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Oligomeric State of the Escherichia coli Metal Transporter YiiP*

YiiP is a 32.9-kDa metal transporter found in the plasma membrane of Escherichia coli (Chao, Y., and Fu, D. (2004) J. Biol. Chem. 279, 17173–17180). Here we report the determination of the YiiP oligomeric state in detergent-lipid micelles and in membranes. Molecular masses of YiiP solubilized with dodecyl-, undecyl-, decyl-, or nonyl-β-D-maltoside were measured directly using size-exclusion chromatography coupled with laser light-scattering photometry, yielding a mass distribution of YiiP homo-oligomers within a narrow range (68.0–68.8 kDa) that equals the predicted mass of a YiiP dimer within experimental error. The detergent-lipid masses associated with YiiP in the mixed micelles were found to increase from 135.5 to 232.6 kDa, with an apparent correlation with the alkyl chain length of the maltoside detergents. Cross-linking the detergent-solubilized YiiP with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) resulted in a dimeric cross-linked product in an EDC concentration-dependent manner. The oligomeric state of the purified YiiP in reconstituted membranes was determined by electron microscopic analysis of two-dimensional YiiP crystals in negative stain. A projection structure calculated from measurable optical diffractions to 25 Å revealed a pseudo-2-fold symmetry within a molecular boundary of −75 × 40 Å, indicative of the presence of YiiP dimers in membranes. These data provide direct structural evidence for a dimeric association of YiiP both in detergent-lipid micelles and in the reconstituted lipid bilayer. The functional relevance of the dimeric association in YiiP is discussed.

YiiP is a member of the cation diffusion facilitator (CDF) family that functions principally as a metal transporter (1, 2). Analysis of genome sequences shows that CDFs represent a ubiquitous protein family, encompassing more than 400 evolutionarily related members found from bacteria and yeast to plants and mammals (3). This protein family is characterized by an N-terminal hydrophobic domain followed by a C-terminal hydrophilic region that is highly variable both in sequence and in length (4). Despite large variability in the hydrophilic region, all CDF family members identified so far, prokaryotic or eukaryotic, are predominantly involved in controls of cytosolic zinc buildup during zinc excesses, either by facilitating zinc efflux from cytosol to the outside of cells or by transporting the cytosolic zinc into intracellular organelles (5). This zinc-transporting function is attributed to the homologous hydrophobic domain, which is thought to be composed of a bundle of six transmembrane segments in an α-helical configuration, a structural theme found in many other membrane channels and transporters, including those in the aquaporin family (6) and those within the ATP binding cassette (ABC) transporter superfamily (7).

The order of subunit oligomerization is an important structural parameter in connection with subunit arrangements and mechanisms of transmembrane activities. Crystal structures of aquaporins revealed a tetrameric structure with four independent transmembrane channels located in the center of each monomer (8), whereas an ABC transporter MsbA was found to be a dimer with a substrate translocation pathway along the dimeric interface (9). Different oligomeric structures of these membrane proteins illustrate how transmembrane active sites may evolve differently from a common six-spanner architecture. Consequently, subunit oligomerization may contribute to the molecular architecture of transmembrane channels and translocation pathways that constitute the structural basis for distinctive transmembrane functions; aquaporins are selective pores that allow transmembrane permeation of water and glycerol by simple diffusion (10), whereas ABC transporters actively move diverse substrates across the membrane at the expense of adenosine triphosphate hydrolysis (11). CDFs operate with an antiport mechanism that utilizes the potential energy in the form of a transmembrane proton gradient to drive the movement of zinc in a reverse direction (2, 12). The oligomeric state of CDF transporters has not yet been established, and little is known about the structure of any CDF besides the fact that most CDFs are predicted to be six-spanning membrane proteins (4). Therefore, determining the quaternary structure of a CDF transporter is paradigmatic for understanding the structural basis of zinc transport.

Two Escherichia coli CDF proteins, YiiP and ZitB, provide unique models for the study of CDFs at the molecular level. The N-terminal hydrophobic domain of YiiP contains ~200 amino acids followed by a C-terminal hydrophilic region of ~100 amino acids. Chromosomal expression of both E. coli CDFs was found to be inducible by zinc in a concentration-dependent manner, suggesting that both CDFs are involved in zinc homeostatic controls against environmental zinc fluctuation (13). YiiP and ZitB were overexpressed, solubilized by non-ionic detergents, and purified to homogeneity.

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1 The abbreviations used are: CDF, cation diffusion facilitator; SEC, size-exclusion chromatography; EDC, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride; ABC, ATP binding cassette; LS, light scattering; UV, ultraviolet; RI, refractive index; DDM, n-dodecyl-β-D-maltoside; UDM, undecyl-β-D-maltoside; DM, decyl-β-D-maltoside; NM, nonyl-β-D-maltoside; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; HPLC, high performance liquid chromatography; TECP, Tris(2-carboxyethyl) phosphine hydrochloride; MES, 4-morpholineethanesulfonic acid.

