An essential component of heme transport in Gram-negative bacterial pathogens is the periplasmic protein that shuttles heme between outer and inner membranes. We have solved the first crystal structures of two such proteins, ShuT from *Shigella dysenteriae* and PhuT from *Pseudomonas aeruginosa*. Both share a common architecture typical of Class III periplasmic binding proteins. The heme binds in a narrow cleft between the N- and C-terminal binding domains and is coordinated by a Tyr residue. A comparison of the heme-free (apo) and -bound (holo) structures indicates little change in structure other than minor alterations in the heme pocket and movement of the Tyr heme ligand from an “in” position where it can coordinate the heme iron to an “out” orientation where it points away from the ligand from an “in” position where it can coordinate the heme between outer and inner membranes. We have solved the first crystal structures of two such proteins, ShuT from *Shigella dysenteriae* and PhuT from *Pseudomonas aeruginosa*. Both share a common architecture typical of Class III periplasmic binding proteins. The heme binds in a narrow cleft between the N- and C-terminal binding domains and is coordinated by a Tyr residue. A comparison of the heme-free (apo) and -bound (holo) structures indicates little change in structure other than minor alterations in the heme pocket and movement of the Tyr heme ligand from an “in” position where it can coordinate the heme iron to an “out” orientation where it points away from the heme pocket. The detailed architecture of the heme pocket is quite different in ShuT and PhuT. Although Arg228 in PhuT H-bonds with a heme propionate, in ShuT a peptide loop partially takes up the space occupied by Arg228, and there is no Lys or Arg H-bonding with the heme propionates. A comparison of PhuT/ShuT with the vitamin B_{12}-binding protein BtuF and the hydroxamic-type siderophore-binding protein FhuD, the only two other structurally characterized Class III periplasmic binding proteins, demonstrates that PhuT/ShuT more closely resembles BtuF, which reflects the closer similarity in ligands, heme and B_{12}, compared with ligands for FhuD, a peptide siderophore.

Successful pathogenic bacteria must acquire nutrients from the host and one such essential nutrient is iron. Numerous Gram-negative bacterial pathogens have developed a sophisticated mechanism for recruiting host heme iron (1, 2). The first step involves a TonB-dependent cell surface receptor that acquires heme. Heme transport across the periplasmic space involves an active transport system, composed of a soluble periplasmic heme-binding protein, an inner membrane heme permease, and an ATPase. Upon crossing the inner membrane, the heme is broken down by heme oxygenase thus releasing iron in the cytosol.

Two of the better characterized heme transport systems in Gram-negative bacteria are from *Pseudomonas aeruginosa* and *Shigella dysenteriae*. *Shigella dysenteriae* is a Gram-negative, non-spore forming bacteria that causes deadly epidemics in many developing regions and nations. This type of bacteria typically resides in the human gastrointestinal tracts and causes an inflammatory disorder of the lower gastrointestinal tract (3).

*Pseudomonas aeruginosa* is widespread in nature-inhabiting soil, water, plants, animals, and humans and is an important cause of diseases, including pneumonia and urinary tract infections, especially in patients with compromised host defense mechanisms (4). Given that the heme transport proteins are unique to these bacteria, heme transport proteins provide potential targets for anti-bacterial agents.

Both *Pseudomonas* and *Shigella* encode a periplasmic binding protein (PBP) required to shuttle heme from the outer membrane to the inner membrane. In *Pseudomonas* PhuT encodes a 33-kDa protein, whereas the *Shigella* ShuT protein at 28.5 kDa is somewhat smaller. Both PhuT and ShuT are members of a broad family of PBPs that are important in maintaining selectivity and specificity of substrate transport (1, 5). PBPs consist of N- and C-terminal domains with a ligand binding cleft between domains. PBPs can be divided into three main classes. Class I and II are defined by having two or three interdomain connections, respectively. The maltose-binding protein belongs to Class I, and this group of PBPs undergoes a fairly large interdomain open/close motion required for substrate binding and release (6). Our crystal structures revealed that ShuT and PhuT belong to a third class that contains a single connecting helix between the two globular domains. The vitamin B_{12}-binding BtuF (5, 7) and FhuD (8), which binds iron-hydroxamic acid siderophores, are the only Class III PBPs whose crystal structures are known. Here we report the crystal structures of heme-free ShuT, partially heme-bound ShuT, and heme-bound PhuT. These structures provide further insights...
Structures of Periplasmic Heme-binding Proteins

