Sequence homology of the Escherichia coli YiiP places it within the family of cation diffusion facilitators, a family of membrane transporters that play a central role in regulating cellular zinc homeostasis. Here we describe the first thermodynamic and mechanistic studies of metal binding to a cation diffusion facilitator. Isothermal titration calorimetric analyses of the purified YiiP and binding competitions among Zn\textsuperscript{2+}, Cd\textsuperscript{2+}, and Hg\textsuperscript{2+} revealed a mutually competitive binding site common to three ions and a set of noncompetitive binding sites, including one Cd\textsuperscript{2+} site, one Hg\textsuperscript{2+} site, and at least one Zn\textsuperscript{2+} site, to which the binding of Zn\textsuperscript{2+} exhibited partial inhibitions of both Cd\textsuperscript{2+} and Hg\textsuperscript{2+} bindings. Lowering the pH from 7.0 to 5.5 inhibited binding of Zn\textsuperscript{2+} and Cd\textsuperscript{2+} to the common site. Further, the enthalpy change of the Cd\textsuperscript{2+} binding to the common site was found to be related linearly to the ionization enthalpy change of the pH buffer with a slope corresponding to the relieving of 1.23 H\textsuperscript{+} for each Cd\textsuperscript{2+} binding. These H\textsuperscript{+} effects are consistent with a coupled deprotonation process upon binding of Zn\textsuperscript{2+} and Cd\textsuperscript{2+}. Modification of histidine residues by diethyl pyrocarbonate specifically inhibited Zn\textsuperscript{2+} binding to the common binding site, indicating that the mechanism of binding-deprotonation coupling involves a histidine residue(s).

Zinc is required by many metalloproteins for catalytic activities, structural stability, and functional regulations (1–3). Low cellular zinc levels inhibit cell growth and division, whereas high zinc levels are toxic. To maintain cellular zinc content within a narrow physiological range, cells rely on a complement of zinc homeostatic mechanisms by the expression of zinc chelation proteins, sequestrations of zinc into intracellular membrane compartments, and controls of zinc entry and exit from cells through several families of zinc transporters (4–9). Among them, zinc transporters in the CDF\textsuperscript{1} family play a major role during zinc excesses, conferring tolerance for zinc and some divalent transition metal ions (10). Seven mammalian CDF proteins, ZnT1–7 (zinc transporter 1–7), have been cloned and characterized. ZnT1 is involved in zinc efflux across the plasma membrane (11), and ZnT2–7 facilitate zinc accumulation in various intracellular compartments (12, 13). Bacterial CDF proteins are distantly related to mammalian counterparts by a homologous hydrophobic domain with six distinct hydrophilic segments (14, 15). Despite large variability in the hydrophilic regions, all CDF family members identified so far, prokaryotic or eukaryotic, appear to transport metal ions exclusively (10). The migration of a metal ion in a transporter involves temporary association of the metal ion with one or more binding sites along a translocation pathway. This combined process of equilibrium binding and energized movement brings about the metal selectivity of the transporter and the mobility of the metal substrate. Therefore, thermodynamic and kinetic studies of CDF proteins are critically important to the understanding of the molecular mechanisms of metal ion transport across biological membranes.

The kinetics of a homologous Escherichia coli CDF protein, ZitB, was studied by stopped-flow measurements of metal ion fluxes across the membrane of proteoliposomes reconstituted from the purified ZitB (16). The ZitB-mediated transport was shown to be a substrate-saturable process that can be described by a two-step reaction of an equilibrium binding followed by a conformational transition that moves the bound metal ion across the membrane. Both Cd\textsuperscript{2+} and Zn\textsuperscript{2+} are effective substrates, the translocation of which requires a proton movement in the reverse direction of the metal transport. This coupling of metal ions to protons provides the energetic basis for zinc efflux in E. coli, where the downhill influx of proton produces free energy to drive efflux of the cytoplasmic zinc.

To gain an energetic and mechanistic understanding of the metal ion binding to CDF proteins, we used isothermal titration calorimetric (ITC) to directly measure the heat exchanges that accompany metal binding to an E. coli CDF protein, YiiP. This calorimetric approach allows the dissecting of the Gibbs free energy of binding \(\Delta G\) into two thermodynamic components: the enthalpy change, \(\Delta H\), and the entropy change, \(\Delta S\) (17). \(\Delta H\) is measured directly from ITC measurements; the binding constant \(K\) and stoichiometry are obtained by a least-squares fit of the binding isotherm to a binding model. \(\Delta G\) is given by \(\Delta G = -RT \ln K\) and \(\Delta S\) is calculated by using the standard thermodynamic expression \(\Delta G = \Delta H - T \Delta S\). These thermodynamic parameters permit the evaluation of enthalpic and entropic contributions to the Gibbs free energy of binding, thereby providing a thermodynamic description of the binding reaction. Furthermore, the coupling between metal ions and protons can be evaluated by the dependence of the metal ion binding on pH and the dependence of the apparent binding enthalpy (\(\Delta H_{\text{app}}\)) on the proton ionization enthalpy (\(\Delta H_{\text{ion}}\)) of the buffer in which the binding reaction takes place (18). In the latter case, \(\Delta H_{\text{app}}\) is the sum of two enthalpic contributions, the metal ion binding enthalpy, \(\Delta H_{\text{bind}}\), and the proton ionization enthalpy of the buffer, which gives rise to a cumulative relationship, \(\Delta H_{\text{app}} = \Delta H_{\text{bind}} + n \Delta H_{\text{ion}}\), where \(n\) is the number of...
protons that are absorbed or released upon metal binding (19).

