Binding and Transport of Metal Ions at the Dimer Interface of the Escherichia coli Metal Transporter YiiP*

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YiiP is a representative member of the cation diffusion facilitator (CDF) family, a class of ubiquitous metal transporters that play an essential role in metal homeostasis. Recently, a pair of \(Zn^{2+}/Cd^{2+}\)-selective binding sites has been localized to two highly conserved aspartyl residues (Asp\(^{157}\)), each in a 2-fold-symmetry-related transmembrane segment 5 (TM5) of a YiiP homodimer. Here we report the functional and structural interactions between Asp\(^{157}\) and yet another highly conserved Asp\(^{49}\) in the TM2. Calorimetric binding analysis indicated that Asp\(^{49}\) and Asp\(^{157}\) contribute to a common \(Cd^{2+}\) binding site in each subunit. Copper phenanthroline oxidation of YiiP\(_{D49C}\) and YiiP\(_{D49C,D157C}\), yielded inter- and intra-subunit cross-links among Cys\(^{49}\) and Cys\(^{157}\), consistent with the spatial proximity of two (Asp\(^{49}-\)Asp\(^{157}\)) sites at the dimer interface. Hg\(^{2+}\) binding to YiiP\(_{D49C}\) or YiiP\(_{D49C,D157C}\) also yielded a Cys49-\(\sim\)Hg\(^{2+}\)-Cys157 biscysteinate complex across the dimer interface, further establishing the interfacial location of a (Asp\(^{49}-\)Asp\(^{157}\))\(_2\) bimetal binding center. Two bound \(Cd^{2+}\) ions were found transported cooperatively with a sigmoidal dependence on the \(Cd^{2+}\) concentration (\(n = 1.4\)). The binding affinity, transport cooperativity, and rate were modestly reduced by either a D49C or D157C mutation, but greatly diminished when all the bidentate cysteine S-ligands. The functional significance of these findings is discussed based on the unique coordination chemistry of aspartyl residues and a model for the translocation pathway of metal ions at the YiiP dimer interface.

The cytosolic zinc pool of a functioning \textit{Escherichia coli} cell is thought to be \(<10^{-12}\) M in free \(Zn^{2+}\) (1). At such a low cytosolic free \(Zn^{2+}\) level, zinc efflux pumps selectively bind and actively move zinc ions across the membrane using energy. The transport activities of two classes of zinc efflux transporters, namely the P-type ATPase ZntA (2) and proton-linked cation diffusion facilitators (CDF)\(^3\) (3) control the overall efflux of excess \(Zn^{2+}\) in \textit{E. coli}. To maintain a rapid flow of zinc ions, zinc should bind, change the transporter conformation at the site of binding and subsequently leave the binding site as rapidly as possible. At present, little is known about the molecular architecture of the metal translocation pathway in any CDF protein, even less about the metal coordination chemistry underlying the dual functionalities of selective binding and rapid movement of metal ions in membrane transporters. Among the divergent cation transporters, a well characterized example is the \(Ca^{2+}\)-ATPases (4–6). Compared with calcium, zinc binds much more strongly, and once bound, zinc protein off-rates are in general five orders of magnitude slower (7). In this light, zinc transporters are anticipated to exploit unusual zinc coordination chemistry, because typical zinc binding in many structural or catalytic sites tends to make zinc ions almost permanent parts of the metalloproteins (8).

CDF is a ubiquitous family of metal transporters found in prokaryotes and eukaryotes (9). The \textit{E. coli} CDF transporter YiiP has emerged as a prototype for structural and functional studies of atypical coordination chemistry for selective metal binding and transport. Structural analysis of YiiP has established that YiiP is a stable homodimer both in the membrane and in detergent micelles (10). Each monomeric subunit contains six transmembrane segments with both N and C termini located on the cytoplasmic side of the membrane (11). Functional analysis of YiiP and its homolog ZitB has indicated that these two CDF proteins are proton-linked antiporters that utilize the free energy derived from the downhill H\(^+\) influx to pump the cytosolic \(Zn^{2+}\) out of the cells (12). Recently, an active metal binding site in YiiP has been localized to a highly conserved Asp\(^{157}\). Topological analysis has further established that Asp\(^{157}\) is embedded within the hydrophobic core of TM5 (11). The local hydrophobic environment of Asp\(^{157}\) suggests that additional negatively charged groups might be needed in collaboration with Asp\(^{157}\) to form a divalent zinc binding site. A likely candidate could be another highly conserved aspartyl residue at position 49 in TM2. Phenotype analyses of two homologous CDF transporters, CzcD from \textit{Ralstonia metallidurans} and ZitB from \textit{E. coli} have demonstrated that mutating Asp\(^{49}\) renders host cells hypersensitive to zinc, probably because of a loss of zinc efflux pumping activities (13). It is not clear, however, whether Asp\(^{49}\) is directly involved in \(Zn^{2+}\) binding and transport, and if so, whether Asp\(^{49}\) and Asp\(^{157}\) contribute to a common active binding site.

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\(^3\)The abbreviations used are: CDF, cation diffusion facilitator; DDM, n-dodecyl-\(\beta\)-d-maltoside; TCEP, Tris(2-carboxyethyl) phosphine hydrochloride; ITC, isothermal titration calorimetry; Cu(Phe)\(_3\), copper phenanthroline; MPB, maleimide polyethylene oxide (PEO)\(_2\) biotin; Ni\(^{2+}\)-NTA, Ni\(^{2+}\)-nitrilo-triacetic acid; HPLC, high performance liquid chromatography; DTT, dithiothreitol; NEM, N-ethylmaleimide; TM, transmembrane.