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detergents, and purified to homogeneity in mixed micelles with detergents and lipids. The purified protein, which appeared to be in a native-like state, was reconstituted into the lipid bilayer and subjected to stopped-flow kinetic analysis (2). The transport of zinc through ZitB was found to be a two-step kinetic process involving an equilibrium metal binding followed by a proton-linked transport that moves the bound metal ion across the membrane. As such, an inwardly orientated proton gradient across the plasma membrane of E. coli serves as the driving force for the efflux of the cytoplasmic zinc. Further mechanistic studies by isotothermal titration calorimetry with purified YiiP revealed a mutually competitive binding site common to Zn$$^{2+}$$, Ca$$^{2+}$$, and Hg$$^{2+}$$ and a set of non-competitive binding sites (1). 

In the present study we sought to determine the oligomeric state of YiiP in an effort to gain structural insights into zinc transport by CDFs.

The oligomeric state of YiiP could be derived from molecular mass measurements (14). Previous studies with analytical SEC showed that the purified YiiP was eluted as a major monodisperse species with a retention time corresponding to an apparent molecular mass of 190 kDa (2). Any oligomeric state of YiiP from a dimeric to a tetrameric form could account for this apparent molecular mass because the retention time, a measure of protein Stoke’s radius and geometry, could vary considerably depending on the amount of detergents and lipids bound to YiiP. To determine definitively the molecular mass of YiiP oligomers in mixed micelles, we used an absolute mass analysis method based on size-exclusion HPLC in conjunction with simultaneous detections of ultraviolet absorption (UV), laser light-scattering (LS), and differential refractive index (RI) of the column effluent (15). This UV-LS-RI method yielded molecular mass estimates for YiiP oligomers in mixed micelles, irrespective of the size, shape, and lipid-detergent constituent of the mixed micelles. Another advantage of this light-scattering analysis method is its applicability to unstable membrane proteins that tend to denature rapidly in detergent micelles. HPLC runs and molecular mass calculations can be completed within 30 min, representing a clear improvement over the analytical ultracentrifugation method that requires several days to attain sedimentation equilibrium (16).

Here we describe the molecular mass analysis of YiiP oligomers in mixed micelles with endogenous lipids and four multiside detergents. The protein masses found were approximately equal to the expected molecular mass of a YiiP dimer. The detergent-lipid masses associated with the YiiP dimer in the mixed micelles varied according to the type of the multiside detergent. To further characterize the oligomeric state of YiiP in mixed micelles, chemical cross-linking was employed using a zero-spacer cross-linker EDC (17). Because the subunit organization in the membrane environment may be different from that in the detergent-solubilized form, the quaternary structure of YiiP was also studied for the membrane-embedded state by two-dimensional crystallization of YiiP and electron microscopic imaging (18). Our results indicate that YiiP is a dimer both in detergent-lipid micelles and in membranes.

**EXPERIMENTAL PROCEDURES**

**Overexpression and Purification of YiiP—**YiiP was overexpressed and purified as described (1). Briefly, a frozen stock of BL21(DE3)pLysS cells (Novagen Inc., Madison, WI) containing pYiiP-TB-His plasmids was inoculated into an auto-inducing medium for unattended induction of protein overexpression. The overnight culture was harvested, and membrane vesicles were prepared and then subjected to detergent solubilization using n-dodecyl-$$beta$$-$$alpha$$-maltoside (DDM, Anatrace, Maumee, OH). The detergent-solubilized YiiP-TB-His was purified by nickel affinity chromatography followed by a desalting step to remove imidazole that was present in the eluate of the N$$^{3}$$-nitritriacetic acid superflo column (Qiagen, Valencia, CA). Next the His tag was removed by overnight thrombin digestion, and the resultant YiiP was further purified by size-exclusion HPLC using a TSK 3000SW column (TosoHaas, Montgomeryville, PA) equilibrated with the following mobile phase: 20 mM HEPES, pH 7.0, 100 mM NaCl, 12.5% glycerol, 0.05% DDM, and 0.25 mM Tris(2-carboxyethyl) phosphine hydrochloride (TECP). The column effluent was monitored by a UV detector (Beckman Coulter, Fullerton, CA), and the purified YiiP was collected as a discrete chromatographic fraction using a Beckman SC1000 fraction collector.

Sample Preparation for Light-scattering Experiments—All protein samples for light-scattering experiments were adjusted to $\sim 1$ mg/ml, dialyzed against the HPLC mobile phase at 10 °C using a 100-kDa molecular mass cutoff DiaPoreDializer (Spectrum Laboratories, Rancho Dominguez, CA), and then centrifuged at 140,000 × g for 30 min to remove any precipitate. The mobile phase was also filtered with a 0.22-$$mu$$ Millipore GS filter and degassed thoroughly. For light-scattering experiments with different detergents, protein samples were dialyzed with frequent changes of the dialysis bulk solution containing one of the following four detergents as indicated: 0.05% DDM, 0.06% undecyl-maltoside (UDM), 0.15% decyl-maltoside (DM) or 0.4% nonyl-maltoside (NM). For light-scattering experiments with YiiP delipidated to varied levels, samples were prepared by passing YiiP through the TSK column successively and collecting the eluted YiiP peak at each passage.

