Inward-facing conformation of the zinc transporter YiiP revealed by cryoelectron microscopy

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YiiP is a dimeric $\text{Zn}^{2+}/\text{H}^{+}$ antiporter from *Escherichia coli* belonging to the cation diffusion facilitator family. We used cryoelectron microscopy to determine a 13-Å resolution structure of a YiiP homolog from *Shewanella oneidensis* within a lipid bilayer in the absence of Zn^{2+} . Starting from the X-ray structure in the presence of Zn^{2+} , we used molecular dynamics flexible fitting to build a model consistent with our map. Comparison of the structures suggests a conformational change that involves pivoting of a transmembrane, four-helix bundle (M1, M2, M4, and M5) relative to the M3-M6 helix pair. Although accessibility of transport sites in the X-ray model indicates that it represents an outward-facing state, our model is consistent with an inward-facing state, suggesting that the conformational change is relevant to the alternating access mechanism for transport. Molecular dynamics simulation of YiiP in a lipid environment was used to address the feasibility of this conformational change. Association of the C-terminal domains is the same in both states, and we speculate that this association is responsible for stabilizing the dimer that, in turn, may coordinate the rearrangement of the transmembrane helices.

membrane protein | secondary transporter | zinc antiporter | FieF

Transition metal ions play either structural or functional roles for approximately half of all cellular proteins (1). Specifically, transition metal ions, such as Cu^{+} , Zn^{2+} , Fe^{2+} , and Co^{2+} , serve as essential cofactors, and their relative concentrations and availability are carefully controlled to ensure that a given protein is loaded with the appropriate metal ion. Heavy metals, such as Cd^{2+} , Hg^{2+} , and Pb^{3+} , often bind these proteins with high affinity and thus displace the physiologically relevant metal ion. To cope with the complexity of this situation, the cell uses an extensive array of pumps, transporters, and binding proteins, all of which have distinct functions in regulating the distribution of the various metal ions.

In the case of Zn^{2+} , homeostasis relies on membrane transporters from a number of different protein families. Primary transport involves ATP-binding cassette transporters (e.g., ZnuB/C) and P-type pumps (e.g., ZntA, ZnuA), which directly couple ATP hydrolysis to the import and export of Zn^{2+} , respectively (2). Secondary transporters come from the family of Zrt, Irt-like proteins (3) and the Nramp broad-spectrum cation transporters (2), both of which are implicated in Zn^{2+} import.

In the present study, we examined YiiP, which belongs to the cation diffusion facilitator (CDF) family and is responsible for the export of Zn^{2+} (4, 5). The CDF family also includes the bacterial homologs ZitB and CztB, as well as eight human homologs designated Znt1–8. Znt3 is associated with loading of Zn^{2+} into synaptic vesicles, and Znt8 mediates Zn^{2+} accumulation by insulin-containing secretory vesicles in pancreatic β cells and thus is associated with type 2 diabetes (6). YiiP was first identified in *Escherichia coli*, and early studies indicated an association with Fe^{2+} transport; thus the name FieF (7). However,

subsequent studies showed YiiP to be a very efficient transporter of Zn^{2+} and Cd^{2+} , but not of Fe^{2+} , Co^{2+} , Cu^{2+} , Mn^{2+} , or Ni^{2+} (8, 9). YiiP was demonstrated to be dimeric both in detergent solutions and in the membrane (10). X-ray crystallography revealed a V-shaped dimer with closely apposed C-terminal domains, but with membrane domains separated by ~ 20 Å. The dimer is stabilized by four Zn^{2+} ions mediating a tight interface between cytoplasmic C-terminal domains and a conserved salt bridge near the membrane surface (11, 12). The transmembrane domain consists of six helices grouped into a bundle of four (M1, M2, M4, and M5) and a bundle of two (M3 and M6). One Zn^{2+} ion is independently bound within the four-helix bundle of each monomer, tetrahedrally coordinated by three aspartates and one histidine. In the mammalian homologs, substitution of one of the aspartate residues with a second histidine eliminated their ability to transport Cd^{2+} (9), thus making them Zn^{2+} -specific transporters.

In the conformation adopted by this X-ray structure, the transport sites were accessible from the periplasmic side of the membrane, consistent with an outward-facing state described by the alternating access mechanism of transport (13). FRET studies suggested a model for transport involving a scissoring of the dimer about the salt bridge, leading to Zn^{2+} release from sites both in the membrane and between the C-terminal domains (12).