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In the present study, we explored how protein tertiary folding and subunit assembly may contribute to the Zn\(^{2+}\)/Cd\(^{2+}\) translocation pathway in YiiP. Toward this end, we first characterized the functional role of Asp\(^{49}\) by examining the effects of D49A and D49C mutations on metal binding and transport. Then, we established the physical proximity between Asp\(^{49}\) and Asp\(^{157}\) by disulfide cross-linking and Hg\(^{2+}\) binding. Our results suggest that the four highly conserved aspartyl residues form a (Asp\(^{49}\)-Asp\(^{157}\))\(_2\) binding center for binding and cooperative transport of two Cd\(^{2+}\) ions at the dimer interface. Further mutation-function characterization identified two neighboring residues, Asp\(^{85}\) and His\(^{153}\), contributing to the bimetal binding center. Because aspartyl residues are frequently observed in multimetal zinc enzymes in which aspartate carboxylates bridge adjacent metal ions by bidentate oxygen-metal coordination (14), we further examined the bridging role of (Asp\(^{49}\)-Asp\(^{157}\))\(_2\). Our experiments led to a model for the translocation pathway of metal ions at the YiiP dimer interface.

**EXPERIMENTAL PROCEDURES**

*Site-directed Mutagenesis*—Construction of the expression plasmid pYiiP-His and site-directed mutagenesis were performed as described previously (15). All DNA sequences were verified by sequencing of both strands.

*Overexpression and Purification*—His-tagged YiiP and mutants were overexpressed in BL21 (DE3) pLyS cells using an auto-inducing growth medium (16). Cells from overnight cultures were harvested by centrifugation. The overexpressed proteins were solubilized, purified by Ni\(^{2+}\)-nitrilotriacetic acid (Ni\(^{2+}\)-NTA) affinity chromatography as described previously (12). His tags were then cleaved proteolytically by overnight thrombin digestion, and metal ions associated with the purified proteins were removed by a brief incubation with 5 mM EDTA, followed by size-exclusion high performance liquid chromatography (HPLC) using a TSK 3000SWXL column (TosoHaas), pre-equilibrated with a degassed HPLC buffer (20 mM HEPES, pH 7.0, 100 mM NaCl, 12.5% glycerol, 0.05% n-dodecyl-β-d-maltoside (DDM), 0.25 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP)). For preparation of N-ethylmaleimide (NEM)-modified protein samples, proteins were incubated with NEM (5 mM) for 30 min at room temperature, followed by a brief incubation with EDTA (5 mM), and then purified by size-exclusion HPLC purification.

*Isothermal Titration Calorimetry (ITC)*—Calorimetric titrations were carried out on a Microcal MCS titration calorimeter (Microcal) at 25 °C as described previously (15). The dilution heat was measured by injecting a titrant into the degassed HPLC buffer and subtracted from the corresponding titration heat generated by injecting the titrant an HPLC-purified YiiP or mutant sample. The titration data were fitted to a binding model consisting of either one or two sets of non-interacting binding sites using a nonlinear least-squares algorithm provided by the Microcal Origin software. The binding enthalpy change ΔH, association constant K\(_{\text{a}}\) and the binding stoichiometry n were permitted to float during the least-squares minimization and taken as the best fit values. Protein concentrations were determined using BCA protein assay (Pierce) and amino acid analysis (W. M. Keck Biotechnology Resource, Yale University).

**Metal Binding and Transport**

Reconstitution and Stopped-flow Transport Assay—Reconstitution and stopped-flow transport experiments were performed in an assay buffer containing 20 mM HEPES, 50 mM K\(_2\)SO\(_4\), pH 6.5 as described previously (12). Briefly, HPLC-purified YiiP and mutants were reconstituted with *E. coli* polar lipids (Avanti Polar Lipids) at a protein/lipid molar ratio of 1:20,000. SDS-PAGE analysis of proteoliposome samples confirmed that approximately the same amount of YiiP or mutants was reconstituted in each experiment. Liposomes were made in parallel to proteoliposomes as a protein-free control sample. Liposomes or proteoliposomes were then loaded with 200 μM fluorescence indicator fluo-3 (Molecular Probes), and 1:1 mixed on a stopped-flow apparatus (KinTek Corp.) with the assay buffer containing CdSO\(_4\) at an indicated concentration. For each data trace, three successive stopped-flow recordings at 525 nm (excited at 490 nm) were collected and averaged, and then normalized to the maximum fluorescence intensity induced by mixing proteoliposomes with 4 mM CdSO\(_4\) in an assay buffer containing 3% n-octyl-β-d-glucoside. Liposome traces were subtracted from the proteoliposome traces for baseline corrections, and the resultant kinetic trace was fitted to an exponential function using the data analysis software SigmaPlot 4.0. The initial rate of the exponential rise was taken as the velocity of Cd\(^{2+}\) influx, v\(_i\). Least-squares fit of v\(_i\) as a function of [Cd\(^{2+}\)] yielded n, K\(_{0.5}\), and V\(_{\text{max}}\) according to Equation 1,

\[
  v_i = \frac{V_{\text{max}} [\text{Cd}^{2+}]^n}{[\text{Cd}^{2+}]^n + K_{0.5}}
\]

(Eq. 1)

where V\(_{\text{max}}\) is the maximum transport velocity, K\(_{0.5}\) is the Cd\(^{2+}\) concentration at half-maximal velocity, and n is the cooperativity of Cd\(^{2+}\) transport.

*Chemical Cross-linking*—Disulfide cross-linking was induced by copper phenanthroline (Cu(Phen))\(_3\) oxidation (17). A Cu(Phen)\(_3\) working solution (10 mM) was freshly prepared by 3:2:15 (v/v) mixing of 200 mM phenanthroline (dissolved in methanol), 100 mM CuSO\(_4\), and ddH\(_2\)O. Overexpressing host cells from a 10-ml overnight cell culture were harvested by centrifugation, washed with a reaction buffer (20 mM HEPES, pH 7.5, 100 mM NaCl), and then ruptured by three passages through an ice-chilled microfluidizer (Microfluidics Co. Newton, MA) at 12,000 psi. The resulting membrane vesicles were collected by centrifugation at 140,000 × g for 45 min, then washed, and resuspended in the reaction buffer. Oxidation reactions were initiated by adding Cu(Phen)\(_3\) to a final concentration of 0.5 mM. After 30 min of incubation at room temperature, the reactions were terminated by adding 10 mM EDTA. Vesicles were washed again to remove EDTA, and then exposed to 1 mM NEM to block any remaining reactive cysteine residue. The Cu(Phen)\(_3\)-treated proteins were solubilized using 1% DDM in 2 ml of solubilization buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, and 20% glycerol). After a brief sonication (1 min) and 30 min of incubation on ice, the solubilization mixtures were pelleted again, and the supernatants were either directly subjected to immunoblot detection using a peroxidase-conjugated monoclonal clone His1 antibody (Sigma), or incubated with Ni\(^{2+}\)-NTA resin for 30 min on ice. The affinity resin was washed with solubilization buffer in the presence of 50 mM imidazole and 0.05% DDM, and eluted using the same...
solution with an elevated imidazole concentration at 500 mM. The resulting purified proteins were analyzed by non-reducing SDS-PAGE on a 15% Tris-HCl polyacrylamide gel and stained with Coomassie Blue.

