Selective Metal Binding to a Membrane-embedded Aspartate in the Escherichia coli Metal Transporter YiiP (FieF)*

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The cation diffusion facilitators (CDF) are a ubiquitous family of metal transporters that play important roles in homeostasis of a wide range of divalent metal cations. Molecular identities of substrate-binding sites and their metal selectivity in the CDF family are thus far unknown. By using isothermal titration calorimetry and stopped-flow spectrofluorometry, we directly examined metal binding to a highly conserved aspartate in the Escherichia coli CDF transporter YiiP (FieF). A D157A mutation abolished a Cd2⁺-binding site and impaired the corresponding Cd2⁺ transport. In contrast, substitution of Asp-157 with a cysteinyl coordination residue resulted in intact Cd2⁺ binding as well as full transport activity. A similar correlation was found for Zn2⁺ binding and transport,suggesting that Asp-157 is a metal coordination residue required for binding and transport of Cd2⁺ and Zn2⁺. The location of Asp-157 was mapped topologically to the hydrophobic core of transmembrane segment 5 (TM-5) where D157C was found partially accessible to thiol-specific labeling of maleimide polyethylene-oxide biotin. Binding of Zn2⁺ and Cd2⁺, but not Fe2⁺, Hg2⁺, Co2⁺, Ni2⁺, Mn2⁺, Ca2⁺, and Mg2⁺, protected D157C from maleimide polyethylene oxide (PEO)2 biotin; HPLC, high pressure liquid chromatography.

Membrane transporters in the cation diffusion facilitator family are found both in eukaryotes and prokaryotes (1). This protein family of more than 400 genetically related members is characterized by a homologous hydrophobic N-terminal domain followed by a hydrophilic conserved Asp residue in the putative TM-5 rendered host cells hypersensitive to zinc, probably because of a loss of zinc efflux pumping activities (17). The Asp appeared to be essential because expression of CzcD158C, CzcD158A, ZitB163C, and ZitB163A mutant proteins conferred no zinc resistance. It is not clear, however, whether this conserved CDF aspartate is directly involved in binding and transport of Zn2⁺ and/or Fe2⁺.

In the present study, we sought to establish the functional role of the equivalent Asp residue in YiiP (Asp-157) by examining the effects of D157A and D157C mutations on metal binding and transport by using direct biophysical measurements. Furthermore, Asp-157 was localized to the hydrophobic core of TM-5 to establish a structural connection with the substrate translocation pathway where metal ions are selected and transported across the membrane. Metal binding to Asp-157 was found to be highly specific, with a strict selectivity for Zn2⁺ and Cd2⁺ over Hg2⁺, Fe2⁺, and other divalent metal ions that are thought to be the frequent CDF substrates. Because Asp-157 is one of the most conserved residues in the CDF family, selective metal binding to Asp-157 has broad structural and mechanistic implications.

**EXPERIMENTAL PROCEDURES**

Site-directed Mutagenesis—Cloning and construction of the expression plasmid pYiiP-His were described previously (15). Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene). A mutant C287S was first prepared using

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3 The abbreviations used are: CDF, cation diffusion facilitator; β-ME, β-mercaptoethanol; DDM, n-dodecyl-β-D-maltoside; TCEP, Tris(2-carboxyethyl) phosphine hydrochloride; ITC, isothermal titration calorimetry; FM, fluorescein 5-maleimide; MBP, maleimide polyethylene oxide (PEO)2, biotin; HPLC, high pressure liquid chromatography.
the pYiiP-His plasmid DNA as template and an anti-parallel pair of primers. The resultant C287S plasmid DNA served as the parent for an additional 13 mutants, each containing a single cysteine substitution mutation at positions 10, 36, 70, 107, 144, 150, 153, 155, 157, 171, 172, 174, or 177. Sequences of these mutants were verified by DNA sequencing of both strands.

Overexpression and Purification—YiiP and mutants were overexpressed with a C-terminal extension containing a thrombin cleavage site followed by six tandem histidine residues to facilitate protein purification. The expression host cells, BL21 (DE3) pLYS, were cultured in an auto-inducing medium for unattended protein overexpression (18). Cells from overnight cultures were harvested, and membrane proteins were extracted using 7% n-dodecyl-β-D-maltopyranoside (DDM) as described previously (15). The detergent-solubilized proteins were absorbed by three passages through a Ni\(^{2+}\)-nitrilotriacetic acid superflow column (Qiagen), which was washed free of contaminants and eluted with an elevated imidazole concentration at 500 mM. The column eluate was immediately applied to a PG-10 gel filtration column (Amerham BioSciences), yielding a desalted sample that was subsequently subjected to overnight thrombin digestion (Novagen) at a ratio of 0.5 units of thrombin per mg of protein. The completeness of thrombin digestion was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometric analysis, showing a complete conversion of the His tagged to a tag-free mass species. The resultant tag-free protein was incubated with 10 mM EDTA for 30 min and then applied to an TSK 3000SWxl size-exclusion HPLC column (TosoHaas), pre-equilibrated with a degassed HPLC buffer (20 mM HEPES, pH 7.0, 100 mM NaCl, 12.5% glycerol, 0.05% DDM, 0.2 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)). The purified protein was collected as a discrete chromatographic fraction using a Beckman SC100 fraction collector.