Here we describe the thermodynamic analyses of metal ion bindings to the purified YiiP. YiiP was over-expressed, solubilized by detergent, and purified free of metal contaminants prior to ITC experiments. Titurations of YiiP were carried out using three group-12 metal ions: Zn$^{2+}$, Cd$^{2+}$, and Hg$^{2+}$. Zn$^{2+}$ is a borderline soft-hard metal ion with a highly concentrated charge, and a high electron affinity, whereas Hg$^{2+}$ is a soft metal ion with a bulky and highly polarizable charge. Cd$^{2+}$, known as a substitute for Zn$^{2+}$ in many zinc enzymes, has an intermediate softness (20). Chemical differences in the Zn$^{2+}$ → Cd$^{2+}$ → Hg$^{2+}$ series resulted in distinct calorimetric responses, serving as thermodynamic signatures to aid the deconvolution of complex binding isotherms. Our results provide mechanistic insights into the molecular recognition that underlies metal ion binding to a metal transporter.

EXPERIMENTAL PROCEDURES

Cloning and Expression Plasmid Construct—The entire open reading frame sequence of YiiP was obtained by PCR using the genomic DNA of E. coli BL21 strain as template and a pair of YiiP-specific primers with a NdeI site incorporated at the 5'-ends of the forward and reverse primer, respectively (forward primer 5'-GGACTAGT-GCTCAGAATCTAATC-3', reverse primer 5'-GGCATCTTGAAGAGCATAGACCGT-3'). The PCR product was double digested using MfeI and BamHI (New England BioLabs, Beverly, MA), yielding two fragments. The larger piece was isolated and further digested using NdeI. The resulting NdeI-MfeI and MfeI-BamHI fragments were inserted between the NdeI and BamHI sites in expression vector pET15b (Novagen Inc., Madison, WI) in frame with an N-terminal 6-histidine affinity tag followed by a thrombin proteolytic cleavage site. The completed expression construct pHis-TB- YiiP was verified by automatic sequencing of both strands.

Overexpression of YiiP—Overexpression of YiiP was achieved using the overnight express autoinduction systems-1 (Novagen) based on an auto-inducing procedure (22). BL21(DE3)pLysS cells (Novagen) were transformed with pHis-TB-YiiP and stored in a noninducing medium at 17°C. Cells were harvested and lysed by three passages through a ice-chilled microfluidizer (Microfluidics, Newton, MA) at 1000 p.s.i., and the resulting membrane vesicles were pelleted by centrifugation at 140,000 × g for 45 min. The pellet was solubilized using a detergent buffer (100 mM NaCl, 20 mM HEPES, pH 7, 7% n-dodecyl-β-D-maltopyranoside (DDM; Anatrace, Maumee, OH), 0.25 mM TCEP, 20% w/v glycerol) with gentle stirring for 40 min at 4°C to achieve a complete extraction of YiiP from membrane vesicles. Insoluble materials were pelletted by centrifugation (140,000 × g for 45 min), and the supernatant was passed through a N3i-nitrirotiacetic acid superfowl column (Qiagen, Valencia, CA) at a flow rate of 2 ml/min. After washing the column with 10 bed volumes of wash buffer (20 mM HEPES, pH 7.5, 300 mM NaCl, 20% w/v glycerol, 0.05% DDM, 0.25 mM TCEP, 30 mM imidazole), YiiP was eluted with an elevated imidazole concentration at 500 mM. The purified His-YiiP was loaded to a 10-kDa cutoff dialysis cassettes (Pierce) for simultaneous dialysis and protein concentration using an external dialysis buffer (20 mM HEPES, pH 8.0, 100 mM NaCl, 20% w/v glycerol, 0.05% DDM, 0.25 mM TCEP) with the addition of 3% polyethylene glycol 35,000. Thrombin (Novagen, Madison, WI) was added into the dialysis cassette at a ratio of 1 unit/mg of His-YiiP to cleave the N-terminal poly-His peptide.

Preparation of Protein Sample for ITC Measurements—Protein aggregates and trace amount of metal contaminants were removed by size exclusion HPLC prior to calorimetric titrations with metal ions. Concentrated YiiP, typically at a concentration of 10 mg/ml, was incubated with 5 mM EDTA for about 20 min before being loaded to a TSK 3000SW (Tosohaas, Montgomery measured over 30–50 consecutive injections) pre-equilibrated with a degassed mobile phase (20 mM HEPES, pH 7.0, 100 mM NaCl, 12.5% glycerol, 0.05% DDM, 0.25 mM TCEP). HPLC purification was performed using a System-Gold HPLC system run at a flow rate of 0.5 ml/min (Beckman Coulter, Fullerton, CA). TCEP was omitted from the mobile phase when YiiP sample was prepared for Hg$^{2+}$ titrations because the presence of this reducing reagent caused significant dilution heats of Hg$^{2+}$. The HPLC-purified YiiP was detected by its UV absorption at 280 nm, collected as a discrete peak fraction, and then immediately used for ITC measurements. For titrations at low pH, YiiP was prepared in an otherwise identical HPLC mobile phase except that 20 mM MES was used to buffer pH at 5.5. For titrations at pH 7.0 in the presence of a different pH buffer, YiiP was purified as described above, except that HEPES was replaced by an indicated buffer. The concentration of the purified YiiP was determined by BCA protein assay (Pierce).