MATERIALS AND METHODS

**ShuT Crystallization**—ShuT and PhuT bacterial transformations, sub-cloning, protein expression, and purification were carried out as previously described (1, 2). Crystals of ShuT belonging to the triclinic space group P1 with unit cell dimensions a = 54.82 Å, b = 73.28 Å, c = 72.38 Å, α = 71.44°, β = 77.8°, and γ = 89.9° were grown by hanging-drop vapor diffusion at room temperature over a reservoir of 0.1 M sodium citrate, pH 4.0, 0.05 M ammonium bromide, and 15% polyethylene glycol 4000. Hanging drops consisted of 2 μl of 15 mg/ml ShuT mixed with an equal volume of reservoir solution. Plate-like crystals with dimensions 600–400 μm by 20–40 μm thick grew within 5–10 days. These crystals tend to grow as large clusters. Although faint brownish in color, they were found to have no observable heme bound. To introduce heme for phasing, a second batch of ShuT crystals was grown in the presence of 1–5 mM hemin chloride (Aldrich), by hanging-drop vapor diffusion at room temperature. These crystals also belong to the triclinic P1 space group with unit cell dimensions a = 54.7 Å, b = 73.31 Å, c = 73.32 Å, α = 70.25°, β = 79.02°, and γ = 90.22°. Crystals with the addition of hemin chloride tend to grow larger and thicker with small satellites within 2 weeks or even as a single crystal, both appearing much darker in color. Based on the estimation of Matthew’s coefficient (9), there are four molecules per asymmetric unit. Cryoprotectant soaking for the first batch of ShuT crystals consisted of a six-step transfer to artificial precipitant solution (0.1 M sodium citrate, pH 4.0, 0.05 M ammonium bromide, and 15% polyethylene glycol 4000) with increasing concentrations of glycerol from 5% to 30%. For the second batch of ShuT crystals, the same precipitant plus increasing concentrations of glycerol from 5% to 30% were used.

**Data Collection for and Phasing Attempts for ShuT**—All data were collected using crystals flash cooled in liquid nitrogen. A high resolution data set (50–2.4 Å) was collected on Stanford Synchrotron Radiation Laboratory beamline 1-5 with a Q4 charge-coupled device detector. A MAD data set was collected at the Fe-K absorption edge, and a fluorescence scan was taken at the Fe-K absorption edge before data collection. Owing to the small size of PhuT crystals, only two x-ray wavelengths, inflection and remote, were used to prevent crystal decay from long x-ray exposures. Optimization of data collection was guided by the STRATEGY function of MOSFLM (10). Two sets of data were collected for two crystals. All data were reduced using DENZO and SCALEPACK, and rejections were performed with SCALEPACK. All data collection and process statistics are shown in Table 1.

**Phase Calculations, Model Building, and Refinement for PhuT**—Two MAD data sets at 3.14- and 2.8-Å resolution were collected using two wavelengths (inflection and remote) at the Fe-K edge of 1.74 Å. Phase information from both MAD data sets was calculated with HKL2MAP (11) and SOLVE (12). HKL2MAP gave a d*/σ value of 1.43 (0.77) for MAD data set 1 and a d*/σ value of 1.26 (0.96) for MAD data set 2. Values in parenthesis correspond to highest resolution shell. Both SHELXD and SOLVE found the same one iron site for two MAD data sets, which was in agreement with the predicted one molecule per asymmetric unit. The Z-score reported by SOLVE for the first MAD data set is 7.07 and for the second MAD data set is 5.31.