We used electron crystallography as an alternative to X-ray crystallography for studying the structure of YiiP within a lipid bilayer (14). We found that the homolog from *Shewanella oneidensis* formed narrow, helical crystals, which were imaged by cryoelectron microscopy (cryo-EM) and used to determine the 3D structure. Based on transport assays and on the Zn^{2+} dependence of crystallization, we concluded that our structure represented the conformation with transport sites occupied by H^{+} instead of Zn^{2+} . We used molecular dynamics (MD) to fit an atomic model to our electron crystallographic density map and to compare this model with the previous structure by X-ray crystallography. This comparison suggests that the EM structure corresponds to the inward-facing state and offers mechanistic insights into the structural basis for the alternating access model of transport.

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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 EMD-5450 and 3J12).

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Fig. 2. Fitting an atomic model to the helical reconstruction. (A) Rigid body docking of the X-ray structure for YiiP (PDB ID code 3H90) to the IHRSR map. The C-terminal domain fits snugly in density protruding from the helical tubes, but the Y-shaped transmembrane domain must undergo major changes to match the map. (B) Results of MDFF fitting of a homology model to the IHRSR map. The transmembrane regions of the monomers have moved closer together, and the cytoplasmic part of the M1-M2-M4-M5 bundle of transmembrane helices has moved away from the M3-M6 pair. The N terminus of YiiP in the X-ray structure is disordered, and the MDFF model includes the slightly longer N terminus from the *S. oneidensis* homolog, which fits nicely into the corresponding density at the cytoplasmic surface of the membrane (under the arrowheads indicating the location of M1, M4, and M5). Residues composing the conserved salt bridge, termed the charge interlock, are shown in yellow near the cytoplasmic membrane surface.

juxtaposition of this structure with the map indicated that YiiP adopted a different conformation (Fig. 24). Sequence comparisons between the *E. coli* protein used for the X-ray structure and the *S. oneidensis* protein used here (Fig. S5) indicate that they have a high degree of sequence similarity (46% identity) with conservation of all three Zn^{2+} -binding sites, as well as the salt bridge that has been proposed to stabilize the dimer (12).

As a first step in producing a structural model consistent with our map, we threaded the sequence for YiiP from *S. oneidensis* onto the X-ray structure of the dimer, which is from *E. coli*. The C-terminal domain of this starting model was docked, and the transmembrane domains were then manually manipulated to match the corresponding portion of the density map (e.g., Fig. S4). The X-ray structure suggested that the transmembrane domain consisted of two relatively independent elements with limited interactions, namely the M3-M6 pair of helices and the four-helix bundle (M1-M2-M4-M5), which harbors the Zn²⁺-binding site (12). Indeed, moving these elements within the membrane produced a much better fit for the backbones of the transmembrane helices (Fig. S4).

As a more rigorous approach to fitting the YiiP model to our EM density map and producing an energetically plausible structure, we applied an MD-based flexible fitting (MDFF) method (25). As suggested by our manual fitting and by the interhelical interactions seen in the X-ray structure, the four-helix bundle formed by M1, M2, M4, and M5 was kept rigid and was allowed to move independent from the M3-M6 helix pair as well as from the C-terminal domain. Both IHRSR and FB maps were used for fitting. After 1.5 ns, both simulations reached a plateau in the cross-correlation between the model and the experimental map (0.94 and 0.92, respectively). The resulting models were very similar to one another, with an rmsd of 2.4 Å for C α atoms (Fig. S4). Given the independence of the two fits, this value is a reasonable statistic for the reliability of the fit, which has been shown to exceed the nominal resolution of the map by as much as 10-fold (26). In our case, our map's 13-Å resolution is sufficient to accurately position helices, but the orientation of side chains and loops are determined solely by MD simulation. Significantly, the

cross-correlation coefficient was substantially lower (0.54) if the four-helix bundle was not allowed to move relative to M3-M6, supporting the existence of a conformational change relative to the X-ray structure.

Conformational Change Reflects Alternating Access Mechanism of Transport.

Transport. Comparison of the X-ray structure and the MDFF model representing the cryo-EM map suggests that this conformational change is relevant to the mechanism of YiiP transport. In particular, pivoting of the four-helix bundle relative to M3-M6, which occurs about a point near the cytoplasmic end of M2, appears to switch access of the Zn^{2+} transport sites from periplasm to cytoplasm (Fig. 3). Specifically, the X-ray structure is characterized by a cavity leading from the transmembrane Zn^{2+} -binding sites to the periplasmic side of the membrane, which has been postulated to be a pathway for binding and releasing the ion (11, 12). In our MDFF/EM model, tilting of the four-helix bundle relative to the M3-M6 pair collapsed this periplasm-facing cavity and

