Crystal Structure of the Heme-IsdC Complex, the Central Conduit of the Isd Iron/Heme Uptake System in Staphylococcus aureus*§

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Pathogens such as Staphylococcus aureus require iron to survive and have evolved specialized proteins to steal heme from their host. IsdC is the central conduit of the Isd (iron-regulated surface determinant) multicomponent heme uptake machinery; staphylococcal cell-surface proteins such as IsdA, IsdB, and IsdH are thought to funnel their molecular cargo to IsdC, which then mediates the transfer of the iron-containing nutrient to the membrane translocation system IsdDEF. The structure of the heme-IsdC complex reveals a novel heme site within an immunoglobulin-like domain and sheds light on its binding mechanism. The folding topology is reminiscent of the architecture of cytochrome f, cellobiose dehydrogenase, and ethylbenzene dehydrogenase; in these three proteins, the heme is bound in an equivalent position, but interestingly, IsdC features a distinct binding pocket with the ligand located next to the hydrophobic core of the β-sandwich. The iron is coordinated with a tyrosine surrounded by several non-polar side chains that cluster into a tightly packed proximal side. On the other hand, the distal side is relatively exposed with a short helical peptide segment that acts as a lip clasp onto almost half of the porphyrin plane. This structural feature is argued to play a role in the mechanism of binding and release by switching to an open conformation and thus loosening the interactions holding the heme. The structure of the heme-IsdC complex provides a template for the understanding of other proteins, such as IsdA, IsdB, and IsdH, that contain the same heme-binding module as IsdC, known as the NEAT (near transporter) domain.

Iron is essential to living organisms for it is used, for instance, by cytochromes in cellular energy transduction processes. Bacterial pathogens require iron to infect cells and proliferate (1) and therefore rely on their host for acquisition of iron. Given the low availability of free iron in tissues and cells, microbial species have evolved specific receptors and carriers capable of high affinity binding to steal heme from host proteins. Most of the research carried out so far on iron/heme uptake systems in bacteria has focused on Gram-negative species. In addition to data from various biochemical experiments, HasA, a heme-siderophore secreted by the pathogen Serratia marcescens, was the first bacterial heme carrier protein to have had its structure elucidated in complex with heme (2). The heme uptake system HemRSTUV, widespread throughout Proteobacteria, has also been studied extensively (3–6). This multicomponent system includes an outer membrane receptor HemR, able to bind free heme or sequester it from host heme proteins, coupled with a periplasmic shutting carrier, HemT, which mediates the transfer to the HemUV complex, responsible for translocation of heme through the inner membrane and final passage to the cytoplasmic recipient HemS. In some cases, the HemR receptor is associated with an auxiliary protein, such as PhuW with PhuR in Pseudomonas aeruginosa or ChaN with ChaR in Campylobacter jejuni (7, 8) (PhuR and ChaR are homologous proteins to HemR; the nomenclature refers to the different species). Several genetic and biochemical investigations, predominantly in Yersinia enterocolitica and Shigella dysenteriae, have contributed to the understanding of this heme uptake system (3–6). Recent structural studies on two components, ChaN and HemS, have added insights into the molecular mechanisms involved in heme binding. The structure of the heme-ChaN complex revealed an unprecedented mode of heme binding with the loading of the ligand accompanied by dimerization of the protein (9). HemS was analyzed both in its apo form and in complex with heme; comparison of the two states unveiled a heme-induced fit conformational change with the N- and C-terminal domains closing onto the ligand (10).

A significant amount of work has also been done to understand the heme uptake process in Gram-positive bacteria, but no structural information is available yet on a carrier protein in complex with heme. In the “hospital superbug” Staphylococcus aureus, the Isd2 iron/heme uptake system (iron-regulated surface determinant) includes proteins for the sequestration, transport, and internalization of heme (reviewed in Refs. 11 and 12). It appears that more than one heme uptake pathway may exist in Staphylococcus, as suggested by the recent identification of the novel heme transport regulators HrtAB (13). In the

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2 The abbreviations used are: Isd, iron-regulated surface determinant system; NEAT, near transporter; GST, glutathione S-transferase; DLS, dynamic light scattering; MES, 4-morpholineethanesulfonic acid; MAD, multiwavelength anomalous dispersion.
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Isd system, the IsdA, IsdB, and IsdH components are attached to the cell wall and transfer their load to IsdC, which is the central conduit in this funnel-like uptake machinery. Passage through the membrane is then mediated by the IsdE/D/F complex, for final delivery to the cytosolic heme oxygenases IsdG and IsdI. IsdA, IsdB, and IsdH are being used to develop vaccines against *S. aureus* to reduce the occurrence of disease (14, 15). Based on sequence comparisons, IsdA, IsdB, IsdH, and IsdC are all believed to contain one or more copies of the same heme-binding structural module, known as the NEAT domain (16). Although biochemical and spectroscopic studies on some components of the system have been carried out (for example, Refs. 17–21), the structural basis of heme binding and the molecular mechanisms of heme transfer are still unexplored. Molecular data on the NEAT domain-containing proteins could also help in the design of better vaccines. Since IsdC is the central conduit of the Isd heme uptake system, we have studied its x-ray crystal structure in complex with heme.