Isothermal Titration Calorimetry—Protein aggregates and trace amounts of metal contaminants were removed by size exclusion HPLC prior to calorimetric titrations as described previously (15). Protein concentrations were determined by BCA protein assay (Pierce). Calorimetric titrations were carried out on a Microcal iTC200 isothermal titration calorimeter (Microcal) at 25 °C. Metal titrants (chloride salts), typically in the concentration range of 0.25–0.5 mM, were dissolved in the same HPLC mobile phase used for protein purification. 1 mM ascorbate was added prior to calorimetric titrations as described previously (15). Protein concentrations were determined as a discrete chromatographic fraction using a Beckman SC100 fraction collector.

Reconstitution and Stopped-flow Transport Assay—HPLC-purified Yiip, Yiip\(^{D287C}\), or Yiip\(^{D375A}\) was reconstituted into liposomes made of E. coli polar lipids (Avanti Polar Lipids) as described previously (14). Control liposomes were prepared following exactly the same procedure without adding protein. 200 \(\mu\)M fluorescence indicator fluozin-1 (Molecular Probes) was encapsulated by two freeze-thaw cycles, followed by gel filtration to remove the untrapped dye. Transport experiments were performed at 8 °C on a stopped-flow apparatus (KinTek Corp.). Proteoliposomes and a transport assay buffer (20 mM HEPES, 50 mM K\(_2\)SO\(_4\), and either Zn\(_2\)SO\(_4\) or Cd\(_2\)SO\(_4\) at an indicated concentration ranging from 0 to 4 mM, pH 7.3) were loaded into two separate syringes of equal volume, and transport reactions were initiated by pushing 60-\(\mu\)l fresh reactants at a 1:1 ratio through the 12-\(\mu\)l mixing cell at a flow rate of 20 ml/s. Zn\(^{2+}\) or Cd\(^{2+}\) concentrations in reaction mixtures were half of the concentrations in the initial transport assay buffers. Stopped-flow traces were the cumulative average of five successive recordings at 525 nm (excited at 490 nm). Liposome traces were collected as base lines and subtracted from proteoliposome traces to yield net fluorescence changes \(\Delta F\). \(\Delta F/\Delta F_{\text{max}}\) was obtained by normalizing \(\Delta F\) to the maximum proteoliposome response elicited by a transport assay buffer containing 4 mM Zn\(_2\)SO\(_4\) or Cd\(_2\)SO\(_4\) plus 2% n-octyl-β-D-glucoside used to solubilized proteoliposomes.

Determinant of Transport Kinetic Parameters—The rate of the fluorescence rise \(k_{\text{obs}}\) was determined by least squares fit of the kinetic trace to a single exponential function using the data analysis software SigmaPlot 4.0 (SPSS Inc., Chicago, IL). The following two-step kinetic scheme (Scheme 1) was used to describe the Yiip transport process (14).

\[
\begin{align*}
M + T_1 & \xrightleftharpoons{K_{\text{obs}}} MT_1 \rightarrow T_2 + M \\
& \text{SCHEME 1}
\end{align*}
\]

where \(T_1\) and \(T_2\) are different conformational states of Yiip, and \(M\) is the metal ion substrate. \(k_1, k_2,\) and \(k_3\) are the rate constants. The dissociation constants of metal binding \(K_p = k_1/k_2\). The relationship among \(k_p, k_y,\) and \(k_3\) is defined as \(K_m = (k_y + k_3)/k_1\). Application of the steady-state condition to the species \(MT_1\) gives Equation 1 as described previously (19).

\[
K_{\text{obs}} = \frac{k_1 k_3 [M]}{k_1 [M] + k_2 + k_3} \tag{Eq. 1}
\]

Thus, Equation 2,

\[
\frac{1}{K_{\text{obs}}} = \frac{1}{k_3} + \frac{1}{K_m} \tag{Eq. 2}
\]

\(k_1, k_2,\) and \(K_m\) were determined by linear regression of \(1/K_{\text{obs}}\) as a function of \(1/[M]\).

MPB Labeling—MPB labeling was carried out with cells that expressed Yiip or a Yiip variant as indicated. Cells (1 ml) from overnight cultures in the auto-inducing medium were pelleted by centrifugation and resuspended in a reaction buffer (0.5 ml) containing 20 mM HEPES, 100 mM NaCl, 2 mM MgCl\(_2\), 10% sucrose, 0.25 mM TCEP, pH 7.5. A freshly prepared MPB stock solution (50 mM) was added to a final concentration of 2 mM, or an equal volume of double deionized water (20 \(\mu\)l) instead of MPB was added to a control sample as indicated. Cells were incubated with MPB at room temperature for 30 min with or without sonication (30 s), and then \(\beta\)-ME (20 \(\mu\)l) was added to quench the untreated MPB. The resulting cells were pelleted again and washed, and membrane proteins were extracted using a solubilization buffer (20 mM HEPES, 100 mM NaCl, 1% DDM, 20% glycerol, 0.25 mM TCEP, pH 7.5) with brief sonication (1 min), followed by a 30-min incubation at 10 °C to achieve complete detergent solubilization. Cellular debris was removed by centrifugation (10,000 × g for 30 min), and supernatants were collected and incubated with 20 \(\mu\)l of Ni\(^{2+}\)-nitrilotriacetic acid superflow resin for 30 min. The resin was washed free of contaminants with 2 ml of wash buffer (20 mM HEPES, 300 mM NaCl, 40 mM imidazole, 12.5% glycerol, 0.05% DDM, and 0.25 mM TCEP, pH 7.0), and then eluted...
with 50 μL of elution buffer (20 mM HEPES, 100 mM NaCl, 500 mM imidazole, 12.5% glycerol, 0.05% DDM and 0.25 mM TCEP, pH 7.0). The purified proteins obtained were immediately subjected to fluorescence labeling.