**Protection of MPB Labeling by Metal Binding**—Cells harboring the overexpression of His-tagged YiiP<sub>D49C</sub>, YiiP<sub>D157C</sub>, or YiiP<sub>D49C/D157C</sub> were pelleted and gently resuspended in a labeling buffer (20 mM HEPES, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 10% sucrose, 0.25 mM TCEP, pH 7.5). The labeling reaction was initiated by adding maleimide polyethylene oxide (PEO)<sub>2</sub> biotin (MPB) to a final concentration of 1 mM to the cell suspensions in the presence of either 0.2 mM EDTA or a metal ion at 0.2 mM as indicated. Cells were incubated with MPB at room temperature for either 1 h or 5 min as indicated, and then 20 mM β-mercaptoethanol was added to quench the unreacted MPB. The resulting cells were pelleted again, washed free of MPB, and then MPB-exposed proteins were solubilized using 1% DDM and purified using the Ni<sup>2+</sup>-NTA resin as described above. The resultant purified proteins were subjected to SDS-PAGE in duplicate. One gel was stained with Coomassie Blue and the other was transferred to a nitrocellulose membrane using a Trans-blot semi-dry transfer cell (Bio-Rad) and exposed to a peroxidase-conjugated monoclonal anti-biotin clone BN-34 antibody (Sigma). MPB labeling was detected using a SuperSignal West Pico chemiluminescent substrate (Pierce).

**RESULTS**

**Correlation between Cd<sup>2+</sup> Binding and Transport**—Asp<sup>49</sup> is a highly conserved aspartyl residue located in the TM2 of YiiP (Fig. 2B). To establish the functional role of Asp<sup>49</sup> in metal binding and transport, we examined the mutation-function correlation using two Asp<sup>49</sup> mutants, YiiP<sub>D49C</sub> and YiiP<sub>D49A</sub>. Cysteine and aspartate are Zn<sup>2+</sup>/Cd<sup>2+</sup>-liganding residues frequently exchangeable in many metalloproteins, whereas alanine is not a Zn<sup>2+</sup>/Cd<sup>2+</sup>-liganding residue. Cd<sup>2+</sup> binding to YiiP, YiiP<sub>D49A</sub>, and YiiP<sub>D49C</sub> were characterized directly by ITC as described under “Experimental Procedures.”

The integrated heat per mole of injected Cd<sup>2+</sup> was plotted as a function of the Cd<sup>2+</sup>/protein molar ratio (lower panel, Fig. 1A), according to the heat flow in response to a sequence of Cd<sup>2+</sup> titrations shown in the upper panel. The titration heat decreased as the Cd<sup>2+</sup> concentration increased progressively to saturated concentrations. Qualitatively, the midpoint of the Yii binding isotherm was at 2.5 stoichiometric units, corresponding to 2.5 Cd<sup>2+</sup> binding sites per subunit. The midpoints for YiiP<sub>D49A</sub> and YiiP<sub>D49C</sub> titrations were around 1.5 and 2.5 stoichiometric units, respectively, indicating that YiiP<sub>D49A</sub> lost one stoichiometric site whereas YiiP<sub>D49C</sub> retained all the 2.5 binding sites.

To quantitatively compare Cd<sup>2+</sup> binding, binding isotherms of YiiP and YiiP<sub>D49C</sub> were fitted with a two-site, whereas YiiP<sub>D49A</sub> with a one-site binding model. As shown in Table 1, the values

**TABLE 1**

Summary of Cd<sup>2+</sup> binding parameters

| Protein     | Site 1      | Site 2      |
|-------------|-------------|-------------|
|             | n           | K<sub>a</sub> | ΔH     | n          | K<sub>a</sub> | ΔH     |
| YiiP        | 1.5 ± 0.1   | 7.9 ± 2.7   | -5.8 ± 0.2 | 1.1 ± 0.1  | 1.1 ± 0.5  | -7.8 ± 0.7 |
| YiiP<sub>D49C</sub> | 1.5 ± 0.3  | 5.5 ± 1.3  | -5.6 ± 0.7 | 1.2 ± 0.3  | 0.8 ± 0.3  | -7.3 ± 3.4 |
| YiiP<sub>D49A</sub> | 1.4 ± 0.1  | 5.4 ± 0.6  | -8.5 ± 0.2 | -9.9 ± 0.1  | -9.9 ± 0.1  | -9.9 ± 0.1  |
| YiiP<sub>D49A/D157A</sub> | 1.4 ± 0.1  | 7.5 ± 0.6  | -8.5 ± 0.2 | -9.9 ± 0.1  | -9.9 ± 0.1  | -9.9 ± 0.1  |
| YiiP<sub>D157C</sub> | 1.3 ± 0.1  | 1.4 ± 0.2  | -5.5 ± 0.1 | 1.2 ± 0.1  | 1.1 ± 0.3  | -6.9 ± 0.3 |
| YiiP<sub>D157A/C287S</sub> | 1.4 ± 0.1  | 5.6 ± 0.5  | -5.5 ± 0.1 | 0.9 ± 0.3  | 1.2 ± 1.1  | -6.1 ± 1.3 |
| YiiP<sub>His123/C307S</sub> | 1.5 ± 0.7  | 7.9 ± 2.0  | -4.9 ± 0.5 | 1.0 ± 0.5  | 0.17 ± 0.41 | -3.3 ± 1.3 |
| YiiP<sub>His123/C307S</sub>NEM | 1.5 ± 0.1  | 7.0 ± 1.2  | -4.9 ± 0.1 | -4.9 ± 0.1  | -4.9 ± 0.1  | -4.9 ± 0.1  |