**Size-exclusion Chromatography and Multitaper Laser-light Scattering Measurement—**The instrument setup used for SEC-light scattering experiments consisted of a System-Gold HPLC system (Beckman Coulter) connected in series with a DAWN DSP light-scattering detector (Wyatt Technology) and an OPTILAB DSP RI interferometric refractometer detector (Wyatt Technology). Analytical size-exclusion chromatography was performed at 20 °C using an TSK 3000SW column (TosoHaas) equilibrated with a mobile phase containing 100 mM NaCl, 50 mM HEPES, pH 7.0, 0.25 mM TECP and one of the following detergents as indicated: 0.05% DDM, 0.06% UDM, 0.15% DM, or 0.4% NM. 100 µl of purified YiiP sample at $\sim 1$ mg/ml was injected into the column and eluted at a flow rate of 0.5 ml/min. The column effluent was monitored in-line with three detectors that simultaneously monitored UV absorption, light scattering, and refractive index, respectively. The resulting three chromatograms were aligned with that of the light-scatter output after corrections for the interdetector volume delays between the UV-LS and RI-LS detector. Detector outputs were digitized and acquired by a Pentium workstation running Astra software (Wyatt Technology).

**Calculation of the Protein Core Mass in Protein-Detergent-Lipid Micelles—**It has been established that the angular deviation of scattered light can be ignored for globular proteins with molecular masses smaller than 5000 kDa (19). The molecular mass of YiiP-detergent-lipid micelles was estimated to be 190 kDa by analytical size-exclusion chromatography. The corresponding SEC light-scattering data, collected simultaneously at 16 different angles between 14 and 163°, remained unchanged within experimental error. Thus, the scattering light signal measured at 90° was used in approximation for all calculations. The second virial coefficient term in the protein mass calculation can also be ignored at protein concentrations encountered in the chromatographic analysis (20). Based on these two approximations, the molecular mass of YiiP ($M_p$) in protein-detergent-lipid micelles can be determined by a three-detector method (15) using the equation,

$$M_p = \frac{k_r(output)_{LS} (output)_{UV}}{A(output)_{LS}}$$  

(Eq. 1)

where $k_r$ is an instrument response factor, (output)$_{LS}$, (output)$_{UV}$ and (output)$_{RI}$ are elution peak intensities, obtained in the unit of volt by LS, UV, and RI detector, and $A$ is the extinction coefficient of the protein.

**Calculation of the Detergent-Lipid Mass in Protein-Detergent-Lipid Micelles—**The amount of detergent and lipid bound to the solubilized YiiP can be calculated according to the value of differential refractive index, $dnc/dcp$, which is defined as the ratio of the change in refractive index of the protein-detergent-lipid complex to the change of the protein concentration. Thus,

$$\frac{dn}{dc_p} = \frac{k_A(output)_{RI}}{(output)_{UV}}$$

(Eq. 2)

where $k_A$ is an instrument response factor (15). The value of $dn/dc_p$ is the sum of weighted contributions of three components in the protein-detergent-lipid ternary complex (15). The differential refractive index of YiiP ($dn/dc_p$) is assumed to be 0.187 mdeg, a constant known for all
**FIG. 1.** Calibration of the UV-LS-RI system with GlpF. A, simultaneous recordings of UV (front), LS (middle), and RI (back) chromatograms. 100 μl of 1 mg/ml pure GlpF sample was loaded into a 50-μl sample loop and injected into a TSK column that was equilibrated and eluted with a DDM buffer as described under “Experimental Procedures.” Column effluent passed sequentially through a UV, LS, and RI detector connected in series. Traces were corrected for interdetector volume delays and normalized to peak maxima. P1 and P2 indicate the position of a major and a minor chromatographic peak at 15.5 and 19.5 min. B, determination of dnc/dcp. The refractive index of the GlpF-detergent-lipid micellar complex (nc) was measured in response to five GlpF concentration (cp) steps using a RI detector operated in the batch mode. Linear regression of nc versus cp, inset, yielded a dnc/dcp value of 0.49 ml/g.  