**Fluorescence Labeling and Western Blot**—The MPB-treated and purified proteins (~0.5 mg/ml) were incubated with 0.1 mM fluorescein 5-maleimide (FM, Molecular Probes) in the presence of 10% SDS at room temperature for 20 min, and then β-ME was added to 10 mM to terminate the reaction. The resulting FM-treated proteins were subjected to SDS-PAGE on an 8–16% Tris-HCl precast polyacrylamide gel (Bio-Rad). The gel was visualized on a UV transilluminator and documented using a BioDoc-It System (Ultraviolet Products). After fluorescence detection, proteins were transferred to nitrocellulose using a Trans-blot semi-dry transfer cell (Bio-Rad) and were exposed to a peroxidase-conjugated monoclonal anti-biotin antibody (Sigma) for detection of MPB labeling by a SuperSignal West Pico chemiluminescent substrate (Pierce). For FM labeling to membrane-bound YiiP variants, cells hosting the overexpression of a YiiP variant were harvested and resuspended in the reaction buffer, and then 1 mM FM was added with a brief sonication. After incubation for 30 min at room temperature, 10 mM β-ME was added to quench unreacted FM. The resulting cells were pelleted and washed, and the FM-treated proteins were purified as described above. Aliquots of purified proteins were either directly subjected to SDS-PAGE or subjected to another exposure of 0.1 mM fresh FM in the presence of 10% SDS, followed by SDS-PAGE. Proteins in the gels were visualized under UV light for fluorescence detection before being stained with Coomassie Blue for estimation of the total amount of proteins.

### RESULTS

**Correlation between Cd²⁺ Binding and Transport**—The transport of metal ions is a sequential process of equilibrium binding and energized movement of metal ions along one or more binding sites in a translocation pathway across the membrane (20). To establish the functional role of Asp-157 in metal binding and transport, we examined the effects of mutating Asp-157 to a cysteine or alanine residue, corresponding to a metal coordination or noncoordination residue. His-tagged YiiP and mutants were overexpressed and purified to homogeneity by nickel affinity chromatography, followed by thrombin cleavage of the affinity tag, EDTA chelation of bound metal ions, and size exclusion HPLC purification to yield protein samples suitable for metal calorimetric titrations (15). Cd²⁺ binding to YiiP, YiiP_D157A, and YiiP_D157C was examined directly by ITC at 25 °C, pH 7.0, as described under “Experimental Procedures.” Examples of heat changes resulting from binding of incremental additions of Cd²⁺ and plots of the integrated heat per mol of Cd²⁺ as a function of the Cd²⁺/protein molar ratio are displayed in Fig. 1A. The heat effects generated by Cd²⁺ binding to YiiP and YiiP_D157C dropped sharply near a stoichiometric equivalence point of 2.5, whereas the midpoint of binding heat changes for YiiP_D157A occurred at 1.5, indicating a loss of 1 eq Cd²⁺-binding site by the D157A mutation. Accordingly, binding isotherms were fitted with a two-site model for YiiP and YiiP_D157C and a one-site model for YiiP_D157A. As shown in TABLE ONE, binding affinities, stoichiometries, and ΔH changes of site 1 and site 2 for YiiP and YiiP_D157C were nearly identical and within experimental errors. Fit of YiiP_D157A binding isotherm to a one-site model
resulted in $K_a = 7.3 \pm 1.9 \text{mM}^{-1}$, $n = 1.5 \pm 0.1$, and $\Delta H = -5.7 \pm 0.2$ kcal/mol, in excellent agreement with the binding parameters of site 1 of both YiiP and YiiP$_{D157C}$. Thus, a D157C mutation caused no change to both sites 1 and 2, whereas a D157A mutation completely abolished Cd$^{2+}$ binding to site 2 but had no effect on site 1. Furthermore, the binding stoichiometries for site 2 were close to unity, suggesting that a D157A mutation disrupted one Cd$^{2+}$-binding site per YiiP subunit.

The ITC data indicated the presence of at least two Cd$^{2+}$-binding sites, each of which could play a structural, functional, or regulatory role. To examine a possible correlation between Cd$^{2+}$ binding to Asp-157 and Cd$^{2+}$ transport, HPLC-purified YiiP, YiiP$_{D157A}$, and YiiP$_{D157C}$ were reconstituted into proteoliposomes, and the kinetics of Cd$^{2+}$ transport was analyzed by stopped-flow measurements of fluorescence changes of an encapsulated Zn$^{2+}$/Cd$^{2+}$-sensitive indicator, fluozin-1, in response to rapid mixtures of proteoliposomes with Cd$^{2+}$ exterior to vesicles. SDS-PAGE analysis of proteoliposome samples confirmed that approximately the same amount of YiiP, YiiP$_{D157A}$, and YiiP$_{D157C}$ was reconstituted into vesicles. Mixing proteoliposomes with external Cd$^{2+}$ evoked rapid and progressive fluorescence increases that were dependent of Cd$^{2+}$ concentrations ranging from 0 to 2 mM in the reaction mixture as described under “Experimental Procedures.” Liposomes prepared in parallel to proteoliposomes only yielded negligible background fluorescence responses. A linear correlation between the initial rates of fluorescence responses and the molar ratios of YiiP/lipid was observed, indicating a linear relationship between the initial rate and the transport activity (data not shown). As shown in Fig. 1B, the fluorescence responses of YiiP and YiiP$_{D157C}$ were comparable in amplitudes within the Cd$^{2+}$ concentration range, in contrast to greatly diminished