Electron density maps produced from either MAD data set alone with density modification using DM were quite noisy with no secondary structure elements clearly visible. SIGMAA (13, 14) was used to combine phases from the two MAD data sets. The combined phases were further improved by density modification using DM (13, 15) with solvent flattening or using SOLOMON (13, 16) with solvent flipping. The electron density maps showed clear features for some major α-helices and β-strands. However, the electron density map from SOLOMON was better showing clearer density for side chains, but it was still very noisy with no secondary structure elements clearly visible.
His tag used for purification. 158 water molecules, 2 sulfate molecules, and 2 glycerol molecules were also included in the final model with a crystallographic R-factor of 19.3% and the free R-factor of 24.7%. Refinement statistics are listed in Table 1.

**ShuT Structure Solution**—A high resolution data set of ShuT was collected at a resolution of 2.0 Å with an x-ray wavelength of 0.999 Å. Molecular replacement in the P1 space group was also carried out with PHASER (20) using PhuT as a search model. Good solutions were found when the full-length polyalanine PhuT was used as the search model. A log likelihood gain value of 3871 and a Z-score of 46.9 were obtained in Phaser for four solutions with minor packing clashes. The model phases were improved by running DM, including solvent flattening, histogram matching, and 4-fold non-crystallographic symmetry averaging. The final density map showed clear densities for side chains that were omitted in the search model.

Electron density map fitting beginning from the polyalanine PhuT model was carried out with the graphic modeling package O (18). Multiple rounds of model building and refinement with simulated annealing at 3000 K in CNS were carried out. Model building was on one chain only, and the models for the other three chains were generated by the non-crystallographic symmetry. Non-crystallographic symmetry restraints were imposed on main-chain atoms until the final stages of refinement. The starting R-factor was 0.420 (R-free = 0.477) and dropped to 0.356 (R-free = 0.414) after one round of anneal and temperature factor refinement. Model building and refinement continued until the final R-factor reduced to 0.21 (R-free = 0.255). The final structure of ShuT consists of 499 water molecules. Final data collection and refinement statistics are summarized in Table 2.
RESULTS AND DISCUSSION

Overall Structures of PhuT and ShuT—The overall fold of PhuT and ShuT share common structural features, having two topologically similar globular domains and a long, rigid α-helix as an interdomain linker (Fig. 1). These structures most closely resemble that of the Class III periplasmic binding protein, BtuF, a vitamin B12-transporting protein (5, 21), and FhuD, an Escherichia coli periplasmic protein that binds hydroxamate-type siderophores (8). Each domain consists of a 5-stranded β-sheet flanked by helices. Although almost all of the secondary structure elements described for the BtuF (5) are conserved in PhuT and ShuT, we do observe a few more small extra hel-
ices in the two new periplasmic binding protein structures. By adopting the nomenclature in BtuF (5), there are additional small helices, α-1'-(47–50) in the N-terminal domain and α-9'-(245–248) and α-9''-(250–254) in the C-terminal domain of PhuT. The r.m.s. deviation between BtuF and PhuT is 1.95 Å for 199 common Ca atoms, and the r.m.s. deviation between BtuF and ShuT is 1.8 Å for 197 Ca atoms, whereas the r.m.s. deviation between ShuT and PhuT for 232 common Ca atoms is 1.4 Å. FhuD is also a Class III PBP, and the r.m.s. deviation between FhuD and PhuT is 2.1 Å for 174 common Ca atoms, whereas the r.m.s. deviation between FhuD and ShuT is 1.9 Å for 165 common Ca atoms.