Fig. 3. (A and B) Comparison of the X-ray and EM models. X-ray structures (A) and MDFF/EM structures (B) equilibrated by MD in an explicit DOPG membrane. Comparison of these two structures illustrates the similarity of the C-terminal domains and scissoring movements of the transmembrane helices. (C and D) Overlay of the helical bundles from a YiiP monomer showing movement of the M1-M2-M4-M5 bundle relative to the M3-M6 pair, the latter of which has been aligned for this comparison. (E) Space-filling representation of the X-ray structure of YiiP (PDB ID code 3H90; in gray), with a cavity (green) leading from the periplasmic side of the membrane. (F) Space-filling representation of the MDFF/EM model (gray) revealing a cavity (green) from the cytoplasmic side of the membrane. The cavities were defined using HOLLOW (37). Residues involved in Zn^{2+} transport are shown in yellow. (Insets) Transport sites shown at higher magnification and at a slightly different orientation.

created a new one leading from the cytoplasmic side of the membrane to the Zn^{2+} -binding sites (Fig. 3).

These comparisons suggest that the X-ray and MDFF/EM structures represent the outward-facing and inward-facing conformations that typify the alternating access mechanism (13, 27) used by many secondary transporters (Fig. 4). In addition, the transmembrane domains of the monomers diverged by $\sim 30^\circ$ in the X-ray structure, but were substantially closer together in the cryo-EM model, producing interactions between the monomers at the periplasmic end of M3, as well as at the cytoplasmic ends of M3 and M6. It is possible that these dimeric interactions within the membrane might coordinate the conformational change of the two monomers.

We used targeted MD simulation to address the dynamics of this conformational change in an explicit DOPG–water environment. After adding Zn^{2+} and equilibrating the inward-facing MDFF/EM structure in the bilayer, we applied a modest steering force to explore the energetic feasibility of making the transition to the outward-facing state. Although the MD structures equilibrated within the membrane environment differed slightly from those determined empirically by EM and X-ray (rmsd of 2 Å and 4 Å, respectively; Fig. S6), a switch in the accessibility of the Zn^{2+} transport sites to water molecules was consistent with the transition from inward-facing to outward-facing states. Thus, this simulation indicates the existence of at least one path for the transition between inward-facing and outward-facing states. Longer equilibration runs are needed to assess the stability of the two conformations in this lipid environment, as well as the influence of ion release and protonation.

We also examined membrane thickness to identify potential mismatches between hydrophobic regions of the protein and the membrane and to assess potential energetic effects on the conformational change. Our analysis considered both total lipids in the simulation, as well as boundary lipids defined to be within 10 Å of the protein, thus revealing a slight thinning of the

membrane next to the protein by 2–3 Å (Fig. S6). Nevertheless, the similarity of membrane thickness in both inward-facing and outward-facing states suggests that there is no significant hydrophobic mismatch that might favor one state over the other.

Discussion

The structural basis for the alternating access mechanism has been described for several secondary transporter families, which rely on internal symmetries within their protein fold (27, 28). In the major facilitator superfamily, typified by GlpT and LacY, a rocker-switch mechanism involves two tandem repeats of a six-helix bundle pivoting about a central ligand-binding site. Transporters with the LeuT fold, such as GlpT_{Ph} and NhaA, are composed of two inverted repeats that swap conformations in order to switch the accessibility of their substrates. YiiP does not contain internal symmetry, although early work suggested that the transport sites might reside at the interface of the homodimer (20).

The structural comparison presented here indicates that each monomer undergoes a unique conformational change to produce alternating access (Fig. 4). Specifically, the four-helix bundle and the M3–M6 helix pair appear to pivot about the ion-binding site in a manner resembling the rocker-switch mechanism. In our MDFF/EM model, the M3–M6 pairs interact along the dimer interface, suggesting a mechanism for coupling the conformational change of twofold related molecules and offering a possible explanation for why YiiP constitutively dimerizes both in detergent and in the membrane (10).

The energy for inducing the conformational change required for alternating access has been postulated to come from the binding energy of the substrate (29). Thus, it stands to reason that the occupancy of transport sites would influence the conformation adopted by YiiP in the two crystal forms. For the X-ray structure, Zn^{2+} was included in the mother liquor and was observed to be bound at the transport sites (11), whereas the low Zn^{2+} concentrations and pH required to produce the EM crystals suggest that the transport sites were either empty or occupied by protons. Thus, we can conclude that some asymmetry is built into the tertiary structure, such that the protein tends to adopt the periplasmic-facing conformation when saturated with Zn^{2+} . This property would facilitate removal of Zn^{2+} from the cytoplasm and would enhance coupling with the proton-motive force, consistent with the role of YiiP family members as Zn^{2+} exporters (2).