**FIG. 2.** Mass analyses of YiiP chromatographic fractions P1 and P2. A, molecular mass distribution across YiiP chromatographic peaks. 50 μg of pure YiiP was injected into a TSK column and eluted as two major chromatographic fractions, P1 and P2, at 16.4 and 19.2 min, respectively. Display of the YiiP elution profile was limited to the regions across the P1 (upper panel) and P2 (lower panel) fraction. Simultaneous measurements of UV, LS, and RI responses allowed for determination of molecular masses of the protein core, Mc (dotted line on the left y axis), and the protein-detergent-lipid micellar complex, Mp (left y axis), based on Equation 1 as described under “Experimental Procedures.” The corresponding RI chromatograms across P1 and P2 are overlaid for reference (right y axis). Data points within the highlighted boxes were taken for calculation of the mean Mc and Mp values. B, MALDI-TOF mass spectra of P1 (upper panel) and P2 (lower panel). P1 and P2 fractions were collected and mixed in series dilutions with the matrix solution, and then mass spectra were taken within a low (500-1000) and high (14000-39000) m/z region encompassing molecular species of detergents, lipids, and YiiP, respectively. The m/z value and charge state of each peak are indicated.
regular proteins (15). The differential refractive indexes of detergents (dn/dc) and lipids (dn/dc) were determined experimentally. Therefore, the weight ratios of the bound detergents β and lipids δ (g/g of YiiP) in the YiiP-detergent-lipid micelles can be derived according to Equation 3.

\[
(dn/dc)_\text{ YiiP} = 0.817 + \beta (dn/dc)_\text{detergent} + \delta (dn/dc)_\text{lipid}
\]  

(Eq. 3)

**Measurements of the Extinction Coefficient A and Instrument Response Factors k1 and k2**—Protein concentrations in the detergent solution were estimated by the BCA assay (Pierce). The UV absorption at 280 nm of the identical protein sample was measured, and the extinction coefficient \(e\) was calculated from the slope of the plot:

\[
\text{Extinction coefficient } e = \frac{\text{A}}{\text{C} \times \text{L}}
\]

where A is the absorbance at 280 nm of the identical protein sample, C is the protein concentration (mg/ml), and L is the light path length (cm)

**Mass Spectrometric Analysis**—A matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometric data were collected using a Voyager Biospectrometry Work station, operated in linear mode.

**EDC Cross-linking**—The HPLC-purified YiiP samples were dialyzed overnight to equilibrate with the following EDC reaction buffer: 100 mM MES, pH 5.5, 100 mM NaCl, 0.1 mM CdSO4, 0.05% DDM, 20% glycerol, and 0.25 mM TCEP. Cross-linking reactions were initiated by adding EDC to 1.0 mg/ml YiiP to a final concentration as indicated. The reaction mixtures were incubated at 25°C for 2 h, and then β-mercaptoethanol was added to 100 mM to terminate the reactions. The reactant reaction mixtures were centrifuged at 14,000 × g for 1 h, and then supernatants were injected to an HPLC system that was equilibrated and eluted with 20 mM HEPES, pH 7.0, 100 mM NaCl, 0.05% DDM, 12.5% glycerol, and 0.25 mM TCEP. Column eluents were monitored by UV absorption, and peak fractions were collected at a retention time corresponding to the peak maximum of the identical YiiP sample before EDC cross-linking. The collected peak fractions were subjected to SDS-PAGE analysis in the presence of 2% β-mercaptoethanol and stained with Coomassie Blue (Molecular Probes) for protein visualization.

**Two-dimensional Crystallization**—The purified YiiP (~1 mg/ml) was reconstituted with *E. coli* polar lipids (Avanti Polar Lipids, Inc.) at various lipid-to-protein ratios ranging from 0.5 to 2.0 (w/w). Lipids were solubilized in 0.05% UDM in a 20 mM HEPES buffer, pH 7.0, containing 100 mM NaCl, 5 mM MgCl2, 0.02% NaN3, and 0.1 mM 0.25 mM TCEP.

The detergent was slowly removed by dialysis against a bulk pH buffer in a controlled manner over 3–5 days (22). Two-dimensional crystals of YiiP with a size ranging from 0.5 to 1 μm were obtained at a lipid-to-protein ratio between 0.5 and 1.0.

**Electron Microscopy and Image Analysis**—A 5-μl droplet of the two-dimensional crystal solution was deposited onto a glow-discharged 300-mesh copper grid covered with a thin layer of continuous carbon film. After a 1-min incubation, excess solution on the grid was blotted with a piece of filter paper, and the grid was washed with 2 drops of deionized water and immersed with a 2% uranyl acetate aqueous solution for 30 s and then blotted and left to air-dry. Electron microscopy was performed with a Jeol-1200EX microscope, and images were recorded on a Gatan 791 CCD camera. Standard image processing (23), including determining image defocus values, Fourier transform, indexing reflections, and unbending lattice distortions, were performed on an SGI Fuel work station with the software packages of MRCC image2000 (24) and Spider (25).