* Binding parameters taken from a previous study for comparison (11).
metal binding and transport

of the association constant $K_a$, stoichiometry $n$ and binding enthalpy change $\Delta H$ of site 1 and site 2 for YiiP and YiiPD$_{D49C}$ were near identical within experimental errors. Fit of YiiPD$_{D49A}$ binding isotherm yielded $K_a$ and $n$ values in excellent agreement with those of site 1 of YiiP and YiiPD$_{D49C}$. The binding enthalpy change for YiiPD$_{D49A}$ decreased to $-8.5$ kcal/mol from $-5.8$ kcal/mol for YiiP, indicative of a more favorable enthalpy for Cd$^{2+}$ binding to site 1. These results indicated that a D49A mutation specifically disrupted Cd$^{2+}$ binding to site 2, while leaving site 1 intact. We also examined Zn$^{2+}$ titrations of YiiP, YiiPD$_{D49A}$, and YiiPD$_{D49C}$ and qualitatively obtained similar results (data not shown). However, as described in earlier studies, titrations of YiiP with Zn$^{2+}$ resulted in a biphasic binding isotherm, precluding a definitive deconvolution of the heat components (15).

To establish the functional role of Asp$_{49}$ in metal transport, we further examined the correlation between Cd$^{2+}$ binding to Asp$_{49}$ and Cd$^{2+}$ transport. HPLC-purified YiiP, YiiPD$_{D49A}$, and YiiPD$_{D49C}$ were reconstituted into proteoliposomes loaded with a Cd$^{2+}$-sensitive fluorescent indicator fluozin-1 for monitoring transmembrane influx of Cd$^{2+}$ as described under “Experimental Procedures.” Upon rapid mixing YiiP proteoliposomes with an assay buffer containing various concentrations of Cd$^{2+}$, the fluozin-1 fluorescence response increased progressively as the Cd$^{2+}$ concentration increased (Fig. 1B). A control experiment with liposomes under identical conditions only showed a background fluorescence response. Thus, the observed proteoliposome responses were derived from Cd$^{2+}$ influx mediated by the reconstructed YiiP. As described previously (12), we also observed a linear relationship between the initial rate of fluorescence rise and the amount of reconstituted YiiP within a YiiP/lipid molar ratio up to 15/20,000 (data not shown). This observation indicated that the rate of Cd$^{2+}$ influx was linearly related to the YiiP transport activity. As shown in Fig. 1B, the overall fluorescence responses of YiiPD$_{D49C}$ and YiiP proteoliposomes were comparable both in rate and amplitude within the Cd$^{2+}$ concentration range examined, while the responses of YiiPD$_{D49A}$ proteoliposomes reduced to a background level (upper panel). The initial rate of YiiP transport was dependent on the Cd$^{2+}$ concentration in a sigmoidal fashion (lower panel), indicative of positive cooperativity. The transport cooperativity $n$, maximum transport velocity $V_{\text{max}}$, and the half-maximal concentration $K_{0.5}$ were obtained by least-squares fit of the concentration dependent data to Equation 1, yielding $n$ values of 1.4 ± 0.1 for YiiP and 1.1 ± 0.1 for YiiPD$_{D49C}$. It appeared that the effect of a D49C mutation is anticooperative. Furthermore, the $K_{0.5}$ value was increased to 0.58 ± 0.07 mM for YiiPD$_{D49C}$ from 0.27 ± 0.02 mM for YiiP whereas the $V_{\text{max}}$ value was marginally decreased to 12.1 ± 0.6 s$^{-1}$ for YiiPD$_{D49C}$ from 14.4 ± 0.4 s$^{-1}$ for YiiP. The modest $K_{0.5}$ values increase was consistent with ITC binding measurement, showing an insignificant decrease of the association constant $K_a$ value of site 2 to 0.8 ± 0.3 $\mu$M$^{-1}$ for YiiPD$_{D49C}$ from 1.1 ± 0.5 $\mu$M$^{-1}$ for YiiP. Taken together, ITC binding and stopped-flow kinetic analyses demonstrated the following mutation-function correlation: substitution of Asp$_{49}$ with a non-liganding Ala residue disrupted a Cd$^{2+}$ binding site, and consequently abolished the transport activity, while substitution of Asp$_{49}$ with a liganding Cys residue retained overall binding capacity and transport activity. Therefore, our result suggested that Asp$_{49}$ is a Cd$^{2+}$ coordination residue required for both metal binding and transport. The stoichiometry of Cd$^{2+}$ binding to Asp$_{49}$ was estimated according to the stoichiometry difference between Cd$^{2+}$ binding to YiiP and YiiPD$_{D49A}$, approximately yielding one Asp$_{49}$ site for each subunit, or a pair of symmetry-related Asp$_{49}$ sites for a YiiP homodimer.

Cd$^{2+}$ Binding to YiiPD$_{D49A}/D157A$—The localization of site 2 to Asp$_{49}$ mirrored an earlier finding that a D157A mutation specifically disrupted site 2 without affecting site 1 (Table 1) (11). These results raised a possibility that both Asp$_{49}$ and Asp$_{157}$ might contribute to a common binding site (site 2), although Asp$_{49}$ and Asp$_{157}$ are topologically located in TM2 and TM5, respectively (Fig. 2B). To further establish a functional connection between Asp$_{49}$ and Asp$_{157}$, we examined the effect of a D49A/D157A double mutation on Cd$^{2+}$ binding. Compared

![Image](https://via.placeholder.com/150)