Heme Binding Cleft in PhuT and ShuT—Spectroscopic studies indicate that the heme is high spin pentacoordinate with the side-chain oxygen of a Tyr residue providing the one protein ligand (1, 2). The spectral properties of ShuT are very similar to catalases, which also use a tyrosinate ligand (22). The crystal structure is fully consistent with the spectral results. The heme is embedded in a cleft between the N- and C-terminal domains (Figs. 1 and 2) and is pentacoordinate with Tyr71 in PhuT from the C-terminal end of β-3 strand in the N-terminal domain providing the heme ligand. A characteristic feature of catalases shared by PhuT is an arginine in the proximal pocket, which H-bonds with the tyrosinate ligand. Arg73 serves this function in PhuT (Fig. 2). One difference is that in PhuT, but not in catalases, Arg73 also H-bonds to the heme propionate. In ShuT, the homolog to the PhuT Arg73 is Lys69. Lys69 is not near the ShuT heme ligand, Tyr67, so if Lys69 H-bonds to Tyr67 to mimic the Tyr71–Arg73 interaction in PhuT, there must be a reorientation of the Lys69 side chain. If, as it appears, ShuT lacks a good H-bonding partner to the Tyr ligand, this could help to explain why ShuT gives a higher Fe–O vibrational frequency obtained from resonance Raman studies (1). The O–Fe bond also is longer in PhuT, 2.3 Å compared with to 2.03 Å in beef liver catalase (2.5-Å resolution, pdb ID: 7CAT (22)) and 1.8 Å in Helicobacter pylori catalase (1.6 Å resolution, pdb ID: 1QWL (23)). The O–Fe–N bond angle ranges from 93 to 98° in PhuT and the two catalases, which is not very significant, but the longer bond distance is important. If ShuT is similar to PhuT, then the longer O–Fe bond is another reason for a higher vibrational frequency in ShuT. This also suggests that the heme is not as tightly held in place in ShuT/PhuT compared with catalases, which is functionally important because the role of ShuT/PhuT is to bind and release heme.

In PhuT the heme propionate groups are pointing outward toward the surface of the protein, and each is bent to the opposite sides of heme plane, respectively (Fig. 2). One propionate group is directly hydrogen bonded to Arg228 from the C-terminal domain. Note that Arg228 is stacked against the distal surface of heme.

There are four molecules present in each unit cell of ShuT. For ShuT crystals that were grown in the presence of hemin chloride, the electron density in molecule A shows partial density for heme (Fig. 2B), whereas molecules B, C, and D show no density for heme. Possible differences between the apo- and heme-bound structures can be derived by comparing the three molecules in the structure that do not have heme bound. In addition, we also solved the structure of ShuT that did not have hemin included in crystallization solutions and here, there are four molecules in the unit cell that are heme-free. The most obvious difference between the heme-free molecules and partially heme-bound molecules is in the orientation of the Tyr67 heme ligand. Tyr67 either points “in” toward the heme pocket where it is in position to coordinate the heme iron, or it flips out...
toward the surface (Fig. 3). For the partially heme-bound crys-
tals, Tyr\textsuperscript{67} points inward in molecule A, because the heme is partially bound, but also points inward in molecule D, even though there is no heme density. In molecules B and C Tyr\textsuperscript{67} points “out.” For the apo crystal, Tyr\textsuperscript{67} points inward in molecules A and B, but outward in C and D. Thus there is considerable flexibility in the orientation of Tyr\textsuperscript{67} in the absence of heme. There also is a slight movement of the loop centered on Gln\textsuperscript{88} toward the heme in the heme-bound molecule. There are, however, no noteworthy changes on the distal side of the heme. A similar Tyr reorientation has been observed in a periplasmic ferric binding protein and has been proposed to play a role in guiding iron into the site (24).

Comparison with BtuF and FhuD—Class one PBPs such as the maltose-binding protein undergo a large change in struc-

FIGURE 5. Comparison of PhuT (left), BtuF (center), and FhuD (right). These are molecular surface representations with the ligands shown as dark stick models. The FhuD cleft is more shallow than in either PhuT or BtuF.