Isothermal Titration Calorimetry—ITC measurements were carried out on a Microcal MCS titration calorimeter (MicroCal, Inc., Northampton, MA). Metal titrants (chloride salts of Zn$^{2+}$, Cd$^{2+}$, or Hg$^{2+}$) were dissolved in the same HPLC mobile phase in which YiiP was purified, and their concentrations were adjusted to be 30–50-fold higher than that of YiiP, typically in the concentration range of 1 to 3 mM. The titrant and YiiP sample were thoroughly degassed before each titration.YiiP samples ranging in concentrations from 0.032 to 0.090 mM were placed in a 1.4-ml reaction cell, and the reference cell was filled with deionized water. All titrations were performed at 25°C. After temperature equilibration, successive injections of an indicated titrant was made into the reaction cell in 5-μl increments at 210–360-s intervals with stirring at 300 rpm to ensure a complete equilibration. The resulting heat of reaction were measured over 30–50 consecutive injections. Control experiments to determine the heats of dilution titration were carried out by making identical injections in the absence of YiiP. The net reaction heat was obtained by subtracting the heat of dilution from the corresponding total heat of reaction. The titration data were deconvoluted based on a binding model containing either one or two sets of noninteracting binding sites by a nonlinear least-squares algorithm using the Microcal Origin software. The binding enthalpy change ΔH, association constant K_a, and the binding stoichiometry n were permitted to float during the least-squares minimization process and taken as the best-fit values.

Reaction with DEPC—YiiP was mixed with 10 mM DEPC dissolved in the HPLC mobile phase with the addition of 2 mM Zn$^{2+}$, Cd$^{2+}$, or EDTA as indicated. After incubation with DEPC for an indicated time, the reactions were terminated by adding 20 mM imidazole to quench the unreacted DEPC. Next, 5 mM EDTA was added to chelate metal ions in the reaction mixture, resulting in metal-free and DEPC-modified YiiP that was further purified by size exclusion HPLC as described above. The protein peak fraction was collected and immediately loaded to the Microcal MCS titration calorimeter for ITC measurements.

RESULTS

Purification—The over-expressed His-YiiP was purified to homogeneity by a one-step nickel affinity chromatography (Fig. 1A). His tag and residual metal contaminants in the purified His-YiiP sample are two major factors that interfere with metal calorimetric titrations. Therefore, the His tag was cleaved by thrombin digestion, and the completeness of the proteolytic cleavage was verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and by Western blot analysis using an antibody specific to poly-His peptide (data not shown). Residual labile metal ions in the YiiP sample were chelated by incubation with 5 mM EDTA for 20 min, but prolonged EDTA incubation caused irreversible YiiP aggregation. The cleaved His tag peptide, metal ion, and EDTA contaminants were removed from YiiP samples by preparative size exclusion HPLC purification. The quality of the resulting YiiP was assessed by analytical size exclusion HPLC, showing a major mono-disperse species with a retention time corresponding to an apparent molecular mass of 190 kDa (Fig. 1B).

Calorimetric Titrations of YiiP with Zn$^{2+}$—The energetics of Zn$^{2+}$ binding to YiiP was examined directly by ITC at 25°C, pH 7.0, as described under “Experimental Procedures.” Examples of heat changes accompanying the binding of incremental additions of Zn$^{2+}$ to YiiP are shown in the upper panels of Fig. 2. Plots of the integrated heat per mole of Zn$^{2+}$ as a function of the molar ratio of Zn$^{2+}$ to YiiP are displayed in the lower
panels. As shown in Fig. 2A, Zn$^{2+}$ titrations began with an exothermic heat reaction, which was followed by a late endothermic reaction. This characteristic exothermic-to-endothermic transition suggests the presence of at least two sets of independent Zn$^{2+}$ binding sites, accounting for the exothermic and endothermic heat reactions, respectively. Furthermore, the dual heat reactions were reduced to a largely endothermic reaction when YiiP was titrated with Zn$^{2+}$ in the presence of either 0.5 mM CdCl$_2$ (Fig. 2B) or 0.25 mM HgCl$_2$ (Fig. 2C). This conspicuous loss of exothermic heat profile in the presence of Cd$^{2+}$ or Hg$^{2+}$ suggests that competitive binding occurs at the Zn$^{2+}$-exothermic binding site (Zn$^{2+}$ site 1) by Cd$^{2+}$ or Hg$^{2+}$. In contrast, binding to the Zn$^{2+}$-endothermic binding site (Zn$^{2+}$ site 2) was less affected by Cd$^{2+}$ and Hg$^{2+}$. Based on this qualitative assessment, Zn$^{2+}$ binding isotherm was fitted to a binding model containing two sets of independent binding sites, with $\Delta H$, $K_n$, and binding stoichiometry $n$ values permitted to float. The fit to this binding model yielded 1.5 and 0.84 equivalents of Zn$^{2+}$ bound to site 1 and site 2 with respective $K_n$ values of 0.33 ± 0.47 μM$^{-1}$ and 6.3 ± 6.1 μM$^{-1}$. The data were of insufficient quality to determine the Zn$^{2+}$ binding parameters with certainty (Table I), in part because of an unresolved enthalpic transition that was evident at the beginning of Zn$^{2+}$ titrations, where the exothermic heat effects increased progressively rather than staying at a steady level (Fig. 2A). This additional heat reaction might be associated with a high affinity binding site on the surface of the YiiP oligomeric complex, because the corresponding transition in the binding isotherm occurred at a stoichiometric equivalence point with a value far less than unity.