**Figure 2. Functional connection between Asp$_{49}$ and Asp$_{157}$.** A, ITC analysis of Cd$^{2+}$ binding to YiiPD$_{D49A/D157A}$. Upper panel, typical calorimetric titrations of 0.5 mM CdCl$_2$ into 0.01 mM YiiPD$_{D49A/D157A}$. Lower panel, integrated heat as a function of the Cd$^{2+}$/protein molar ratio. Filled circles, open squares, and open diamonds represent data points calculated from titrations of YiiPD$_{D49A/D157A}$, YiiP, and YiiPD$_{D49A}$, respectively. Solid lines represent best fits of the binding isotherms with fitting parameters summarized in Table 1. Note that the arrow indicates the leftward shift of the YiiPD$_{D49A/D157A}$ binding isotherm, B, schematic representation of the YiiP transmembrane topology (11). Positions of Asp$_{45}$, Asp$_{49}$, His$_{153}$, Asp$_{157}$, Cys$_{127}$, and Cys$_{287}$ in the topology model are marked.
with YiiP and YiiP_{D49A/C127V/C287S} exhibited a binding isotherm characteristic of the YiiP_{D49A} behavior, with a midpoint shifted leftward by one stoichiometric unit from the midpoint of the YiiP binding isotherm (Fig. 2A). Fitting the YiiP_{D49A/D157A} binding isotherm to a one-site model yielded $K_a = 7.5 \pm 0.6 \mu M^{-1}$, $n = 1.4 \pm 0.1$, in excellent agreement with the binding parameters for site 1 of YiiP, YiiP_{D49A}, andYiiP_{D157A} (Table 1). The fitted $\Delta H$ value slightly decreased from $-8.5$ kcal/mol for YiiP_{D49A} to $-9.9$ kcal/mol for YiiP_{D49A/D157A}. Nevertheless, it was evident that the D49A/D157A double mutation disrupted site 2 while retaining the overall binding capacity of site 1. The same disruptive effect on site 2 by D49A and D157A and D49A/D157A mutations suggested that Asp$^{49}$ and Asp$^{157}$ belong to a common binding site (site 2).

**Disulfide Cross-link**—The functional connection between Asp$^{49}$ and Asp$^{157}$ suggested that tertiary folding of the YiiP polypeptide chain might bring together these two aspartyl residues to a common binding site. To establish a possible physical proximity between Asp$^{49}$ and Asp$^{157}$, we examined whether copper phenanthroline oxidation could induce Cys$^{49}$-Cys$^{157}$ disulfide linkage. The cross-linking experiments were performed on a YiiP_{D49C/D157C/C287S/C127V} quadruplet mutant. The two native Cys$^{287}$ and Cys$^{127}$ were removed to prevent their adventitious contributions to the cross-linking reaction. The YiiP_{C287S/C127V} double mutation caused no detectable change in protein expression, Cd$^{2+}$ binding to site 2 and transport activity.$^4$ As shown in Fig. 3, YiiP_{D49C/D157C/C287S/C127V} purified from membrane vesicles without copper phenanthroline exposure only yielded a single protein band on a 15% non-reducing gel (lane 1), corresponding to a monomeric YiiP species as indicated. In contrast, copper phenanthroline exposure yielded three additional protein bands with distinct mobility (lane 2). Adding 5 mM dithiothreitol (DTT) to the copper phenanthroline-treated protein sample prevented all aberrant mobility (lane 3), indicating that the observed band shifts reflected the formation of disulfide cross-links. Thus, the major lower protein species moving in front of the monomeric band was interpreted as a more compact monomeric species caused by an intrasubunit D49C-D157C cross-link as indicated. The cross-linked dimers could be fully reduced to the monomeric form by DTT treatment (lanes 4 and 7), yet an additional dimeric species appeared after copper phenanthroline treatment (lanes 5 and 8), yet an additional dimeric species appeared after copper phenanthroline oxidation (lanes 5 and 8). The cross-linked dimers could be fully reduced to the monomeric form by DTT treatment (lanes 6 and 9). As expected, copper phenanthroline oxidation did not cause fast-moving protein species in front of the monomeric protein (lanes 5 and 8), because a single cysteine residue in either triple mutant was not able to form an intrasubunit cross-link. Interestingly, the dimeric (YiiP$_{D49C/C287S/C127V}$)$_2$ in lane 5 moved slightly faster than (YiiP$_{D157C/C287S/C127V}$)$_2$ in lane 8. Thus, the observed mobility difference between these two dimeric species accounted for the two distinct upper (YiiP$_{D49C/D157C/C287S/C127V}$)$_2$ bands shown in lane 2. As a control experiment, copper phenanthroline treatment of YiiP$_{C287S/C127V}$ under the identical condition did not yield any cross-linked protein species (lane 11).

A relatively high concentration of copper phenanthroline (0.5 mM) was used in this study because membrane vesicles in the reaction mixture absorbed a significant amount of copper ions. The actual Cu(phen)$_3^-$ concentration in the reaction mixture was found to be only 0.043 mM as estimated by a fluorescence metal ion indicator Phen Green-SK (Molecular Probes) according to a standard curve generated using a set of known Cu(phen)$_3^-$ concentrations. We also performed cross-linking reactions using 0.05 mM copper phenanthroline with $\sim$10-fold less membrane vesicles. Under this condition, the cross-linked products were only detectable by immunoblot detection using an antibody against the polyhistidine tag. Nevertheless, this less stringent oxidation condition yielded essentially the same results except that the dimeric (YiiP$_{D49C}$)$_2$ and (YiiP$_{D157C}$)$_2$