| Titrant | Protein          | Site 1                        | Site 2                        |
|---------|------------------|-------------------------------|-------------------------------|
|         |                  | $n$  | $K_a$ ($\mu$M$^{-1}$) | $\Delta H$ (kcal/mol) | $n$  | $K_a$ ($\mu$M$^{-1}$) | $\Delta H$ (kcal/mol) |
| CdCl$_2$ | YiiP             | 1.5 $\pm$ 0.1 | 7.9 $\pm$ 2.7 | $-5.8 \pm 0.2$ | 1.1 $\pm 0.1$ | 1.1 $\pm 0.5$ | $-7.8 \pm 0.7$ |
|         | YiiP$_{D157A}$   | 1.5 $\pm$ 0.1 | 7.3 $\pm$ 1.9 | $-5.7 \pm 0.2$ | 1.2 $\pm 0.1$ | 1.1 $\pm 0.3$ | $-6.9 \pm 0.3$ |
|         | YiiP$_{D157C}$   | 1.4 $\pm$ 0.1 | 7.5 $\pm$ 1.8 | $-5.5 \pm 0.1$ | 1.2 $\pm 0.1$ | 26 $\pm 14$ | $-2.7 \pm 0.2$ |
| ZnCl$_2$ |YiiP             | 0.6 $\pm$ 0.2 | 0.0069 $\pm$ 0.0029 | 22 $\pm 86$ | 2.0 $\pm 0.1$ | 4.2 $\pm 14$ | $-3.1 \pm 0.2$ |
|         | YiiP$_{D157A}$   | 0.7 $\pm$ 0.1 | 0.061 $\pm$ 0.011 | 3.4 $\pm 1.7$ | 1.1 $\pm 0.1$ | 26 $\pm 14$ | $-2.7 \pm 0.2$ |
|         |YiiP$_{D157C}$   | 0.6 $\pm$ 0.1 | 0.0079 $\pm$ 0.0021 | 35 $\pm 11$ | 1.9 $\pm 0.1$ | 14 $\pm 29$ | $-1.7 \pm 0.9$ |

**FIGURE 2.** Correlation between Zn$^{2+}$ binding and transport. A, ITC analysis of Zn$^{2+}$ binding. Titrations were made with 5-$\mu$l injections of 0.5 mM ZnCl$_2$ into 0.01 mM YiiP, YiiP$_{D157A}$, and YiiP$_{D157C}$ as indicated. Negative or positive heat effects correspond to an exothermic or endothermic reaction, respectively. Solid lines represent best fits of Zn$^{2+}$ binding isotherms to a two-site model with fitting parameters summarized in TABLE ONE. B, kinetics of Zn$^{2+}$ transport. YiiP and mutants were reconstituted into lipids at a molar ratio of 1:20,000 (protein/lipid). Stopped-flow traces of YiiP, YiiP$_{D157A}$, and YiiP$_{D157C}$ as indicated were fluorescence responses to 0, 0.25, 0.5, 1, 2, or 4 mM ZnSO$_4$ in a transport assay buffer. Inset, plot of $1/K_{max}$ as a function of $1/[M]$. The solid line represents linear regression of the kinetic data.
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responses of YiiP_D157A, that were reduced to a background level. This significant impairment of the transport activity was attributed to the removal of a metal coordination group by a D157A mutation, because an Asp → Cys substitution only caused insignificant kinetic changes within experimental errors. Linear regression of $1/K_{as}$ versus $1/[M]_t$ using kinetic data from three experiments (Fig. 1B, inset) yielded $K_{as}$ values of 266 ± 20 and 304 ± 32 μM for YiiP and YiiP_D157C, respectively. The $k_3$ values for YiiP and YiiP_D157C were identical and within experimental errors (26 ± 6 s⁻¹).

Correlation between Zn²⁺ Binding and Transport—In contrast to the pure exothermic heat reactions during Cd²⁺ titrations, Zn²⁺ titrations shown in Fig. 2A displayed a mixed heat reaction that began with exothermic and followed by late endothermic heat changes. In addition to the main exothermic-to-endothermic transition, another enthalpic transition was evident at the beginning of Zn²⁺ titrations where exothermic heat effects increased progressively. The overall profile of the YiiP_D157C binding isotherm was comparable with that of YiiP, but the binding isotherm of YiiP_D157C showed a leftward shift of the exothermic-to-endothermic transition by about 1 stoichiometry unit, qualitatively corresponding to the loss of one exothermic binding site. To a first approximation, Zn²⁺ binding isotherms were fitted with two sets of independent binding sites, accounting for the endothermic and exothermic heat reactions with the respective binding stoichiometries of 0.6 ± 2.4 and 2.0 ± 0.1 for YiiP, 0.6 ± 1.8 and 1.9 ± 0.1 for YiiP_D157C, and 0.7 ± 3.1 and 1.1 ± 0.1 for YiiP_D157A (TABLE ONE). Compared with YiiP, YiiP_D157C appeared to retain all Zn²⁺-binding sites, whereas YiiP_D157A was short of one exothermic site. The presence of multiple heat transitions precluded fitting of Zn²⁺ binding isotherms with certainty, as indicated by significant fitting errors. Thus a quantitative comparison of Zn²⁺ binding parameters was not amenable. Nevertheless, it was evident that a Zn²⁺ exothermic binding site was disrupted in YiiP_D157A, whereas the same site remained intact in YiiP_D157C. Furthermore, multiple transitions observed in the YiiP_D157A binding isotherm indicated the presence of at least two additional Zn²⁺-binding sites after disruption of the Asp-157 site. In corroboration of the effects of Asp-157 mutations on Zn²⁺ binding, the rate of Zn²⁺ transport was unchanged with $k_3$ values of 34 ± 5 and 33 ± 3 s⁻¹ for YiiP and YiiP_D157C, respectively, making a sharp contrast to YiiP_D157A responses that were reduced to the background level (Fig. 2B). The $K_{as}$ values estimated from three experiments were 310 ± 32 and 358 ± 36 μM for YiiP and YiiP_D157C, respectively, indicating no significant change in Zn²⁺ binding.