**EXPERIMENTAL PROCEDURES**

**Cloning and Overexpression**—The gene encoding IsdC (National Center for Biotechnology Information number gi:13700931) was amplified from *S. aureus* genomic DNA (strain RN6390B) by PCR using the forward and reverse primers: IsdC forward, 5′-cgacctgtagcggatcgagctggactttcgc-3′, and IsdC reverse, 5′-ccgattaagcgtttacgttattcgc-3′. The codons for the signal peptide and cell wall-anchoring motifs were omitted. The PCR-amplified product was cloned into the T7-based expression vector pGAT2 (22) and then transformed into *Escherichia coli* BL21-DE3 cells (Novagen). An overnight culture of 5 ml of Luria Bertani medium was used to inoculate 0.5 liters of YT broth supplemented with carbenicillin (Melford) to a final concentration of 50 μg/ml. Cultures were shaken in 2-liter baffled flasks at 220 rpm at 37 °C until they reached an absorbance at 600 nm of 0.6–1.0; protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM, and cultures were then transferred at 30 °C and further grown overnight. Cells were harvested by centrifugation at 4600 rpm for 30 min and stored frozen at −20 °C. Once resuspended in a buffer containing 50 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM imidazole, cells were lysed by sonication, and the lysate was centrifuged twice at 15,000 rpm for 30 min.

**Purification**—Protein purification was carried out, taking advantage of the double tag (His6 and GST) provided by the expression vector, first by metal affinity chromatography (nickel-nitriilotriacetic acid, Qiagen) and then by glutathione-Sepharose affinity chromatography (Amersham Biosciences). The His-GST tag was removed by proteolytic cleavage with thrombin (10 units/mg of protein, Cambridge Biosciences) at room temperature overnight. Thrombin was removed by incubation with *p*-amino-benzamide-agarose (Sigma) (2 μl/unit of thrombin); cleaved His-GST tags and uncleaved protein were separated from the cleaved IsdC preparation by means of a further glutathione-Sepharose affinity chromatography step. IsdC was reconstituted with its heme ligand by incubating it with a 2-fold molar excess of hemin chloride (Sigma) at 4 °C for 30 min before removing excess hemin by gel filtration.

**Size-exclusion Chromatography**—Analytical gel filtration was carried out using a Superdex 75 column (Amersham Biosciences) coupled to a fast performance liquid chromatography system (ÅKTA). The column was equilibrated with 500 mM NaCl, 50 mM Tris-HCl, pH 8. A sample volume of 1 ml at a concentration of 3 mg/ml was injected into the column and run at a flow rate of 0.5 ml/min. Protein elution was followed by absorption measurements at 280 nm and also at the Soret absorbance peak of 400 nm. The column was calibrated with an Amersham Biosciences Biotechnologies low molecular weight gel filtration kit. IsdC elutes with a volume equivalent to a molecule of about 30,000 Da. However, the calculated molecular mass of IsdC is 17,500 Da, thus making it plausible that IsdC may be a dimer. Given the long molecular shape of IsdC and that changing conditions such as concentration of protein and salts made no difference to any peaks in terms of a possible monomer-dimer equilibrium, we believe that IsdC is not likely to be a dimer, exactly as observed in the case of gel filtration work on IsdA (21). Therefore, further analysis was carried out using dynamic light scattering (DLS).

**Dynamic Light Scattering**—Experiments were carried out at room temperature in a 1-ml quartz cuvette (path length 1 cm) containing IsdC at 0.5 mg/ml in phosphate-buffered saline buffer. Prior to the DLS study, protein samples were passed through a 0.2-μm filter and centrifuged at 14,000 rpm for 10 min. Data were collected with a Viscotek model 802 DLS instrument. Measurements were programmed using the software OmniSIZE 2.0 (Viscotek Europe Ltd.) such that each experiment was averaged over 10 runs, each for 20 s. The results were processed with the OmniSIZE software. Results from five experiments give a radius of 47.3 nm (± 2.5 nm). The monomer has a maximal width of 50 nm as measured from the crystal structure, and the dimer has a width of 80 nm. The DLS data therefore indicate that IsdC is a monomer in the conditions tested.