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$^4$ Y. Wei and D. Fu, unpublished data.
could not be distinctly resolved after proteins were transferred to the nitrocellulose membrane. To further validate the cross-linking result, we tested a collection of nine single cysteine variants. As shown in Fig. 3B, D45C, D49C, H153C, D157C, and Cys287 were cross-linked by 0.5 mM copper phenanthroline while Cys127, S144C, D179C, and E200C were not able to be cross-linked under the same oxidation condition. Among the four non-reactive positions, Cys127 is located in the middle of TM4, S144C is located in the connecting loop between TM4 and TM5, D179C is at a highly conserved position in the connecting loop between TM5 and TM6, and E200C is in the C-terminal hydrophilic domain. This set of positions provided negative controls for the five reactive positions. Among them, the observation of D49C-D49C and D157C-D157C intersubunit disulfide linkages suggested that both aspartyl residues are located at the dimer interface. The observation of D49C-D157C intrasubunit disulfide linkage, as shown in Fig. 3A, further indicated physical proximity between the Asp49 and Asp157. Therefore, the four highly conserved aspartyl residues, two from each subunit, may be located within the disulfide bonding distance at the dimer interface. D45C and H153C were cross-linked, probably because of their closeness to Asp49 and Asp157, respectively. Assuming that both TM2 and TM5 adopt a α-helical configuration, Asp45 is located one helical turn from Asp49 toward the outer leaflet, while His153 is one helical turn from Asp157 toward the inner leaflet of the cell membrane. We also performed cross-linking reactions using 0.5 mM 1,1-methanediyl bismethanethiosulfonate (18) instead of 0.5 mM copper phenanthroline under otherwise identical conditions. Dimeric cross-links were observed for D45C and H153C single cysteine mutants, consistent with the copper phenanthroline results. In contrast, we did not observe dimeric D49C-D49C and D157C-D157C cross-links, suggesting that D49C and D157C are not accessible to the cross-linker whereas D45C and H153C are located at more accessible regions. Cys287 is located in the C-terminal domain and the observation of the Cys287-Cys287 intersubunit cross-link may reflect their physical proximity.

**A** D49C Mutation Generates a Biscysteinate Hg²⁺ Binding Site at the Dimer Interface—A pair of cysteine residues may form a biscysteinate Hg²⁺-binding site if they are positioned within a disulfide bonding distance. To further establish the interfacial location of Asp49, we examined whether a D49C mutation generated a biscysteinate Hg²⁺-binding site across the dimer interface. As shown in Fig. 4, Hg²⁺-titrations of YiiP_C287S yielded a binding isotherm with a stoichiometry of 1.0 ± 0.1 (Table 2). A D49C/C287S mutation caused a rightward shift of the binding isotherm, which could be described by a two-site binding model. The fitted parameters for the first site agreed well with that of YiiP_C287S. The second site had a stoichiometry of 0.6 ± 0.1 (Table 2), corresponding to an Hg²⁺ binding site shared by two subunits in a YiiP homodimer. Thus, the D49C mutation generated a biscysteinate Hg²⁺-binding site at the dimer interface. In contrast, titrations of YiiP_D49C/D157C/C287S exhibited a binding isotherm similar to that of YiiP_D49C/C287S (Fig. 4), indicating that a D157C mutation did not generate additional Hg²⁺ binding sites. This observation was consistent with our previous finding that Cys157 is not accessible to Hg²⁺ binding (11).
Metal Binding and Transport

Probing the Local Environment of the (Asp$^{49}$-Asp$^{157}$)$_2$ Bimetal Binding Center—To explore the local environment of the (Asp$^{49}$-Asp$^{157}$)$_2$ cluster, we introduced D → C mutations and examined metal protection of thiol-specific labeling of MPB to the reporter cysteiny1 residues. There are two native cysteines in YiiP, at position 127 and 287, respectively. Earlier studies have established that neither cysteine residue can be labeled from the extracellular side by the membrane impermeant MPB when the hydrophobic barrier of the plasma membrane is intact (11). Thus, the protection of different divalent metal ions was examined in intact cells with a 5-min exposure to MPB in the absence of either EDTA or a divalent metal ion as indicated. The labeling reactions were set for 5 min, and then quenched by 20 mM β-mercaptoethanol. The MPB-treated proteins were purified and subjected to SDS-PAGE analysis. MPB labeling was detected by immunoblot (upper gel), and a duplicated gel was stained with Coomassie Blue (lower gel). B, Hg$^{2+}$ protection of MPB labeling. MPB labeling to an indicated YiiP mutant was carried out with intact cells in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 0.2 mM HgCl$_2$. The labeling reactions were set for 60 min. C, a schematic illustration of Hg$^{2+}$ protection of MPB labeling to Cys$^{49}$ and Cys$^{157}$. Note that Hg$^{2+}$ binding to Cys$^{49}$ protects Cys$^{157}$ from MPB labeling by blocking the MPB accessibility to Cys$^{157}$.

Additional Coordination Residues Contribute to the (Asp$^{49}$-Asp$^{157}$)$_2$ Bimetal Binding Center—Asp$^{49}$ and Asp$^{157}$ are the two most conserved residues in the CDF protein family (9). Their functional roles in metal binding and transport suggest that the (Asp$^{49}$-Asp$^{157}$)$_2$ cluster is located within the translocation pathway. In the entire transmembrane spanning domain, only three residues, Asp$^{45}$, Cys$^{127}$, and His$^{153}$ can meet the topological and chemical expectations for a role as a metal coordination residue. Cys$^{127}$ is a variable residue and a C127V mutation caused no detectable change in Cd$^{2+}$ transport and binding to the (Asp$^{49}$-Asp$^{157}$)$_2$ binding site. Both Asp$^{45}$ and His$^{153}$ are somewhat conserved, each located one helical turn above or below the (Asp$^{49}$-Asp$^{157}$)$_2$ binding center as depicted in Fig. 2B. Cd$^{2+}$ titrations of YiiP$_{D45C/C287S}$, or YiiP$_{H153C/C287S}$ revealed a binding isotherm that could be fitted to a two-site model (Fig. 6). Compared with YiiP, the D45C/C287S mutation caused little or no change to both binding sites while the H153C/C287S mutation reduced the $K_a$ value of site 2 by 7-fold without significantly affecting site 1 (Table 1). Interestingly, both D45C/C287S and H153C/C287S mutations conferred NEM sensitivity to site 2. As shown in the top and middle panels of Fig. 6, incubation of protein samples in 5 mM NEM for 30 min caused a loss of one binding component for both YiiP$_{H153C/C287S}$ and YiiP$_{D45C/C287S}$. The fitted parameters for the remaining sites were consistent with that of site 1, indicating that NEM selectively disrupted site 2 (Table 1). Control experiments with YiiP or YiiP$_{C287S}$ showed no detectable NEM effect on the respective Cd$^{2+}$ binding isotherm, thereby localizing the NEM-sensitive residue to Cys$^{45}$ or Cys$^{153}$. The effects of D45C and H153C mutations on Cd$^{2+}$ transport were examined by stopped-flow analysis of Cd$^{2+}$ influx into reconstituted proteoliposomes. As shown in the bottom panel of Fig. 6, both mutations minimally affected the transport kinetics. Fits of the Cd$^{2+}$-concentration-dependent data yielded $K_a$ values of 0.37 ± 0.01 mM, 1.3 ± 0.1, and 13.3 ± 0.7 s$^{-1}$ for YiiP$_{D45C/C287S}$ and 0.43 ± 0.07 mM, 1.4 ± 0.2 and 14.2 ± 1.0 s$^{-1}$ for YiiP$_{H153C/C287S}$. In contrast, incubating proteoliposomes with 5 mM NEM for 30 min...
Aspartyl Residues Bridge Two Metal Binding Sites at the Dimer Interface—Aspartyl residues are frequently found in metal binding clusters where they bridge a pair of metal ions by providing two oxygen ligands, each interacting with one of the two adjacent metal ions (14). If certain carboxylates in (Asp49- and Asp157), playing bridging roles in bimetal binding and transport, a D49C mutation resulted in a functional transporter reduced Cd²⁺ influx to the background level. Taken together, our data suggested both Asp⁴⁹ and His¹⁵³ contribute to binding and transport of Cd²⁺.