Topological Mapping of Asp-157—A mechanistic understanding of the functional role of Asp-157 depends heavily on the ability of mapping it to a reliable topology model. The membrane topology of any CDF transporter has not yet been determined experimentally, but six segments of hydrophobic residues are suggested by YiiP hydrophathy analysis. To determine whether each of these hydrophobic segments actually traversed the membrane and, if so, to determine their membrane spanning polarity, we introduced a series of cysteine substitution mutations, each located at the beginning or end of a hydrophobic segment. When intact cells were exposed to an impermeant thiol-specific probe, the probe accessibility to a reporter cysteine residue could indicate its extracellular or intracellular location. Two maleimide derivatives, FM and MPB, were used in this study. The maleimide group in FM is directly attached to a bulky and charged fluorescein moiety, and the maleimide in MPB is tethered to its biotin moiety through a linear polyethylene oxide chain (Fig. 3A). The bulkiness and the hydrophilic nature of both maleimide derivatives make them impermeant to the cytoplasmic membrane (21). YiiP contains two native cysteine residues at positions 127 and 287. FM or MPB labeling to Cys-127 was not detected when YiiP_C287S was exposed to 1 mM FM or MPB, even in the presence of 10% SDS (Fig. 3B). Thus a panel of single cysteine substitution mutants was constructed on the YiiP_C287S background. Only one reactive cysteine residue is present in each of the following YiiP variants at a position as numbered: S10C/C287S, S36C/C287S, N70C/C287S, S107C/C287S, S107C/C287S, S107C/C287S, S144C/C287S, R177C/C287S, Cys-287 (equivalent to wild type YiiP). All these mutants were found to be fully functional by stopped-flow analysis, thereby ascertaining the structural and functional relevance of the topological mapping.

MPB labeling was carried out with intact cells (lane 1) or sonicated cells (lane 2) as described under “Experimental Procedures.” As a control, FM labeling was performed with purified YiiP_C287S without MPB pretreatment (lane 3). MPB-treated YiiP_C287S was purified and then labeled with FM in the presence of 10% SDS. FM and MPB labeling were detected by UV transillumination (middle) and Western blot detection (bottom). Approximately an equal amount of protein (3 μg) was loaded to each lane as indicated by Coomassie Blue staining (top), but neither fluorescence transillumination nor Western blot detection yielded any visible signal. C, alternating patterns of FM and MPB labeling. MPB labeling was carried out in intact cells (lane 1) and sonicated cells (lane 2). Lane 3 is FM labeling to purified proteins without MPB pretreatment. Based on the accessibility of these seven cysteine residues to MPB and FM labeling, a topology model is depicted with both N and C terminus in the cytoplasm.
Cys-287 (bottom panel). The MPB reactivities in the former three positions and the lack of MPB reactivity in the latter four positions mirrored the pattern of FM fluorescence labeling, showing a background level of fluorescence in Fig. 3C, lane 1, for S36C, S107C, and R177C, as opposed to an intense fluorescence signal in lane 1 for S10C, N70C, S144C, and Cys-287. Because MPB labeling was directed to cysteine residues on the extracellular surface, whereas FM labeling was directed to any cysteine residue that survived the first run of MPB labeling, the location of an MPB- or FM-reactive residue was interpreted to be extracellular or intracellular, respectively. Correlating MPB and FM labeling with the positions of seven cysteine residues in a sequence from the N to C terminus revealed an alternating pattern of MPB reactivities and a reversed alternating pattern of FM reactivities, both of which were consistent with a topology model depicted in Fig. 3C (middle panel). In a control experiment shown in Fig. 3C, lane 2, of each gel, MPB labeling with sonicated cells yielded a strong and uniform MPB chemiluminescence signal regardless of the cysteine position. The subsequent FM labeling showed a background level of fluorescence signal, indicating the completeness of MPB labeling to all seven cysteine residues when the membrane barrier was disrupted by sonication. Likewise, FM labeling of the purified YiiP variants without MPB pretreatment yielded a strong and uniform fluorescence signal at all positions (Fig. 3C, lane 3), indicating that the seven cysteine residues were fully reactive with FM when the plasma membrane was disrupted by detergent solubilization. Taken together, these control experiments ensured that the alternating pattern observed with intact cells truly reflected the transmembrane location of the cysteine residues. The alternating accessibility of positions Ser-144 and Arg-177 established a transmembrane spanning domain (TM-5) between these two positions with an N → C polarity from the cytoplasm to periplasm. Therefore, Asp-157 was localized to TM-5.