**RESULTS**

**Calibration of the UV-LS-RI System**—The instrument response factor \(k_1\) was determined by calibrating the outputs of UV, LS, and RI detector according to Equation 1 using GlpF, a 29-kDa aquaporin that was solubilized and purified following the identical procedure employed for the solubilization and purification of YiiP. Size-exclusion HPLC analysis of GlpF revealed a major peak (P1) with a retention time at 15.5 min followed by a minor peak (P2) at 19.5 min (Fig. 1A). It appeared that the LS and RI detector had more pronounced responses to the minor peak; particularly, the height of the RI output at P2 rose to ~80% of the height at P1. In a set of control experiments, injections of detergent-lipid mixtures only caused base-line-level UV responses but elicited significant LS and RI responses in a concentration-dependent manner. It is known that the RI response reflects the concentration of all solutes, including those with and without chromophores, whereas UV absorption is only derived from the UV absorbivity of chromophore-containing species. The negligible UV response to P2 fraction was indicative of a non-protein nature of the P2 fraction, likely attributed to empty detergent-lipid micelles. On the other hand, the P1 fraction with its dual UV and RI responses corresponded to GlpF in mixed micelles with detergents and bound lipids that may have survived the purification process. Therefore, system calibration was carried out using outputs of UV, LS, and RI detectors across the P1 maxima. A \(k_1\) value of 115.2 was obtained that allowed the outputs of UV, LS, and RI to be converted to the theoretical value of the GlpF tetrameric molar mass (116 kDa). The instrument response factor \(k_2\) was calculated based on an experimentally measured dn/dcpe value of GlpF. Off-line measurements of \(n_\text{e}\) showed a series of plateaus when back-flushing the RI detector with five evenly spaced GlpF dilutions from a 2.4 mg/ml stock solution (Fig. 1B). The RI output collected from each concentration plateau was plotted against the GlpF concentration, producing a value of 0.49 mg/l
for $dn/dc_p$ (Fig. 1B, inset). Using this measured $dn/dc_p$ value, a $k_2$ value of 0.13 was obtained that allowed conversion of RI and UV outputs to the actual $dn/dc_p$ value according to Equation 2.

**Molecular Mass of YiiP Oligomers in Mixed Micelles—YiiP-TB-His was overexpressed, solubilized with DDM, and purified by metal affinity chromatography in the form of (YiiP-TB-His)-DDM-lipid micelles. The His tag was cleaved by thrombin digestion at a cleavage recognition sequence LVPR that was inserted between the His tag and the C-terminal sequence of YiiP, resulting in a tag-free YiiP variant with a predicted molecular mass of 32.6 kDa. Size-exclusion HPLC analysis of the purified YiiP-DDM-lipid micelles revealed two major chromatographic peaks (P1 and P2) with peak maxima eluted at a retention time of 16.4 and 19.2 min, respectively (Fig. 2A).** Compared with the GlpF chromatograms under the identical chromatographic condition, YiiP P1 was characteristic of protein-detergent-lipid micelles with both strong UV and RI responses, whereas YiiP P2 was characteristic of empty detergent-lipid micelles with a strong RI response accompanied by a base-line level UV response. The retention time of YiiP P1 was longer than that of GlpF P1, suggesting a relatively smaller hydrodynamic radius for YiiP-detergent-lipid micelles.

Molecular identities of P1 and P2 fractions were determined by MALDI-TOF mass spectrometry. P1 and P2 fractions were collected and analyzed directly in the presence of detergents and lipids. As shown in Fig. 2B, sample analyzed in this fashion yielded high quality MALDI-MS spectra over a wide range from 0.5 to 40 kDa. Because non-covalent subunit interactions were too weak to survive the MALDI process, the native YiiP oligomer was detected in the form of a monomer. Analysis of P1 fraction revealed two $m/z$ peaks (16289.7, 32556.4) in a higher $m/z$ range, corresponding to the expected $m/z$ values of YiiP in double (16287.5)- and single (32575.0)-charge state. The spectrum at a lower $m/z$ range showed two major mass species of 510.3 and 714.9, consistent with $m/z$ values of the singly charged DDM (510.6) and an E. coli lipid that typically ranges from 700 to 800. Thus, MALDI-TOF analysis confirmed the molecular identity of P1 as mixed micelles of YiiP, DDM, and bound lipids. Analysis of the P2 fraction exhibited an $m/z$ profile in the lower $m/z$ range similar to that of the P1 fraction, but the expected YiiP mass peaks in the higher $m/z$ range were absent. This result confirmed that P2 was composed of empty detergent-lipid micelles.