**Calorimetric Titrations of YiiP with Cd$^{2+}$**—In contrast to the mix of endothermic and exothermic reactions during Zn$^{2+}$ titrations, the heat effects generated by the Cd$^{2+}$ binding were purely exothermic with a transition occurring at a stoichiometric equivalence point of 2 (Fig. 3A). Thus, this binding isotherm suggests the involvement of two sites for Cd$^{2+}$ binding. Cd$^{2+}$ titrations in the presence of 0.25 mM HgCl$_2$ also yielded a pure exothermic reaction but showed a midpoint of binding heat changes at 1 stoichiometric equivalent (Fig. 3B). This apparent loss of one equivalent Cd$^{2+}$ binding site suggests that one of the two Cd$^{2+}$ binding sites is blocked by Hg$^{2+}$ binding, whereas the other is not affected. Therefore, a binding model for two sets of independent binding sites was used to fit the corresponding competitive and noncompetitive Cd$^{2+}$ bindings. All parameters ($n$, $K_n$, and $\Delta H$) were allowed to float during the least-squares minimization process, resulting in an excellent fit correspond-
Fig. 3. ITC analyses of Cd2⁺ binding to YiiP. A, titrations with 5-μl injections of 2 mM CdCl₂ into 0.05 mM YiiP in a 1.4-ml reaction cell containing 20 mM HEPES, pH 7.0, 100 mM NaCl, 12.5% glycerol, 0.05% DDM, 0.25 mM TCEP. B, experiment identical to A except that 0.25 mM HgCl₂ was added to the reaction cell before titrations. C, experiment identical to A except that 0.5 mM ZnCl₂ was added to the reaction cell before titrations. The solid lines represent the best-fit to a binding model including two set of independent sites in A or one set of independent site in B. The resulting fit parameters are summarized in Table I.

**Table I**

Summary of thermodynamic parameters for metal ion binding to YiiP at 25 °C at pH 7.0

| Site               | Titrant | Competing metal ion | $K_a$ (μM⁻¹) | $n$ | $ΔH$ (kcal/mol) | $ΔG$ (kcal/mol) | $TΔS$ (kcal/mol) |
|--------------------|---------|---------------------|--------------|-----|----------------|-----------------|-----------------|
| Site 1 (common site) | ZnCl₂   | None                | 0.33 ± 0.47  | 1.5 ± 0.2 | -4.5 ± 0.3     | -7.5            | +3.0            |
|                    |         | CdCl₂               |              |       | Not observed   |                 |                 |
|                    |         | HgCl₂               |              |       | Not observed   |                 |                 |
|                    | CdCl₂   | None                | 8.7 ± 0.45   | 1.2 ± 0.1 | -6.5 ± 0.1     | -9.4            | +3.0            |
|                    |         | HgCl₂               |              |       | Not observed   |                 |                 |
|                    | HgCl₂   | None                | 960 ± 460    | 1.0 ± 0.1 | -22.5 ± 0.2    | -12.2           | -10.3           |
|                    |         | CdCl₂               |              |       | Not observed   |                 |                 |
|                    |         | ZnCl₂               |              |       | Not interpretable |               |                 |
| Site 2             | ZnCl₂   | None                | (6.3 ± 6.1) × 10⁻³ | 0.84 ± 2.3 | 12.5 ± 380      | -5.2            | +17.7           |
|                    |         | CdCl₂               |              |       | Not interpretable |               |                 |
|                    |         | HgCl₂               |              |       | Not interpretable |               |                 |
|                    | CdCl₂   | None                | 0.30 ± 0.05  | 0.84 ± 0.05 | -6.1 ± 0.7    | -7.4            | +1.3            |
|                    |         | HgCl₂               |              |       | Not interpretable |               |                 |
|                    | HgCl₂   | None                | 1.8 ± 0.9    | 1.3 ± 0.1 | -4.1 ± 0.3     | -8.5            | +4.4            |
|                    |         | CdCl₂               |              |       | Not interpretable |               |                 |
|                    |         | ZnCl₂               | 2.7 ± 0.3    | 1.5 ± 0.1 | -18.3 ± 0.2    | -8.7            | -7.6            |

The binding of $K_{a1} = 8.70 ± 0.45 \text{ μM}^{-1}$, $n_1 = 1.2 ± 0.1$, $ΔH_1 = -6.5 ± 0.1$ kcal/mol; $K_{a2} = 0.30 ± 0.05 \text{ μM}^{-1}$, $n_2 = 0.84 ± 0.05$, $ΔH_2 = -6.1 ± 0.7$ kcal/mol. The binding stoichiometries for both sites are close to unity, consistent with the qualitative assessment that there are two independent binding sites for Cd²⁺ binding. One tight (site 1) and one weaker (site 2) site can be mathematically distinguished by a 29-fold difference in their binding affinities. The Cd²⁺ binding isotherm obtained in the presence of 0.25 mM Hg²⁺ was fit to a one-site model, resulting in best-fit parameters ($K_a = 0.50 ± 0.05 \text{ μM}^{-1}$, $n = 0.77 ± 0.03$, and $ΔH = -7.6 ± 0.3$ kcal/mol) in agreement with those of site 2. A comparison between the binding parameters obtained from free YiiP and Hg²⁺-prebound YiiP suggests that site 1 is a common competitive binding site for both Cd²⁺ and Hg²⁺.

Cd²⁺ titrations in the presence of 0.5 mM Zn²⁺ broadened the binding isotherm and caused a greatly reduced exothermic heat effect, indicative of a diminished Cd²⁺ binding to the Zn²⁺-prebound YiiP (Fig. 2C). The resulting featureless isotherm precluded fits of any binding model, but it was clear that Zn²⁺ binding obstructed Cd²⁺ binding to both site 1 and site 2.