Localization of Asp-157 to the Hydrophobic Core of TM-5—We further determined whether Asp-157 is located in the membrane-embedded region of TM-5 by fine topological mapping. The boundary of the hydrophobic core of TM-5 was probed by examining FM reactivities of a set of(YiiP variants, each containing a single reactive cysteine residue at one of the following positions: 150, 153, 155, 157, 171, 172, and 174. All these mutants are active Zn$^{2+}$/Cd$^{2+}$ transporters (data not shown). FM labeling to sonicated cells overexpressing one of the seven YiiP variants revealed a sharp positional discontinuity in fluorescence intensities as shown in Fig. 4A (bottom panel); intense labeling to positions 150 and 153 on the cytoplasmic side, followed by marginal labeling to three central positions 155, 157, and 171, yet again intense labeling to positions 172 and 174 on the periplasmic side. FM labeling to purified YiiP variants in the presence of 10% SDS yielded uniform fluorescence (Fig. 4A, middle panel), and Coomassie Blue staining of the same gel indicated that approximately equal amounts of proteins were loaded to each lane (top panel). Thus, the discontinuous pattern of FM labeling in the membrane was due to neither a lack of FM reactivity for the three central positions nor different levels of protein expression but to a change of local environments surrounding the cysteine residues as TM-5 traversed the plasma membrane through a membrane-embedded region. Accordingly, the region from position 155 to 171 was localized to the hydrophobic core of TM-5, within which is located Asp-157 close to the cytoplasmic boundary (Fig. 4B).

MPB Labeling to D157C—We also evaluated the accessibility of six TM-5 positions to MPB labeling. As shown in Fig. 4C, for the three peripheral positions, position 172 was labeled in both intact (I) and sonicated cells (S), whereas positions 150 and 153 were only labeled in sonicated cells, consistent with the extracellular location of position 172 and intracellular locations of positions 150 and 153. For the three central positions, positions 155 and 171 were not accessible to MPB labeling, consistent with the finding that these two positions are located in a membrane-embedded region. However, D157C exhibited a reduced but positive reactivity toward MPB in both intact and sonicated cells. This observed MPB reactivity was contrary to the marginal FM labeling to...
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D157C as shown in Fig. 4A, suggesting that the maleimide group in a more flexible MPB might reach a buried D157C that was not accessible to the maleimide group directly attached to a rigid and charged fluorescein moiety of FM (Fig. 3A). The observed partial MPB labeling to D157C appeared to be position-specific because MPB labeling to two neighboring cysteine residues at positions 155 and 171 was not detectable.

Selective Metal Binding to D157C—Because YiiPD157C exhibited intact Zn$^{2+}$/Cd$^{2+}$ binding and full Zn$^{2+}$/Cd$^{2+}$ transport activity, the selectivity and binding affinity of metal binding to Cys-157 may reflect steric hindrance to MPB labeling.(YiiPD157C/C287S in the membrane was indicated by metal protection of Cys-157 from MPB-labeling, because metal binding to a membrane-embedded Cys-157 is expected to incur steric hindrance to MPB labeling. YiiPD157C/C287S in the membrane was exposed to MPB labeling with a brief sonication in the presence of one of the following divalent cations: Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$, Fe$^{2+}$, Ni$^{2+}$ and Co$^{2+}$, each at a concentration of 0.2 mM. The MPB-treated YiiPD157C/C287S was then purified and subjected to Western blot analysis. Among the nine divalent metals tested, only Zn$^{2+}$ and Cd$^{2+}$ appeared to abolish the corresponding Western blot signals (Fig. 5A, lower panel), whereas Coomassie Blue staining of a duplicate gel indicated that approximately an equal amount of protein was loaded to each lane (Fig. 5A, upper panel). Additional experiments were carried out to examine the concentration dependence of Zn$^{2+}$ or Cd$^{2+}$ protection. MPB labeling was carried out with sonicated cells in the presence of 0.5 mM HgCl$_2$ into 0.01 mM YiiP or YiiPD157A as indicated. The MPB-labeling to the maleimide group directly attached to a rigid and charged fluorescein moiety of FM (Fig. 3A) to the maleimide group directly attached to a rigid and charged fluorescein moiety of FM (Fig. 3A).

**FIGURE 6.** ITC analyses of Fe$^{2+}$ and Hg$^{2+}$ binding. A, titrations with 5-µl injections of 0.25 mM FeCl$_2$ into 0.01 mM YiiP or YiiPD157A as indicated. B, titrations with 5-µl injections of 0.5 mM HgCl$_2$ into 0.01 mM YiiP or YiiPD157A as indicated. The solid lines represent the best fits to a two-site binding model with fitting parameters summarized in TABLE TWO.

| Titrant  | Protein   | $n$  | $K_c$ (µM$^{-1}$) | $ΔH$ (kcal/mol) | $n$  | $K_c$ (µM$^{-1}$) | $ΔH$ (kcal/mol) |
|----------|-----------|-----|------------------|----------------|-----|------------------|----------------|
| FeCl$_2$ | YiiP      | 1.3 ± 0.2 | 0.45 ± 0.09 | -16 ± 1.4 | 0.8 ± 0.3 | 4.6 ± 3.9 | -26 ± 0.6 |
|         | YiiPD157A | 1.2 ± 0.1 | 0.48 ± 0.13 | -19 ± 0.6 | 0.8 ± 0.2 | 6.5 ± 1.0 | -28 ± 0.2 |
| HgCl$_2$ | YiiP      | 0.81 ± 0.11 | 530 ± 200 | -21.9 ± 0.2 | 1.2 ± 0.2 | 2.3 ± 0.9 | -47 ± 0.3 |
|         | YiiPD157A | 0.78 ± 0.12 | 480 ± 190 | -25.0 ± 0.4 | 1.2 ± 0.2 | 2.7 ± 1.4 | -55 ± 0.6 |
Selective Metal Binding and Transport

