Mn\textsuperscript{2+} (only at higher concentration). No inhibition was observed by addition of Li\textsuperscript{+}, Na\textsuperscript{+} or Mg\textsuperscript{2+}. The modest activations by Li\textsuperscript{+}, Na\textsuperscript{+} or Mg\textsuperscript{2+} are perhaps caused by non-specific ion electrostatic interaction with the protein due to their similar activation and their independence of cation concentration.
**Fig. S4 | Experimental electron density maps of the Se heavy atoms in the YfkE mutant structures.** Stereo views of the backbone of one YfkE molecule in yellow ribbons superimposed with an anomalous Fourier map calculated from the SeMet dataset of five methionine mutants: (A) L29M; (B) L92M; (C) L106M; (D) L224M; (E) V247M. The maps are displayed as green meshes contoured at $5\sigma$ (A-D) or $4\sigma$ (E). Identified Se heavy atom positions are showed as Cyan spheres. New Se sites introduced by methionine mutations are showed as Magenta spheres.

![Experimental electron density maps](image)

**Fig. S5 | Topology of a YfkE protomer.** Topology and structural organization of TM helices showing anti-parallel pseudo twofold symmetry formed by trimer-forming TMs 4-5 & 9-10 (blue), α-repeat TMs 2-3 & 7-8 (red), exterior TMs 1 & 6 (yellow) and distal TM0 (grey). The central blue dashed line represents the pseudo twofold symmetry. Two cytoplasmic loops unresolved in the structure are depicted as black dashed line: 1) between TMs 1 and 2a, 2) between TMs 5 and 6.

![Topology of a YfkE protomer](image)

**Fig. S6 | Molecular packing in the YfkE protein crystal lattice.** (A) A top view. (B) A side view from the membrane plane. Two anti-parallel YfkE trimers colored in red or green stack together with their large cytoplasmic surfaces facing each other. TM0 helices from two adjacent YfkE trimers form weak anti-parallel interaction.

![Molecular packing in the YfkE protein crystal lattice](image)
Fig. S7 | **Stereo view of the central cavity of a YfkE trimer.** Three copies of TMs 4 and 9 (in **grey, yellow or purple**) from a YfkE trimer form a vase-like hydrophobic cavity opening on the extracellular surface along the threefold axis. Residues forming the cavity are depicted as sticks with their N/O atoms in **blue/red**. The surface representation of the cavity was calculated by MOLE (14).

![Stereo view of the central cavity of a YfkE trimer](image)

Fig. S8 | **Disulfide crosslinking on the trimeric interface.** (A) YfkE trimer interface formed by hydrophobic residues from TMs 4'-5' of one protomer (**green**) and from TMs 9-10 of its adjacent protomer (**red**). Two pairs of residues (I133/A322 and I162/I343) were chosen for disulfide crosslinking based on their proximal localizations on the trimer interface. The distance of their C alpha atoms in the pair is 6.1 Å (I133/A322) or 7.3 Å (I162/I343), which are similar to the distance of two cysteine residues forming a disulfide bond (6.5-7.2 Å) in antibody structure (PDB: 1UWG) (15). The disulfide crosslinking is mimicked with the dashed lines. (B) Western blot analysis of unpurified crosslinked YfkE mutant proteins on the crude *E. coli* membranes before (-) and after (+) incubation with 0.5 mM iodine. 1: YfkE wild type; 2, 3: I133C/A322C; 4, 5: I162C/I343C; 6: I133C/I343C. (C) Disulfide crosslinking of detergent-purified proteins visualized by comassie staining. 1: YfkE wild type; 2: I133C/A322C; 3: I162C/I343C.
Fig. S9 | Localization of carboxylate residues in a YfkE protomer. Each YfkE protomer (cartoon) has 16 Asp/Glu residues (sticks and dots). The residues E72 and E255 from TMs 2 (orange) and 7 (blue) are embedded in the membrane in contrast to other carboxylate residues located on the periplasmic or cytoplasmic surface.

Fig. S10 | Protein characterization of YfkE mutants. (A) Western blot analysis of YfkE wild type or mutants showing their comparable expression yields on the everted vesicles. Western blot was performed with same amount of the vesicles in each sample using an anti-His tag antibody HRP conjugate. (B) Size-exclusion chromatography of the purified wild type and mutant proteins eluted from Superdex 200 10/300 GL column indicating the similarity of their protein monodispersities. The samples from Ni-NTA affinity chromatography were loaded with the same volume without adjusting protein concentration. Two protein standards, aldolase (158 KDa) and ferritin (440 KDa),
were eluted at the volume of 12.5 and 10.5 ml on this gel filtration column, respectively. Therefore, the YfkE protein may exist as an oligomer in the solution given its molecular weight of 37 kDa and estimated DDM micelle size of 70 KDa (16). (C) CD spectra of the purified YfkE wild type and alanine mutant proteins indicating their similar α helical conformations.

**Fig. S11 | Proposed Ca\(^{2+}\) translocation pathway.** (A) Surface representation of the pores on the periplasmic (green) or the cytoplasmic side (red) of YfkE obtained with the program MOLE (14). One YfkE protomer is drawn as grey cartoons viewed from the membrane plane. Their corresponding radius along the pores is displayed as (B) green curve or (C) red curve.

**Fig. S12 | Hydrophobic interface between TMs 2b-3 and TM8.** TMs 2b-3 tilt on TM8. Their TM interface formed by hydrophobic residues (blue). The residues at the Ca\(^{2+}\) binding site of the “umbrella” is colored in green and highlighted with dots. TM8 is stabilized by TM5 and the membrane-embedded loop between TMs 8 and 9. The side chain of M301 inserts deeply into the helical interface.
**Fig. S13 | Localization of the residue H256 in a YfkE protomer.** Each YfkE protomer (grey ribbon) has six histidine residues depicted as pink sticks and dots, among which five are located on the periplasmic or cytoplasmic surface of the molecule. H356 is the only histidine residue embedded in the membrane and along the Ca$^{2+}$ translocation pathway.
### SI Appendix, Tables

#### Table S1 | Statistics of data collections and structural refinements

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<td>$R^3$</td>
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**Note:** Values in parentheses are for the highest resolution shell. The Se-derivative data is the L77M peak dataset in Table S2, but was processed in space group $R^3$. 
Table S2 | Statistics of data collections of selenomethionine-derivative YfkE crystals for phasing

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<th>L77M</th>
<th>L29M</th>
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<th>L106M</th>
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Note: Values in parentheses are for the highest resolution shell.
SI Appendix, References