The presence of a lipid bilayer in the EM crystals may exert an additional effect on the conformation. The lateral tension, surface charge, and, in the case of our helical crystals, the close packing of YiiP dimers combined with the high curvature (Fig. S7) could serve to pry open the membrane domain to produce the inward-facing conformation. However, the converse is equally plausible, that protonation of the transport site favors the inward-facing conformation, thus inducing high curvature as the membrane forms during reconstitution. The latter explanation is consistent with our inability to relax the curvature by altering either lipid species or other components of the crystallization buffer.

Of note, the dimeric structure of the C-terminal domain is not affected by this conformational change. This dimer interface is mediated by four Zn^{2+} ions, and the persistence of this dimer, together with evidence of very-high-affinity Zn^{2+} binding, suggest that residual Zn^{2+} still may be bound even under our crystallization conditions. Specifically, isothermal titration calorimetry indicated Zn^{2+} binding with an affinity too high to be resolved by this technique (19). Furthermore, Zn^{2+} sites at the interface of C-terminal domains produced significantly higher anomalous X-ray signals than other sites, indicating higher occupancy and thus higher affinity (11).

Finally, when we used EDTA to strip YiiP of its Zn^{2+} , we found that the protein remained stable and tubes grew with moderate EDTA concentrations (0.5 mM), but that prolonged

Fig. 4. Alternating access mechanism for $\text{Zn}^{2+}/\text{H}^{+}$ antiport by YiiP. This mechanism involves inward-facing (A and B) and outward-facing (C and D) conformations, both of which are able to bind Zn^{2+} or H^{+} . The proton motive force provides a driving force for exporting Zn^{2+} from the cytoplasm. The conformation in A corresponds to that observed in the helical tubes, whereas the conformation in C corresponds to the X-ray structure solved in the presence of Zn^{2+} .

incubation at higher concentrations (5 mM) led to protein precipitation. A similar result was previously reported with a *E. coli* homolog (19), suggesting that very-high-affinity metal ion sites may be essential to protein stability. Although the conserved salt bridge at the membrane surface, dubbed the charge interlock, has been implicated in formation of the YiiP dimer (12), it seems possible that the stability of the YiiP dimer also relies on Zn^{2+} binding to the C-terminal domain. Indeed, structures of C-terminal domain constructs from the homologous CzcB lacking the charge interlock have shown that the precise geometry of the dimer can be achieved simply by binding of Zn^{2+} at the interface (30). Interestingly, Zn^{2+} can be removed from these constructs, and although the apo form continues to produce dimers, these dimers adopt variable angles between monomers and demonstrate little interaction across the face of the domain (31). This observation suggests that tethering the monomers with the charge interlock contributes to the high affinity of these Zn^{2+} -binding sites in the dimer, which in turn would maintain a fixed architecture for the C-terminal domains.

An unresolved question is how Zn^{2+} is delivered to the transport sites. As with Cu^+ , the levels of free Zn^{2+} in the cytoplasm have been shown to be virtually zero (32). Unlike Cu^+ , however, cytoplasmic metallochaperones for delivering Zn^{2+} to transporters and metalloproteins have not yet been identified (33). Intriguingly, the $\alpha\beta\alpha\beta$ fold of the C-terminal domain from YiiP is related to that of soluble Cu^+ metallochaperones, and an overlay of the first four elements supports the idea of structural homology. This homology raises the possibility that the C-terminal domain acts as a tethered metallochaperone that binds Zn^{2+} and transfers it to the transport sites. Intriguingly, ATP-dependent Cu^+ pumps also carry metallochaperone domains on their N termini, although evidence supports their role in regulation rather than in ion transfer (34).

A regulatory role for the C-terminal domain of YiiP also has been proposed, with Zn^{2+} causing allosteric changes in that domain that promote binding and transport by the transmembrane domain (12). The hypothesized changes in the C-terminal domain are not supported by our EM structure, however, and the implication that Zn^{2+} is bound with extremely high affinity makes it difficult to envision how these ions could be transferred to the transport sites. Instead, we favor the idea that Zn^{2+} acts as a structural element that stabilizes the YiiP dimer, which in turn may facilitate or coordinate the conformational change that accompanies the alternating access mechanism (e.g., by interaction of the M3-M6 pair across the dimer axis). Clearly, more work is needed to clarify both the role of Zn^{2+} binding by the C-terminal domain and the mechanism by which Zn^{2+} is delivered to transport sites in a physiological environment that chelates any and all free Zn^{2+} ions.