ONE. It appears that Asp-157 and Cys-157 are largely interchangeable, likely due to the similar space filling volume of both residues. Further supporting evidence for a direct Zn\(^{2+}\)/Cd\(^{2+}\)-Asp-157 coordination comes from the observation that replacing Asp-157 with a noncoordination Ala residue completely disrupted a Cd\(^{2+}\)/Zn\(^{2+}\) site. Comparing Cd\(^{2+}\) binding isotherms of YiiP\(_{\text{Asp-157}}\) and YiiPD\(_{\text{D157A}}\) suggests that a D157A mutation disrupts site 2 of Cd\(^{2+}\) binding while leaving site 1 unchanged. Thus, site 2 can be assigned to position 157 with an equivalent \(K_a\) value of 0.91 \(\mu M\) for an aspartate or cysteine residue. The Cd\(^{2+}\) binding affinity obtained by direct ITC measurements agrees well with the micromolar range binding affinity estimated based on Cd\(^{2+}\) protection against MPB labeling to YiiPD\(_{\text{D157C}}\). A similar Zn\(^{2+}\) binding affinity is suggested by the Zn\(^{2+}\) protection analysis. It is noted that the affinity of Zn\(^{2+}\)/Cd\(^{2+}\) binding to Asp-157 is several orders of magnitude lower than Zn\(^{2+}\)/Cd\(^{2+}\) affinities found in many zinc metalloproteins (22). Zinc exchanges from those metalloproteins in general occur over a period of hours or days. Exchange rates of this order are not suitable for a more rapid on-off binding reaction required to keep the flow of a zinc ion when it is transported. Thus, a lower Zn\(^{2+}\)/Cd\(^{2+}\) affinity is anticipated to reconcile metal ion mobility in YiiP. In this regard, the Asp-157/Cys functional convertibility at position 157 may reflect a loose metal coordination interaction resulting from some side chain flexibility within the binding site. Likewise, the Asp-157-binding site is sufficiently flexible to accommodate various ionic radii from 0.74 Å for Zn\(^{2+}\) to 0.97 Å for Cd\(^{2+}\).

The correlation between Zn\(^{2+}\)/Cd\(^{2+}\) binding and transport distinguishes Asp-157 from a regular metal coordination residue. Besides supplying an oxygen donor for coordination binding, Asp-157 also acts as a pivotal structural component that couples metal binding to a transport process. Thus, the local chemical environment surrounding Asp-157 may contribute to binding-transport coupling. Asp-157 was localized to TM-5 by coarse topological mapping using a set of cysteine substitution mutations introduced such that each pair of cysteine residues was positioned in two solvent-accessible regions flanking each of the six putative TMs in YiiP. Thiol-specific labeling using permethane probes MPB and FM revealed an alternating pattern of thiol reactivities when intact cells were probed, indicating that the polypeptide chain of YiiP traverses the membrane six times with both N and C termini in the cytoplasm. A fine topological mapping then focused on TM-5 by using cysteine-scanning mutagenesis to probe the positional dependence of cysteine thiol reactivities toward a water-soluble probe, FM, which reacts more readily with solvent-exposed cysteines than with those embedded in the membrane. A central region of low and two peripheral regions of higher FM reactivities were identified with two sharp boundaries occurring between residues 153–155 and 171–172. The lower FM reactivities could be attributed to inaccessibility (23) and/or elevated \(pK_a\) of cysteine residues (24) in a hydrophobic environment. Thus Asp-157 was localized to a stretch of membrane-embedded residues. The mechanistic implications of a membrane-embedded Asp-157 are 2-fold. 1) The transport of metal ions is thought to be associated with a global protein conformational change originating from the metal-binding site. The hydrophobic surroundings of Asp-157 can greatly strengthen the Asp-157-Zn\(^{2+}\)/Cd\(^{2+}\) interaction and facilitate electrostatic propagation of a local conformational change through a hydrophobic medium. 2) Zn\(^{2+}\) transport in YiiP is coupled to antiport of proton(s). The localization of Asp-157 to a hydrophobic environment raises the possibility that the \(pK_a\) of this membrane-embedded aspartate may be sufficiently elevated to remain protonated in the absence of Zn\(^{2+}\)/Cd\(^{2+}\) binding. Upon Zn\(^{2+}\)/Cd\(^{2+}\) binding, Asp-157 may be deprotonated to trigger Zn\(^{2+}\)/Cd\(^{2+}\) transport with a separation of the released proton moving in an opposite direction. In agreement with this speculation, our recent ITC study indicated that binding one Cd\(^{2+}\) to YiiP yielded 1.23 protons (15). Further experiments are underway to investigate the involvement of Asp-157 in binding-deprotonation coupling.