Size-exclusion HPLC in conjunction with simultaneous UV, LS, and RI detections was used to determine molecular masses of the YiiP homo-oligomer in the mixed micelles using the instrument response factor $k_r$ over 15 sampling points across the two chromatographic peaks, P1 (from 16.26 to 16.51 min) and P2 (from 19.05 to 19.30 min) (Fig. 2A). The mass distribution of the YiiP oligomer across P1 appeared to be homogeneous with a range from 64.6 to 70.1 kDa with an average molecular mass of 68.4 ± 1.4 kDa, suggesting that YiiP is a monodisperse, irreversibly associated dimer. Examination of the mass distribution across P2 showed that the mass ($M_p$) declined from the leading edge of P2 to a steady lower level below 1 kDa and then climbed up along the trailing edge of the P2. The marginal masses around the P2 maximum were in agreement with the masses of chromophore-containing species in empty DDM-lipid micelles. The higher masses on both edges of the P2 were likely caused by peak overlaps with the P1 on the leading edge and possibly with another unidentified solute peak that typically emerged along the trailing edge of the P2.

To further examine the oligomeric state of YiiP solubilized in three more different maltoside detergents with alkyl chain lengths ranging from 9 to 11, we replaced the DDM in the purified YiiP sample with one of the following detergents: NM, DM, and UDM. No protein aggregation was observed after extensive detergent exchange. Size-exclusion HPLC chromatograms of YiiP with all maltoside detergents tested exhibited a similar profile, with two peaks designated as P1 and P2. The retention time of the P1 maximum increased progressively from 16.5 to 16.9 min with the decrease of the alkyl chain length from 11 to 9, suggesting a correlation between the hydrodynamic radius of YiiP-detergent-lipid micelles and the size of the maltoside detergents. Nevertheless, the molecular masses of the YiiP oligomer in different detergent-lipid micelles appeared to remain constant within experimental error with an average...
value of 68.4 kDa regardless of variations of the micellar size (Table I). This result demonstrated that YiiP in different detergent-lipid micelles contains a protein core with a constant molecular mass corresponding to the molecular mass of a dimer.

Molecular Mass of the YiiP-associated Detergents and Lipids—The molecular masses of detergents and lipids in the mixed YiiP micelles can be derived from Equation 3. $dn/dc_p$ values of YiiP in mixed micelles with bound lipids and one of the four maltoside detergents were calculated using the measured RI and UV outputs according to Equation 2. The actual values of $dn/dc_d$ and $dn/dc_c$ were measured off-line for E. coli polar lipids and for maltoside detergents, yielding a $dn/dc_c$ value of 0.138 and $dn/dc_d$ values ranging from 0.138 to 0.142 for different maltoside detergents. Taking an average $dn/dc$ value of 0.140 for both detergents and lipids, Equation 3 can be simplified to the following: $dn/c_dc_p = 0.187 + 0.140\delta$, where $\delta = \delta_c + \delta_l$ is the combined weight ratio of the bound detergents and lipids in the YiiP micellar complex. Calculation of $\delta$ values for YiiP solubilized with NM, DM, UDM, or DDM showed a progressive increase, corresponding to an increase of bound detergent-lipid masses from 135.5 to 232.6 kDa (Table I). Accordingly, the masses of micellar complexes increased with the alkyl chain length from 204.4 kDa for the nonyl chain to 235.6 kDa for the decyl chain, 259.9 kDa for the undecyl chain, and 301.0 kDa for the dodecyl chain. The dependence of the micellar mass increase on the alkyl chain length mirrored the dependence of the micellar size increase on the alkyl chain length.

Delipidation of YiiP—The chromatographic fraction $P_1$ reflected YiiP in mixed micelles, whereas $P_2$ represented the lipid removed from YiiP during the chromatographic process. To examine YiiP delipidation and the corresponding changes in the micellar mass and protein stability, we monitored changes in the $P_1$ and $P_2$ height during a delipidation process consisting of successive passages of a DDM-solubilized YiiP sample.
through a SEC column that was equilibrated and eluted with a DDM solution. Throughout the four successive SEC passages, UV outputs at P2 maxima remained at a base-line level due to the low UV absorptivity of detergents and lipids (Fig. 3, A–D). LS outputs showed significant signals at P2 maxima in the first two SEC passages and then declined to a base-line level in the third and fourth passages. The LS outputs were expected to be biased toward the P1 fraction, where larger micelles eluted. On the other hand, RI detector responded to lipids and detergents with a dn/dc value (0.140 ml/g) comparable with that of proteins (0.187 ml/g). Thus, heights of P1 and P2 in the RI chromatograms approximately reflected the actual concentration difference between the two fractions. RI outputs at P2 maxima exhibited a progressive decrease in parallel to the process of delipidation from an initial P2/P1 ratio of 2.5 to 0.9 in the second SEC passage and, finally, to a steady low ratio of 0.4 and 0.3 in the succeeding passages. The continuous decline of the relative P2 height toward a lower steady level suggested that the first two SEC passages removed a majority of YiiP-associated lipids, whereas the third and fourth SEC passages appeared to have nearly depleted the bound lipids. In contrast to the progressive decline of the RI outputs at P2 maxima, the protein mass (Mp) in YiiP-DDM-lipid micelles remained constant, and the combined masses of detergents and lipids (Mp+Δm) in the mixed micelles changed slightly between 238.4 and 225.4 kDa (Table II). The relative stable Mp+Δm values suggested that delipidation was likely a process of detergent replacement in exchange for bound lipids. Further delipidation after four successive SEC passages began to induce significant protein aggregation. It appeared that a certain amount of lipids was required to sustain YiiP in a native-like structure since RI outputs at P2 maxima remained at a low but detectable level even after multiple SEC passages.