**Calorimetric Titrations of YiiP with Hg²⁺**—The binding of Hg²⁺ was shown to block an exothermic Zn²⁺ binding site (Zn²⁺ site 1) or a high affinity Cd²⁺ binding site (Cd²⁺ site 1). To further examine whether the binding of Zn²⁺ or Cd²⁺ could mutually block the Hg²⁺ binding, Hg²⁺ titrations were carried out to examine Hg²⁺ binding to free-YiiP and to YiiP pre-bound with Zn²⁺ or Cd²⁺. Similar to Cd²⁺ titrations at neutral pH, Hg²⁺ titrations showed a pure exothermic reaction with two distinct phases (Fig. 4A). In the first phase the exothermic heat effects dropped rapidly and then relaxed in the second phase to the dilution level, which was considerably higher than when titrated with Zn²⁺ or Cd²⁺. After correction for heats of Hg²⁺ dilution, an excellent fit to the Hg²⁺ binding isotherm was obtained for a model containing two sets of independent bind-
ing sites with all six fit parameter floating freely. The resulting $K_A$ value for the tight binding site ($Hg^{2+}$, site 1) differs by 550-fold from that of the weaker site ($Hg^{2+}$, site 2) (Table I). Furthermore, the fit of the $Hg^{2+}$ binding isotherm obtained from the $Cd^{2+}$-prebound YiiP (Fig. 4B) showed only a single $Hg^{2+}$ binding site with three best-fit parameters ($K_A, n$, and $\Delta H$) in agreement with those of $Hg^{2+}$ site 2 (Table I). This result suggests that $Cd^{2+}$ binding blocks $Hg^{2+}$ site 1 but has little effect on $Hg^{2+}$ site 2.

The $Hg^{2+}$ binding isotherm obtained from $Zn^{2+}$-prebound YiiP showed that the rapidly declining phase of the exothermic $Hg^{2+}$ heat effect was abolished (Fig. 4C). The binding isotherm was complicated, containing at least two low affinity binding sites. Thus, we were not able to quantitatively assess the effect of $Zn^{2+}$ on individual $Hg^{2+}$ binding site. However, $Zn^{2+}$ binding was clearly shown to inhibit the high affinity $Hg^{2+}$ binding ($Hg^{2+}$ site 1).

Taken together, calorimetric titrations revealed at least two binding sites for each of $Zn^{2+}$, $Cd^{2+}$, and $Hg^{2+}$. All three metal ions bind to a common site (site 1) in a mutually competitive fashion. This common binding site is thermodynamically distinguishable as the exothermic binding site for $Zn^{2+}$ and the higher binding affinity sites for $Cd^{2+}$ and $Hg^{2+}$, respectively. The relationship between the second sites is less clear. The $Cd^{2+}$ site 2 and $Hg^{2+}$ site 2 are independent of each other, but $Zn^{2+}$ binding to the $Zn^{2+}$ second site(s) appeared to interfere with bindings to both $Cd^{2+}$ and $Hg^{2+}$ second site.

The pH Dependence of Metal Ion Binding to YiiP—To test the involvement of histidine residues in metal ion binding, we examined the pH dependence of $Zn^{2+}$, $Cd^{2+}$, and $Hg^{2+}$ titrations. The binding of a metal ion to the imidazole group ($pK_a = 6.2$) of a histidine is coupled to the release of a proton. Acidic conditions would prevent proton release, thereby inhibiting metal binding. YiiP was purified with MES buffer at pH 5.5, and ITC experiments were performed in the identical MES mobile phase. $Zn^{2+}$ titrations exhibited a predominantly endothermic reaction except that the early midrange of titrations consisted of biphasic heat effects, a fast endothermic process followed by a slow exothermic process (Fig. 5A). Deconvolution of this complicated binding isotherm was difficult. However, a comparison with the $Zn^{2+}$ binding isotherms obtained at pH 7.0 (Fig. 2A) revealed a conspicuous loss of the exothermic heat profile, indicating that $Zn^{2+}$ binding to the common site ($Zn^{2+}$ site 1) was significantly inhibited at pH 5.5. The heat effects generated by $Cd^{2+}$ titration remained purely exothermic, but the amplitude of the binding enthalpy was also significantly reduced (Fig. 5B). The best-fit of the $Cd^{2+}$ binding isotherm to a two-site binding model yielded $K_{1a} = 3.15 \pm 0.79 \mu M^{-1}$, $n_1 = 0.31 \pm 0.01$, and $\Delta H_1 = -0.06 \pm 0.16 \text{ kcal/mol}$; $K_{2a} = 0.15 \pm 0.01$ $\mu M^{-1}$, $n_2 = 1.34 \pm 0.02$, and $\Delta H_2 = -3.0 \pm 0.08 \text{ kcal/mol}$. In addition to the decreases in binding enthalpy that occurred at both sites, the binding stoichiometries decreased from 1.2 to 0.31 for the high affinity site but increased from 0.84 to 1.34 for the low affinity site (Table I). The major pH effect on $Cd^{2+}$ binding appears to occur at the high affinity site because of the significant reduction of both $\Delta H_1$ and $n_1$. Nevertheless, a quantitative evaluation is not possible based on a simple comparison of fit parameters obtained in two pH buffers of different proton ionization enthalpies. In contrast to the strong pH dependences of $Zn^{2+}$ and $Cd^{2+}$, lowering the pH to 5.5 had little effect on $Hg^{2+}$ binding. The $Hg^{2+}$ binding isotherm obtained at pH 5.5 (Fig. 5C) was similar to that obtained at pH 7.0 (Fig. 4A). The best-fit to two sets of binding sites yielded $K_{1a} = 380 \pm 90$ $\mu M^{-1}$, $n_1 = 0.71 \pm 0.01$, $\Delta H_1 = -22.8 \pm 0.2 \text{ kcal/mol}$; $K_{2a} = 0.4 \pm 0.07$ $\mu M^{-1}$, $n_2 = 0.94 \pm 0.02$, $\Delta H_2 = -9.9 \pm 0.3 \text{ kcal/mol}$.
elementary $\Delta H_{\text{app}}$ values, corresponding to the binding to site 1 and site 2, respectively. The resulting $\Delta H_{\text{app}}$ is related to the ionization enthalpy of the pH buffer in which Cd$^{2+}$ titrations were performed (Fig. 6) (23). Linear regressions yielded a slope of $-1.23$ for site 1 and $-0.15$ for site 2. According to the accumulative relationship, $\Delta H_{\text{app}} = \Delta H_{\text{bind}} + n \Delta H_{\text{ion}}$, the slopes of these linear regressions indicate that 1.23 protons are released from Cd$^{2+}$ site 1, whereas 0.15 protons are released from Cd$^{2+}$ site 2 upon Cd$^{2+}$ binding at neutral pH. These results indicate that Cd$^{2+}$ binding to the common site is coupled to a deprotonation process.