CDF transporters were initially identified as Zn\(^{2+}\)/Cd\(^{2+}\)/Co\(^{2+}\) pumps (25, 26), and they subsequently were shown to transport Cd\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), and Fe\(^{2+}\). Categorization of CDF proteins based on multiple sequence alignment suggested three distinct subgroups, each apparently associated with a broad range of substrate specificities but with different metal preferences (3). The E. coli CDF transporters ZitB and YiiP belong to group 2 and group 3, respectively. ZitB functions as a Zn\(^{2+}\)/Cd\(^{2+}\) efflux pump, whereas the substrate specificity of YiiP remains obscure. YiiP (FieF) was recently implicated in a role of iron efflux pump based on the observations that both zinc and iron induced yiiP transcription and that overexpression of YiiP led to a loss of cytosolic iron content in E. coli and an increase of iron tolerance in a Δfur strain. However, only accumulation of Zn\(^{2+}\) but not Fe\(^{2+}\) was observed in reconstituted YiiP proteoliposomes (12). In the present study, both MPB protection analysis and direct ITC measurement indicated that Fe\(^{2+}\) did not bind to Asp-157 that was localized to the active site of the Zn\(^{2+}\)/Cd\(^{2+}\) transporter. Nevertheless, Fe\(^{2+}\) calorimetric titrations suggested the presence of two independent Fe\(^{2+}\)-binding sites in YiiP, both with modest binding affinities (2.2 and 0.22 \(\mu M\)). The functional connection of these putative Fe\(^{2+}\)-binding sites to Fe\(^{2+}\) transport has yet to be established.

Contrary to the general expectation that YiiP is a broad range metal ion transporter, metal binding to Asp-157 is highly specific. Calorimetric measurements of metal binding to YiiP\(_{\text{Asp-157}}\) and YiiPD\(_{\text{D157A}}\) showed that a D157A mutation disrupted Zn\(^{2+}\) or Cd\(^{2+}\) binding to Asp-157 but caused no detectable change to Fe\(^{2+}\) or Hg\(^{2+}\) binding. Fe\(^{2+}\) and Hg\(^{2+}\) represent neighboring metal ions in the same transition period and in the same element group, respectively. The selectivity of Asp-157 for Zn\(^{2+}\)/Cd\(^{2+}\) over Fe\(^{2+}\) and Hg\(^{2+}\) is consistent with the selectivity of Cys-157 for Zn\(^{2+}\)/Cd\(^{2+}\) over Fe\(^{2+}\) and Hg\(^{2+}\), as well as Co\(^{2+}\), Ni\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\), and Mg\(^{2+}\), as suggested by metal binding protection of Cys-157 from MPB labeling. The lack of Hg\(^{2+}\) protection is noteworthy because the cysteine thiolate is a very strong Hg\(^{2+}\) donor group. It is difficult to see how selection of Zn\(^{2+}\)/Cd\(^{2+}\) by Asp-157 and Cys-157 is so specifically based on chemical consideration for metal ions, because the electrostatic binding by Zn\(^{2+}\)/Cd\(^{2+}\) is a property shared with Mg\(^{2+}\) and Ca\(^{2+}\), whereas the Lewis acid strength, and thus the donor group preference of Zn\(^{2+}\)/Cd\(^{2+}\), is within the same range shared by Co\(^{2+}\), Ni\(^{2+}\), Fe\(^{2+}\), and Mn\(^{2+}\). The mechanism of metal selectivity may be better appreciated in terms of coordination chemistry in the chemical context of the immediate binding site neighborhood. To a first approximation, the hydrophobic surroundings of Asp-157 may entail steric and electrostatic hindrances as suggested by the observation that Cys-157 was only partially accessible to thiol-specific modification by a maleimide group tethered to a flexible and neutral MPB as opposed to a rigid and charged FM molecule.

Previous ITC analyses of metal binding to YiiP suggested the presence of at least one mutually competitive binding site common to Zn\(^{2+}\), Cd\(^{2+}\), and Hg\(^{2+}\) and a set of noncompetitive binding sites (15). In the present study, we physically assigned Asp-157 to one of the noncompetitive binding sites, because metal binding to Asp-157 is specific to Zn\(^{2+}\) and Cd\(^{2+}\) but not to Hg\(^{2+}\). After removing the Asp-157 site by a D157A mutation, a reduced Cd\(^{2+}\) binding isotherm could be fitted with one set of independent binding sites with a binding stoichiometry of 1.5,
suggesting that one or two additional Cd^{2+}/H11001-binding sites are present in YiiP_{D157A}. In light of our recent finding that YiiP is a homodimer (27), the 1.5 stoichiometric equivalence of Cd^{2+}/H11001 binding may be interpreted as two independent Cd^{2+}/H11001-binding sites, one is located in each subunit and the other at the dimeric subunit interface. The molecular identities of these additional Cd^{2+}/H11001-binding sites and their roles in metal transport have yet to be determined. The stoichiometries of Zn^{2+}/H11001 and Cd^{2+}/H11001 binding to Asp-157 are both close to unity, corresponding to two independent Asp-157-binding sites per YiiP homodimer. Because Zn^{2+}/H11001/Cd^{2+} binding to Asp-157 is directly linked to Zn^{2+}/H11001/Cd^{2+} transport, transport of Zn^{2+}/H11001/Cd^{2+} in a YiiP dimer may occur in two independent translocation pathways, each located in the center of a YiiP monomer. Alternatively, two Cd^{2+}/H11001-binding sites may be located in a common translocation pathway at the dimer interface, thus transport of two Cd^{2+} ions may occur in a shared translocation pathway. Further structural analysis of YiiP is underway to test these hypotheses.

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