**EDC Cross-linking of YiiP**—The order of YiiP oligomer was further examined by chemical cross-linking using EDC, a zero spacer cross-linker that catalyzed the formation of amide bonds between nearby pairs of a primary amine and a carboxyl group. SEC chromatograms of the EDC reaction mixtures showed a major peak eluted at a position corresponding to the native YiiP species (Fig. 4A). SEC chromatograms showed a progressive decrease of the peak height in an EDC concentration-dependent manner. SDS analysis of the peak fractions of the cross-linked species revealed an oligomeric product at ~55 kDa, consistent with a dimer, and some monomeric species migrating as two distinct protein bands at ~25 and ~30 kDa (Fig. 4B). The dimeric band increased with the EDC concentration accompanied by a decrease of the 25-kDa band, demonstrating the conversion of YiiP monomer to dimer. In addition, the protein band at 30 kDa was also increased with the EDC concentration, probably due to the formation of the intermediate N-acrylurea cross-linking side-product. Inconsistent with this finding, a MALDI-TOF analysis revealed heterogeneity of the EDC labeling, as indicated by a mass upward-shift from 32.6 to 33.1 kDa and a broadening of the m/z peaks (Fig. 4C). Finally, to examine the possibility that higher order cross-linked species might be too rare to be detected due to a relatively low efficiency of EDC cross-linking, we performed EDC cross-linking with YiiP-TB-His and used Western blot for protein detection (data not shown). No higher than a dimeric form of the minor peak decreased with increased EDC concentrations. This apparent loss of YiiP aggregates after EDC cross-linking was probably a result of the ultracentrifugation process that precipitated the cross-linked YiiP aggregates, because the pellet size was found to increase accordingly after EDC treatments.

**Electron Microscopic Imaging of YiiP Two-dimensional Crystals**—To reveal the physiologically relevant subunit assembly of YiiP in the membrane, we carried out two-dimensional crystallization of YiiP and examined its oligomeric structure by electron crystallography. The purified YiiP was reconstituted with *E. coli* polar lipids by equilibrium dialysis for detergent removal. Two types of crystals were found in the lipid bilayer; they are the tubular form and the single layered sheet. At a suitable ratio of YiiP to lipid (0.5–1), a small fraction (~10%) of the tubular structures was evident that contained YiiP packed with a helical symmetry. The same crystallization condition also yielded single layered sheets with size ranging from 0.2–2 µm (Fig. 5A). A small area (~0.5 × 0.5 µm) of the sheet was imaged at a higher magnification to reveal crystal lattice orientations (Fig. 5B). The power spectrum of the crystalline sheet showed reflections displaying order to ~25 Å (Fig. 5C). Indexing the reflections suggested an orthogonal unit cell (a = 7.5 ± 0.2 nm; and b = 4 ± 0.2 nm). The order of the lattice was slightly improved by an unbending procedure. Amplitudes and phases were extracted computationally from the power spectrum of the lattice-straightened images and used to calculate a projection structure with no symmetry enforced. The molecular boundary of the YiiP oligomer was apparent, showing a dimension of 40 Å in width and 75 Å in length (Fig. 5D). Within the molecular boundary were observed two distinct electron density areas arranged around the center of the oligomer with a pseudo-2-fold perpendicular to the membrane plane. The size of each electron density area was estimated on the order of 40 × 35 Å,
approximately corresponding to the size of a 35-kDa protein. Therefore, the YiiP projection structure indicated that YiiP is a dimer in membranes.