Aspartyl residues are frequently found in metal binding clusters where they bridge a pair of metal ions by providing two oxygen ligands, each interacting with one of the two adjacent metal ions (14). If certain carboxylates in (Asp⁴⁹, Asp¹⁵⁷), acting as bridging residues, replacing bidentate carboxylate oxygen ligands with monodentate thiolate sulfurs is expected to disconnect the bridging interaction, leading to the disruption of the bimetal binding. As noted above, a D49C mutation reduced the cooperativity of YiiP transport from 1.4 to 1.1, with only marginal effect on the cooperativity of YiiP transport from 1.4 to 1.1. Likewise, a D157C mutation also reduced the cooperativity of YiiP transport to 0.99 ± 0.08 with modest effects on Vmax (13.5 ± 0.9 s⁻¹) and Ks (0.50 ± 0.08 mM) (data not shown). In sharp contrast, a D49C/D157C double mutation was found to selectively disrupt the bimetal binding center (site 2). As shown in Fig. 7A, Cd²⁺ titrations of YiiPDD49C/D157C yielded a binding isotherm shifted leftward by one stoichiometric unit as compared with those of YiiP and YiiPD49C. Fitting the YiiPDD49C/D157C binding isotherm with one-site model yielded Ks = 1.4 ± 0.2 μM⁻¹, n = 1.3 ± 0.1, and ΔH = −5.5 ± 0.1 kcal/mol, in agreement with the site 1 binding parameters of YiiP, YiiPDD49C, and YiiPDD157C (Table 1). Corresponding to the loss of site 2 in YiiPDD49C/D157C, the fluorozin-1 responses of YiiPDD49C/D157C proteoliposomes were greatly diminished (Fig. 4B). The residual transport activity was apparently correlated with a weak Cd²⁺ binding component characterized by a high titration heat plateau as the titration proceeded toward saturation (upper panel, Fig. 7A). Fitting the YiiPDD49C/D157C concentration-dependent data to Equation 1 revealed that the double cysteine mutation converted Cd²⁺ transport into a negative cooperative process (n = 0.73 ± 0.25) with significant alternations of both Vmax (2.7 ± 1.8 s⁻¹) and Ks (3.5 ± 6.28 mm). Because of a greatly reduced site 2 binding affinity in YiiPDD49C/D157C, the maximum experimental Cd²⁺ concentration (4 mM) was not sufficiently high to elicit a quasi-static-state response. The fitted parameters for YiiPDD49C/D157C transport were of less certainty. Nevertheless, it was evident that a D49C/D157C double mutation caused severe impairments of the site 2 binding capacity, and consequently metal transport. Considering that cysteine substitution of either Asp⁴⁹ or Asp¹⁵⁷, only marginally compromised Cd²⁺ binding, it appeared that two of the four aspartates in (Asp⁴⁹-Asp¹⁵⁷) may play bridging roles in bimetal binding and transport at the dimer interface.

**DISCUSSION**

The experiments described herein establish a strong correlation between YiiP transport and metal binding to two highly conserved aspartyl residues (Asp⁴⁹ and Asp¹⁵⁷). Direct metal binding and indirect metal protection analyses both demonstrated a Cd²⁺-Asp⁴⁹ interaction characteristic of general coordination chemistry of Cd²⁺ binding. In sharp contrast to the disruptive effect of a D49A mutation on Cd²⁺ binding and transport, a D49C mutation resulted in a functional transporter
Further evidence that Asp49 and Asp157 contribute to a common mutation has the same disruptive effect specific to site 2, providing metal ions, the thiol reactivity of Cys49 responded to metal ions although cysteine is a preferred coordination residue for soft Zn\(^{2+}\) in a selective fashion. Among the divalent cations tested, only Cd\(^{2+}\) binding sites (site 2). This result mirrors an earlier finding that a D157A mutation selectively disrupts Cd\(^{2+}\) binding to site 2 without affecting site 1 (11). Moreover, the D49A/D157A double mutation has the same disruptive effect specific to site 2, providing further evidence that Asp49 and Asp157 contribute to a common binding site. In corroboration with the Asp\(^{49}\) Asp\(^{157}\) functional connection, the formation of the Cys\(^{49}\)-Hg\(^{2+}\)-Cys\(^{49}\) bis-cysteinate binding, and Cys\(^{49}\)-Cys\(^{49}\), Cys\(^{157}\)-Cys\(^{157}\) intersubunit and Cys\(^{49}\)-Cys\(^{157}\) intrasubunit disulfide cross-links established physical proximities among Cys\(^{49}\)s and Cys\(^{157}\)s across the dimer interface. Our results lead to the localization of active metal binding to the dimer interface of YiiP, thereby demonstrating for the first time that the functional unit of a representative CDF transporter is a homodimer.