FIGURE 6. Comparison of the binding cleft in PhuT and BtuF. A and B, the ligand binding in PhuT (A) and BtuF (B). A superimposition of the ligand binding pocket of PhuT and BtuF is shown in Panel C. The B12 ring in BtuF rotates 45° relative to the heme in PhuT. The proximal (lower) part of the binding clefts are very similar with the exception that the Tyr ligand in PhuT occupies the position of the dimethylbenzimidazole ligand in BtuF.
ture upon ligand binding as the domains close down around the ligand. The ligand-free and -bound structures of BtuF show that the change upon vitamin B$_{12}$ binding is modest, resulting in at most a 1-Å shrinking of the ligand binding pocket (5). Because the ShuT apo and partially heme-bound structures are in the same crystal form, it is possible that the crystal lattice prevents important structural changes required for heme binding. Even so, because ShuT/PhuT are structurally so similar to BtuF, it is doubtful that heme binding leads to major changes in structure other than a reorientation of the Tyr heme ligand and a slight movement of the loop centered on Gln$^{88}$ toward heme. However, the conformational changes in the heme binding pocket, such as the reorientation of the Tyr heme ligand, may play a role in the heme binding and/or release processes. By analogy with BtuF heme binding probably has no large effect on structure, then differences in the heme pocket between PhuT and ShuT are not the result of heme binding but rather a major difference in heme pocket architecture.

Fig. 5 provides a comparison of the binding cleft in PhuT, BtuF, and FhuD. Both PhuT and BtuF form well defined deep clefts, whereas FhuD forms a depression on the molecular surface. The less well defined pocket in FhuD may reflect a lack of selectivity in FhuD compared with PhuT and BtuF. In addition, the ligands for PhuT and BtuF are relatively flat aromatic groups with a large surface area that requires a deep cleft for binding. Another similarity is the positioning of the B$_{12}$ or heme relative to the “proximal” ligand. In BtuF B$_{12}$ binds in the “base-on” conformation with the N3B nitrogen of the dimethylbenzimidazole coordinated to the cobalt. In PhuT the heme ligand, Tyr$^{71}$, occupies approximately the same position. Therefore, the BtuF pocket is somewhat larger because, unlike heme where the protein provides a metal ligand, the B$_{12}$ substrate provides its own dimethylbenzimidazole ligand. A more detailed comparison of the pockets shows that the B$_{12}$ ring is rotated $\sim$45° relative to the heme in PhuT (Fig. 6). Even so, the major elements of secondary structure on the proximal ligand binding side of the substrate are conserved. For example, the loop centered on Trp$^{66}$ in BtuF corresponds to the loop containing the PhuT heme ligand, Tyr$^{71}$ (Fig. 6). The main difference is that this loop in BtuF is positioned closer to the ligand thus allowing Trp$^{66}$ to interact with the vitamin B$_{12}$ ring. However, like the comparison between ShuT and PhuT, the architecture on the opposite distal surface of the substrate is quite different. The main difference is centered on Arg$^{228}$ in PhuT, which approximates to Gly$^{216}$ in BtuF. PhuT has four extra residues in this loop that enables Arg$^{228}$ to dip into the distal cavity where it can interact with the heme propionate.

In summary, the ShuT and PhuT structures illustrate a very similar architecture on the proximal Tyr ligand binding side of the heme. This was not unexpected given the stereochemical restrictions in forming a Tyr-Fe coordination bond. The conservation of secondary structural elements on the ligand binding side extends to BtuF as well, although the proximal side elements of secondary structure must reposition relative to PhuT/ShuT to accommodate the B$_{12}$ dimethylbenzimidazole ligand. However, the substantial differences on the opposite distal side of the heme between ShuT and PhuT were unexpected. Such diversity could not be tolerated in heme enzymes where the distal pocket often provides essential catalytic groups that are evolutionarily conserved. However, the role of PhuT and ShuT is to bind and deliver heme and not to catalyze critical reactions, and thus, diversity in the heme pocket can be tolerated.

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