**Effect of DEPC on Metal Ion Binding**—The coupling between Cd$^{2+}$ binding and a deprotonation process at neutral pH is consistent with the involvement of histidine residues in the binding reaction. The role of the histidine residues was further investigated by examining zinc calorimetric titrations before and after His-specific modification by DEPC (24). The control experiment in which YiiP was titrated with Zn$^{2+}$ before DEPC modification showed a mix of exothermic and endothermic reactions with a characteristic exothermic-to-endothermic transition occurring in the midrange of titrations (Fig. 7A). Because the exothermic reaction was assigned to Zn$^{2+}$ binding to the common binding site, changes in the exothermic profile were used as a thermodynamic signature to assess the effect of DEPC modification on the common binding site. A prolonged incubation with DEPC (12 h) abolished the exothermic reaction (Fig. 7B), whereas a short incubation (0.5 h) resulted in a loss of the general exothermic profile along with a broadening of the binding isotherm (Fig. 7C). To examine the specificity of the inhibitory effect of DEPC, DEPC modifications were carried out in the presence of 2 mM Zn$^{2+}$ or Cd$^{2+}$. Both Zn$^{2+}$ and Cd$^{2+}$ binding prevented the loss of the exothermic profile to varied degrees after 0.5 h of DEPC incubation (Fig. 7D and E). Taken together, the DEPC inhibitory effect and the binding protection suggest the involvement of a histidine residue(s) in the common binding site. Furthermore, the cross-protection by Cd$^{2+}$ on the exothermic Zn$^{2+}$ binding site again indicates that the exothermic Zn$^{2+}$ binding site is a common binding site for both Zn$^{2+}$ and Cd$^{2+}$.

**DISCUSSION**

The experiments described herein provide direct energetic measurements of heat flow derived from the interactions between YiiP and its physiological substrate Zn$^{2+}$ and two related group-12 metal ions, Cd$^{2+}$ and Hg$^{2+}$. Deconvolution of binding isotherms indicates that the observed heat flows are the results of cumulative contributions from bindings to at least two thermodynamically distinguishable binding sites (site 1 and site 2) for each metal ion. The physical identities of these hypothetical binding sites were deduced from comparing the changes of binding isotherms in the absence and presence of a competing metal ion at a saturation concentration. The Cd$^{2+}$ or Hg$^{2+}$ titrations under mutually competing conditions yielded a reduced binding isotherm that could be fit to a on-site mode with binding parameters matching that of their respective second sites. These quantitative analyses are internally consistent with the assignments that Cd$^{2+}$ site 1 and

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**FIG. 5.** ITC analyses of metal ion binding at pH 5.5. All experiments were performed at 25°C in 20 mM MES, pH 5.5, 100 mM NaCl, 12.5% glycerol, 0.05% DDM, and 0.25 mM TCEP (omitted for Hg$^{2+}$ titration). A, titrations of 0.09 mM YiiP with 5-μl aliquots of 2 mM ZnCl$_2$. B, titrations of 0.09 mM YiiP with 5-μl aliquots of 2 mM CdCl$_2$. C, titration of 0.045 mM YiiP with 5-μl aliquots of 0.75 mM HgCl$_2$. The solid lines represent the best-fit to a binding model including two sets of independent sites in B.

**FIG. 6.** Coupling of Cd$^{2+}$ binding to the deprotonation of YiiP. Titrations with 5-μl injections of 2 mM CdCl$_2$ into 0.09 mM YiiP in a 1.4-ml reaction cell containing 100 mM NaCl, 12.5% glycerol, 0.05% DDM, 0.25 mM TCEP, and an indicated pH buffer, pH 7.0, at 20 mM. The solid and dotted lines represent least-squares fits of the site 1 and site 2 binding enthalpies to the linear function, $\Delta H_{\text{app}} = \Delta H_{\text{bind}} + n \Delta H_{\text{ion}}$. 