Materials and Methods

YiiP Expression, Purification, and Crystallization. YiiP (Q8E919) from *S. oneidensis* was expressed in a 3-L culture of *E. coli*, solubilized with 30 mM n-dodecyl β -D-maltoside, and purified by nickel-nitrilotriacetic acid affinity chromatography. After cleaving the deca-histidine tag with tobacco etch virus protease, the protein was further purified by size-exclusion chromatography, producing a pooled fraction with ~6 mg in 3 mL, which was used for 2D crystallization without further concentration. For crystallization, YiiP was mixed with detergent-solubilized DOPG at a lipid:protein mass ratio of 1:1 and dialyzed against 20 mM N-[tris(hydroxymethyl)methyl]-2-

aminoethanesulfonic acid (pH 7.0), 5 mM MgCl_2 , 100 mM NaCl, and 5 mM NaN_3 . Crystals started growing after 1–2 d and were generally harvested after 5–7 d. To test the effect of Zn^{2+} on crystallization, citrate and N-(2-acetamido)iminodiacetic acid were used to buffer free Zn^{2+} concentrations between 10 nM and 1 mM. All experimental procedures are described in more detail in *SI Materials and Methods*.

Reconstitution and Transport Assays. Reconstitution and stopped-flow transport assays were conducted using procedures described by Wei and Fu (8). Purified YiiP was reconstituted in *E. coli* polar lipids at a protein:lipid mass ratio of 1:2,000 by dialysis against 20 mM Hepes (pH 6.5) and 50 mM K_2SO_4 at 4 °C for 4 d. Proteoliposomes were loaded with the Zn-sensitive fluorophore FluoZin-1 by freeze-thaw, and then extruded through 0.4- μm polycarbonate membranes. After removal of free FluoZin-1 with a desalting column, liposomes were mixed with solutions containing either ZnCl_2 (20 μM –8 mM) or CdCl_2 (20 μM –4 mM) using a stopped-flow apparatus coupled to a fluorimeter with excitation at 490 nm and emission at 525 nm. Protein-free liposomes were used to measure background, and fluorescence signals were normalized to those produced with 4% β -octyl-D-glucoside and either 8 mM ZnCl_2 or 4 mM CdCl_2 . Initial rates were fitted with the Hill equation, and pH dependence was measured by incubating liposomes in buffers at pH 6.5–9 overnight before the transport assays were conducted.

EM, Image Analysis, and Computational Methods. Images of frozen-hydrated crystals were recorded on film and digitized with an effective pixel size of 2.73 Å on the specimen scale. Helical symmetry was deduced by assigning Bessel orders to layer lines in the Fourier transform and by calculating corresponding values for the azimuthal rotation ($\Delta\phi$) and rise (Δz) relating one subunit to the next. IHRSR reconstruction was performed in SPARX (35), taking into account the D3 symmetry of these helical crystals. The IHRSR cycle was iterated until the Euler angle assignments for individual segments ceased to change. FB reconstruction was performed using EMIP (<http://cryoem.nysbc.org/EmIP.html>), which makes use of the strategies developed by Beroukhim and Unwin (23, 36).

To determine the correct hand for the map, asymmetric units (shown in gray in Fig. 3) were extracted from maps generated with alternate handedness and compared with the X-ray structure of YiiP (Fig. S8). For MDFF (25), a homology model of YiiP from *S. oneidensis* was built with MODELER 9v7 using the X-ray crystal structure of YiiP from *E. coli* (PDB ID code 3H90) as a template. An external potential was defined as $\zeta(1 - \phi/\phi_{\text{Max}})$, where ϕ/ϕ_{Max} is the normalized density value from the map and ζ is a scaling factor. An all-atom simulation was conducted in vacuo using the CHARMM force field. In the first step, with $\zeta = 1$, harmonic potentials of elastic constant $k = 500$ kcal/(mol·Å²) were applied to (i) the four-helix bundle formed by M1, M2, M4, and M5; (ii) the M3 and M6 helices at the dimeric interface; and (iii) the C-terminal cytoplasmic domain for 0.5 ns. Then the harmonic restraints were released, and an additional 1-ns fitting was performed using a scaling constant of $\zeta = 0.3$. Targeted MD was performed in an explicit DOPG lipid bilayer solvated by TIP3P water. After a 3-ns equilibration, the C_α rmsd for the transmembrane domain of the MD model relative to the X-ray structure was biased toward 0 for 1.0 ns using a harmonic force with an elastic constant of 1,500 kcal/(mol·Å²). After steering, the system was re-equilibrated without biases or restraints for an additional 3 ns.

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