**DISCUSSION**

The experiments described herein represent the first quaternary structure analysis of a metal transporter in the CDF family. YiiP was purified in a native-like structure as a dimeric complex with detergents and bound lipids. The presence of detergents and lipids complicates the conventional chromatographic analysis of the molecular mass of the protein core in mixed micelles, thus precluding definitive determination of the oligomeric state. By coupling SEC with laser light-scattering photometry, molecular masses of the protein core can be determined independent of the amount and type of the bound detergents and lipids. The molecular masses of the YiiP oligomer were determined in mixed micelles with four different maltoside detergents and at four different levels of protein delipidation, yielding molecular masses that all approximately equal to 68 kDa. The mass measurement could be slightly overestimated due to a small but positive mass contribution derived from the UV absorbitivity of lipids and detergents in the micellar complexes. Taking into account of the expected experimental error, the measured molecular mass of the YiiP oligomer is consistent with the theoretical value of a YiiP dimer. Further evidence for dimeric association came from EDC cross-linking of DDM-solubilized YiiP, showing that the native-like chromatographic fraction of the EDC-treated YiiP contained a mixed population of cross-linked and non-cross-linked YiiP homodimers. The conversion of the non-cross-linked to the cross-linked species was evidently dependent on the EDC concentration, indicating that EDC catalyzed the formation of a covalent intersubunit linkage across at least one homodimer interface (26). No cross-linked species higher than dimer was observed with a Western blot, precluding the existence of a higher oligomeric order. Taken together, both light-scattering and cross-linking analyses established that YiiP is a homodimer in the detergent-lipid micelles. The YiiP quaternary structure in membranes was directly visualized by electron microscopy of two-dimensional crystals formed by reconstituting the purified YiiP into its native *E. coli* lipid bilayer. The projection structure of the crystals displayed a single dimer of YiiP in the unit cell with overall dimensions of 40 × 35 Å for a YiiP monomer. The relevance of the crystal packing for the native structure was ascertained by a 1,11-bis-maleimidotetraethyleneglycol cross-linking experiment performed on membrane vesicles that were prepared from *E. coli* cells hosting YiiP overexpression. Only the dimeric cross-linked YiiP species was observed (data not shown). These experiments lead to the conclusion that YiiP exists as a homodimer in the membrane.

An important aspect of membrane protein solubilization and purification is the detergent-protein interaction that plays a central role in maintaining the protein in a native-like structure after being extracted from biological membranes. Equilibrium chromatography studies with radioactive detergents showed a systematic trend that related the detergent binding to the size of the hydrophobic sector of a group of membrane proteins (27). It appeared that detergent binding reflected the number of detergent molecules required to cover the hydrophobic surface of the membrane proteins. Contrary to the earlier findings that the detergent binding level was inversely related to the bulkiness of the detergent molecule (27), we found a characteristic increase of the maltose binding with the increasing alkyl chain length. Our data suggests that the binding affinity of a detergent molecule may be an additional parameter that contributes to the detergent binding capacity. In this way, a maltose detergent with a longer alkyl tail would be expected to bind to the hydrophobic surface better, thereby having a higher binding occupancy or molar binding ratio. It has been observed that the molar binding ratio of a maltoside detergent is correlated to the stability of YiiP in detergent micelles, further suggesting that a longer alkyl tail may better shield the hydrophobic surface from solvent exposure, probably due to a higher detergent binding affinity. Despite the discrepancy noted above, our light-scattering analysis yielded a DM binding molar ratio (175 mol/mol of protein) in excellent accordance with the values determined by equilibrium chromatography for the binding of DM to different membrane proteins: bacteriorhodopsin (207 mol/mol of protein), Ca$^{2+}$-ATPase (152 mol/mol of protein), reaction center (148 mol/mol of protein), and cytochrome oxidase (215 mol/mol of protein) (27). Our results, in corroboration with these earlier studies, indicate that the principal mechanism of detergent solubilization and stabilization can be attributed to detergent-protein interactions that shield the hydrophobic protein surface from solvent exposure.

In the absence of a high resolution YiiP structure, information on its quaternary structure provides clues as to the molecular organization of the functional unit in YiiP. Comparing the monomer size of YiiP with projected dimensions derived from known crystal structures of membrane channels and transporters, YiiP monomer is comparable with the size of GlpF (38 × 38 Å), a 6-spanner water-glycerol channel (8), but smaller than the following: GlpT (40 × 50 Å), a 12-spanner glycerol 3-phosphate transporter in the major facilitator superfamily (28); BtuC (30 × 60 Å), a 10-spanner membrane-spanning subunit of an ABC transporter (29); CIC (55 × 55 Å), a 14-spanner chloride transporter (30). Of particular interest is the fact that the crystal structures of GlpF, GlpT, and CIC, although they are genetically unrelated, all revealed an internal repeat pattern in which the N-terminal half of the polypeptide is structurally related to the C-terminal half by a pseudo-symmetry axis either perpendicular or in parallel to the membrane normal. The two halves of the polypeptide chain wrap around a common center to form a transmembrane channel or substrate translocation pathway along the domain interface. This 2-fold architecture appears to make no exception for the ABC transporters, because BtuC only represents half of the ABC transporter, which in general contains two copies of BtuC-like subunits fusing into a single polypeptide chain. In the sense of size and protein mass similarities, a YiiP monomer is close to half of GlpT or CIC. This raises the possibility that two copies of YiiP are assembled into a homodimer to make a complete transporter structure. If this is the case, the metal ion translocation pathway in YiiP is likely located at the dimer interface, the equivalent of the domain interfaces in the GlpT and CIC structure. However, there is no clear genetic evidence in support of such a gene fusion event in the evolution of the CDF family. Thus, we cannot exclude the possibility that the YiiP structure may harbor two independent translocation pathways, each located in the center of a YiiP subunit. Further structural analysis is under way to address these questions.

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