ITC analysis indicated that Cd\(^{2+}\) binds to (Asp\(^{49}\)-Asp\(^{157}\)) in each subunit with a stoichiometry near unity, thus the (Asp\(^{49}\)-Asp\(^{157}\))\(^{2}\) cluster forms a bimetal binding center at the dimeric interface. In general, this binding of metal ions in pair is opposed by electrostatic and entropic considerations, but the hydrophobic environment of (Asp\(^{49}\)-Asp\(^{157}\))\(^{2}\) may act in favor of bimetal binding. The two bound Cd\(^{2+}\) ions and four aspartyl carboxylates, each potentially carries a full negative charge, may allow an overall electrostatic balance in the middle of the transmembrane spanning domain. In support of the bimetal binding, Cd\(^{2+}\) transport showed a sigmoidal dependence on the Cd\(^{2+}\) concentration, a characteristic of positive cooperativity of Cd\(^{2+}\) binding. It appears that the...
Asp45)-(Asp49-Asp157)₂-(His153-His153) bimetal binding center. Nevertheless, cysteine substitution of all four aspartates in the (Asp49-Asp157)₂ cluster disrupted the bimetal binding and significantly impaired Cd²⁺ transport. This observation may reflect different roles that cysteines and aspartates may play in a bimetal binding center, in which a bidentate Asp carboxylate can potentially coordinate with two metal ions simultaneously whereas a monodentate Cys thiolate can only coordinate with one of the two metal ions. Thus, if an aspartate is involved in coordination of two metal ions in the bimetal binding center, a cysteine substitution is expected to disrupt the bidentate bridging interaction. To account for the ability of Cd²⁺ binding to (Asp49-Cys157)₂ and (Cys49-Asp157)₂ as well as the inability of Cd²⁺ binding to (Cys49-Cys157)₂, we consider protein conformational dynamics and propose that the putative (Asp45-Asp49)-(Asp49-Asp157)₂-(His153-His153) bimetal binding center may be only partially accessible to Cd²⁺ binding at any time by alternating between an extracellularly facing (Asp45-Asp49)-(Asp49-Asp157)₂ and an intracellularly facing (Asp49-Asp157)₂-(His153-His153) site (Fig. 8). Each site contains only six coordination residues, and two of four aspartates in the (Asp49-Asp157)₂ cluster may act as bridging residues to provide two additional oxygen ligands. Thus the six coordination residues take up all eight coordinates as depicted in Fig. 8.

In accordance with this model, cysteine substitution of two aspartyl residues, either by a D49C or D157C mutation, only marginally affected metal binding whereas cysteine substitution of four aspartyl residues disconnected the bridging interaction in the bimetal binding center, leading to the disruption of Cd²⁺ binding. Likewise, NEM modification of Cys45 or Cys153 disrupted only one-half of the bimetal binding sites, but may also locked the protein into a conformation in which the remaining intact half is not accessible to Cd²⁺ binding. It is noted that cysteine substitution of either D49C or D157C caused a more pronounced reduction of Cd²⁺ transport cooperativity as compared with cysteine substitution of Asp49 or His153. The (Asp49,Asp157)₂ cluster may play a more significant role in cooperative transport of two Cd²⁺ ions at the dimer interface. As a proton-antipporter, the unligated YiiP is expected to adopt a protonated conformation. The bridging interaction between Cd²⁺ binding sites may permit the first Cd²⁺ ligation to deprotonate its neighboring site, priming the unligated site for the entry of the second Cd²⁺. Previous ITC analysis has indicated that Cd²⁺ binding to YiiP is directly coupled to deprotonation. Whether (Asp49,Asp157)₂ is directly involved in coupling with deprotonation remains to be determined.

Zinc biology is fundamentally different from those of dominant cations in biological systems, such as K⁺, Na⁺, and Ca²⁺. Free Zn²⁺ is only available in trace amounts (probably <10⁻⁹ × 10⁻¹² M). To counterbalance this, zinc binds to most protein ligand centers at least 1000 times more strongly than Ca²⁺. On the other hand, a characteristic of transport systems, as oppose to those of structural or catalytic metalloproteins is that the former must bind and release metal ions quickly to keep the flow of a metal ion when it is transported. This rapid on-off process is in general coupled with protein conformational changes. In the absence of a chemical energy input from ATP hydrolysis, the bridging interaction found in YiiP could propagate a local binding energy input to a more extensive bimetal binding center. The binding of two metal ions could further increase the energy capacity to switch the protein conformation without relaying on an increased metal binding strength. Thus this low affinity, bimetal binding could help facilitate the movement of metal ions along the translocation pathway. At present, most of the zinc coordination chemistry has been learned from static zinc binding sites. Our experiments suggest that, instead of using the canonical S- and N-ligands, the metal transporter YiiP takes advantage of the fast on-off kinetics of Zn²⁺-O coordination and adopts a bridging coordination system to achieve the dual functionalities of selective binding and rapid release. It is noted that while the affinity of Zn²⁺/Cd²⁺ binding to (Asp49-Asp157)₂ is in the micromolar range, several orders of magnitude lower than the nanomolar to picomolar range binding affinities found in many zinc metalloproteins (20), it is in the same micromolar range as Ca²⁺ binding affinities in Ca-ATPases (21). Accordingly, the rate of Zn²⁺/Cd²⁺ transport by YiiP (~15 s⁻¹) is also comparable to that of Ca²⁺ transport by Ca-ATPases (22). Oxygen atoms emerge as the preferred ligand for Na⁺, K⁺, and Ca²⁺ (23) in membrane channels and transporters, and now for Zn²⁺/Cd²⁺ selective binding in YiiP. Despite this apparently universal ligand preference, oxygen atoms could be arranged into different coordination geometries to confer extremely precise selectivity, for example, between Na⁺ and K⁺ (24). Therefore, the relatively weaker Zn²⁺-O coordination in YiiP could facilitate rapid binding/release, whereas at the same time retain the fidelity of discriminatory effects against dominant cations that are several orders more abundant than free zinc in a living cell.

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