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The experiments described herein provide direct energetic measurements of heat flow derived from the interactions between YiiP and its physiological substrate Zn$^{2+}$ and two related group-12 metal ions, Cd$^{2+}$ and Hg$^{2+}$. Deconvolution of binding isotherms indicates that the observed heat flows are the results of cumulative contributions from bindings to at least two thermodynamically distinguishable binding sites (site 1 and site 2) for each metal ion. The physical identities of these hypothetical binding sites were deduced from comparing the changes of binding isotherms in the absence and presence of a competing metal ion at a saturation concentration. The Cd$^{2+}$ or Hg$^{2+}$ titrations under mutually competing conditions yielded a reduced binding isotherm that could be fit to a one-site mode with binding parameters matching that of their respective second sites. These quantitative analyses are internally consistent with the assignments that Cd$^{2+}$ site 1 and
Hg$^{2+}$ site 1 overlap a common binding site, whereas site 2 of Cd$^{2+}$ and Hg$^{2+}$ are two independent binding sites. Classification of the Zn$^{2+}$ binding sites was aided by the characteristic exothermic heat reaction attributed to the Zn$^{2+}$ binding to its site 1. This thermodynamic signature was abolished by the binding of Cd$^{2+}$ or Hg$^{2+}$, indicating that Zn$^{2+}$ site 1 overlaps a binding site common to both Cd$^{2+}$ and Hg$^{2+}$, the common competitive binding site. Binding affinities to site 1 are 1.23 nM, 0.12 μM, and 1.04 nM for Zn$^{2+}$, Cd$^{2+}$, and Hg$^{2+}$, respectively. The relationship among the second sites of the three metal ions remains obscured. Cd$^{2+}$ or Hg$^{2+}$ titrations in the presence of a saturated concentration of Zn$^{2+}$ only yielded a low affinity binding isotherm that could not be fit to any binding model. Qualitative assessments suggested that Zn$^{2+}$ binding inhibited both site 1 and site 2 for Cd$^{2+}$ and Hg$^{2+}$. Because Cd$^{2+}$ site 2 is independent of Hg$^{2+}$ site 2, the inhibitory effects of Zn$^{2+}$ on both Cd$^{2+}$ and Hg$^{2+}$ site 2 suggest that Zn$^{2+}$ might have additional binding sites. This possibility is also reflected by the poor fit of the Zn$^{2+}$ binding isotherm to a two-site model, indicative of inadequate modeling with two binding sites. However, deconvolution of multiple binding sites is not conclusive without additional fitting constraints, precluding a definitive assignment for all Zn$^{2+}$ binding sites.

Metal binding is likely coupled to a deprotonation process involving three major ligand groups in proteins: imidazole, sulphydryl, and carboxylate (25). If this is the case, the binding reaction would be a pH-dependent process. Because an extreme acidic or alkaline condition would denature YiiP, the pH dependence of metal binding was examined at pH 5.5 and 7.0, representing the protonation and deprotonation pH of the histidine imidazole. Lowering the pH from 7.0 to 5.5 caused a significant reduction of binding enthalpies associated with Zn$^{2+}$ and Cd$^{2+}$ binding to the common binding site, suggesting the involvement of histidine residues. This finding is consistent with the inhibitory effect of DEPC modification on the binding isotherm of Zn$^{2+}$ titrations, showing a specific inhibition of the exothermic reaction. The deprotonation associated with Cd$^{2+}$ binding was further indicated by the linear relationship between the total heat exchange and the ionization heat of the pH buffer. The slope of this linear relationship suggests that 1.23 protons are released upon Cd$^{2+}$ binding to the common binding site. It is not known to what extent the histidine residue in the binding site is deprotonated at pH 7.0. These 1.23 protons released upon Cd$^{2+}$ binding could come from the deprotonation process of two possible ligand groups: a partially protonated histidine imidazole and a cysteine thiol that is expected to be fully protonated at neutral pH. Interestingly, in a parallel titration experiment with Hg$^{2+}$, the lower pH had little effect on the binding of Hg$^{2+}$ to both site 1 and site 2. The lack of pH dependence of the Hg$^{2+}$ binding to the common binding site contradicts the pH dependence of Zn$^{2+}$ and Cd$^{2+}$ binding to the same site. This apparent discrepancy is likely attributed to the difference in coordination chemistry. Zn$^{2+}$ and Cd$^{2+}$ binding generally takes up a 4- or 5-coordinate geometry, whereas the binding of Hg$^{2+}$ prefers a lower coordination number of 2 or 3 (30). The lower coordination number of Hg$^{2+}$ binding may allow of a high Hg$^{2+}$ binding affinity without the need to interact with the histidine that is essential to Zn$^{2+}$ and Cd$^{2+}$ coordination. In this case, a sulphydryl group is another likely ligand group in the common site in addition to the imidazole of a histidine, because sulfur is a preferred donor atom for Hg$^{2+}$ coordination in a linear or trigonal geometry (31). The identification of determinant residues in the common binding site is under way by site-specific labeling of histidine and cysteine residues.

Dissection of the Gibbs free energy of binding into entropic and enthalpic changes provides thermodynamic details of the molecular interactions between YiiP and metal ions. The binding enthalpy change primarily reflects the strength of metal ion-YiiP interactions relative to that of metal ion-solvent interactions, whereas the binding entropy change depends on the gain of solvation entropy because of metal ion desolvation relative to the loss of protein conformational entropy (freedom) upon metal ion binding. The binding enthalpy change at the common binding site is −10.3 kcal/mol for Hg$^{2+}$, becomes slightly more favorable at −6.5 kcal/mol for Cd$^{2+}$, but leaps to −22.5 kcal/mol for Zn$^{2+}$. The corresponding TAS values are 3.0 kcal/mol for both Zn$^{2+}$ and Cd$^{2+}$ binding and a negative value of −10.3 kcal/mol for Hg$^{2+}$ binding to the same site. Therefore, the binding of Zn$^{2+}$ and Cd$^{2+}$ to the common site are driven both enthalpically and entropically, whereas the binding of Hg$^{2+}$ is driven only by enthalpy. The unfavorable entropy change during Hg$^{2+}$ binding is dominated by a much more pronounced, favorable enthalpy change. The modest thermodynamic difference between Zn$^{2+}$ and Cd$^{2+}$ binding implies that Cd$^{2+}$ and Zn$^{2+}$ bind to the common binding site through liganding to a set of mostly shared donor groups with similar, if not identical, coordination geometry. On the other hand, Hg$^{2+}$ appears to adopt a different thermodynamic approach to reach a much higher binding affinity. The distinctive thermodynamic character of Hg$^{2+}$ binding suggests a much stronger bond formation and a greater degree of structural rigidification as compared with Zn$^{2+}$ and Cd$^{2+}$ binding. These differences, corroborated by the pH independence of the Hg$^{2+}$ binding to the common binding site, suggest that the binding of Hg$^{2+}$ to the common binding site may involve a different set of donor groups arranged in a different coordination geometry.

A comparison of the binding of three metal ions to their respective second sites indicates that the binding of Cd$^{2+}$ and Hg$^{2+}$ is favored both enthalpically and entropically, whereas the binding of Zn$^{2+}$ to its site 2 is associated with an unfavorable enthalpy change, leaving the entropic force to drive the
Thermodynamics of Metal Binding to the Zinc Transporter YiiP

Metal ion binding to a CDF protein represents the first step of the transport process. A stopped-flow analysis of the homologous E. coli CDF protein ZitB suggested that the CDF transport process is consistent with the following two-step scheme,

\[
\begin{align*}
M + T_1 & \rightleftharpoons \text{MT}_1 \rightarrow M + T_2 \\
k_1 & \quad k_2 & k_1 & \quad k_2
\end{align*}
\]

**Scheme 1**

where \( T_1 \) and \( T_2 \) correspond to the inward facing and outward facing conformations of the transporter, and \( M \) is the metal ion (16). The kinetic step \( \text{MT}_1 \rightarrow M + T_2 \) represents the translocation of a metal ion across the membrane. The relationship among three rate constants \( k_1, k_{-1}, \) and \( k_2 \) is defined by \( K_m = (k_2 + k_{-1})/k_1 \). \( K_m \) for both Zn\(^{2+}\) and Cd\(^{2+}\) are in the order of 100 \( \mu \text{M} \) and the transport turnover rate \( k_2 \) is in the order of 5 \( \text{s}^{-1} \) (16). Kinetic studies of YiiP showed that Zn\(^{2+}\) and Cd\(^{2+}\) are both effective transport substrates with kinetic parameters in the same order of magnitude of the ZitB parameters. In this study, calorimetric titrations of YiiP with Zn\(^{2+}\) and Cd\(^{2+}\) revealed multiple binding sites with binding affinities in the order of the micromolar to submicromolar range. It is not known which one of these binding sites is involved in the transport process and the binding affinity of which conformational states corresponds to the values determined by calorimetric titrations. Nevertheless, our thermodynamic analyses provide an estimate of the transport constant \( K_d \) (\( k_{-1}/k_1 \)). If it is assumed \( K_d = 1 \mu \text{M}, K_m = 100 \mu \text{M} \) and \( k_2 = 5 \text{s}^{-1} \), the corresponding on and off rates of the metal ion-CDF complex can be calculated as follows: \( k_1 = 50,000 \text{s}^{-1} \text{M}^{-1} \) and \( k_{-1} = 0.05 \text{s}^{-1} \). \( k_{-1} \) is slower than \( k_1 \) by a factor of 100, indicating that the metal-protein complex (\( \text{MT}_1 \)) will undergo a \( \text{MT}_1 \rightarrow T_2 + M \) conformational transition with a likelihood of being 100-fold greater than that of releasing it back to the cytosol (\( \text{MT}_1 \rightarrow T_1 + \text{M} \)). Thus, the transport process is nearly irreversible once a metal ion is loaded to the binding site. However, the binding of metal ion appears to be a highly unfavorable kinetic process under the physiological condition. It has been estimated that intracellular zinc binding capacity exceeds the total number of zinc ions in a cell. This overcapacity sets the biologically available zinc pool at an extraordinarily low level (26). The zinc concentration in the cytoplasmic milieu is estimated to be less than \( 10^{-10} \text{M} \) (27). The rate of zinc binding at this concentration is \( 5 \times 10^{-6} \text{s}^{-1} \) (equals to \( k_1 \times 10^{-10} \)), giving a half-time of \( 2.3 \times 10^{-4} \text{s} \) leading to a CDF in the order of 40 h. Therefore, the kinetic barrier of zinc binding is quite prohibitive under zinc homeostatic conditions. The function of CDF transporters may not be driven by Zn\(^{2+}\) but rather, in a kinetically controlled substitution fashion. Several recent studies have suggested that a class of metallochaperone proteins might be responsible for catalyzing metal ion transfers from protein donors to acceptors with high metal binding affinity, while retaining a fast transfer kinetics (21, 28, 29). This traffic of metal ions in the cell allows the delivery of metal ions through specific protein-protein interactions to overcome the kinetic binding barrier at an extremely low free metal-ion concentration. Likewise, the efflux or sequestration of cytosolic zinc through CDF transporters may require a cytoplasmic zinc trafficking factor that can capture cytoplasmic zinc and deliver it to the binding site of a CDF to initiate the transport process.

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