**Crystallization**—Crystals were obtained using the sitting drop vapor diffusion method with samples of IsdC at 95 mg/ml in 50 mM Tris-HCl, pH 8, 500 mM NaCl mixed in equal volumes (2 + 2 μl) with reservoir solution containing 26% polyethylene glycol 550 mono-methyl-ether, 18 mM zinc sulfate, and 0.1 mM MES, pH 6.0. IsdC crystals typically grew to a size of 40 × 60 × 130 μm after 2 weeks. Before flash-freezing in liquid nitrogen, crystals were soaked in 27.5% polyethylene glycol 550 mono-methyl-ether, 19 mM zinc sulfate, and 0.1 mM MES, pH 6.0, for cryo-protection.

**Data Collection and Phasing**—Diffraction data were collected at liquid nitrogen temperature at the beamlines ID14 and ID29, European Synchrotron Radiation Facility (ESRF) (Grenoble, France), and processed with the programs MOSFLM (23) and SCALA (24) of the CCP4 suite (25). IsdC crystals belong to the orthorhombic space group P212121 and contained a molecule of about 30,000 Da. The iron peak and inflection at the iron resonance edge, and data sets were collected at...
1.739 (peak), 1.741 (inflection), and 1.722 Å (remote). The heme iron was located with SHELXD (26), and phase calculations were carried out with SHARP/autoSHARP (27). A high resolution native data set was collected at 0.933 Å. Diffraction data statistics are reported in supplemental Table 1 online.

Model Building and Refinement—The model was built manually using COOT (28) and then refined against the high resolution native data set using restrained and Translation-Libration-Skew (TLS) tensors refinement in REFMAC5 (29). The optimal number of TLS groups was determined using the TLSMD server (30). The final model consists of residues 28–150 for chain A and residues 28–146 for chain B and has excellent geometry and no Ramachandran outliers. The construct used for the overexpression of IsdC also includes residues 151–188, but these are not part of the model because only weak, broken peaks of electron density were obtained for this region, and the maps could not be interpreted. Visual inspection of the sequence reveals that this stretch of polypeptide chain is almost exclusively made up of small, polar, and charged amino acids, consistent with this region forming random coil, disordered structure. Data processing and model refinement statistics are summarized in Table 1. Surface area accessibility calculations were carried out using the program AREAIMOL (31). All structural figures were prepared using PyMOL (DeLano Scientific).

Homology Modeling—The sequences of IsdA (residues 65–184) and IsdC (residues 30–150) were aligned using CLUSTAL W (32). Based on this alignment and on the structural data of the heme-IsdC complex, a model of IsdA was constructed using MODELLER (33). The best generated models were energy-minimized and then evaluated in PROCHECK (34). Further docking studies were performed using GOLD 2.0 (Genetic Optimization for Ligand Docking) (35). Coordinates for heme were subjected to docking calculations using the best IsdA model with the docking site defined as the three-dimensional space within 20 Å of His-83. Docking calculations were performed with default settings and with atom types as defined in INSIGHTII (Accelrys Inc.). The best scoring 10 results were viewed in SILVER (35), and the two most common conformations are shown in supplemental Fig. 3.

RESULTS AND DISCUSSION

The structure of the heme-IsdC complex was determined using phases from Fe-MAD experiments, and atomic coordinates were refined against data to 1.5 Å resolution (Table 1 and Fig. 1). Two molecules are present in the asymmetric unit, but their conformations do not differ significantly (1 Å root mean square deviation for main chain atoms and 1.4 Å for all atoms). Despite the association between the molecules in the lattice, IsdC is a monomer as determined by gel filtration and dynamic light scattering. Over 35 amino acids are missing from the model (see “Experimental Procedures”), which amounts to almost 20% of the scattering matter; this corresponds to a highly disordered C-terminal tail presumably important to provide IsdC with a flexible foot extending from the cell wall matrix.

The overall structure resembles the architecture previously unveiled by the NMR work on the apo N-terminal NEAT domain of IsdH, termed IsdHNE (20), although the two proteins share only 16% of their amino acid sequence. Comparison of the IsdC and IsdH structures by least-squares fitting of 50 equivalent Cα atoms gives a root mean square deviation of about 2 Å (see structure-based sequence alignment in supplemental Fig.
Therefore, it can now be accepted that all Isd proteins distantly related by the NEAT signature indeed share the same structural module. The fold is characterized by eight elements of extended secondary structure, split between a three-stranded sheet and a four-stranded sheet that pack into a twisted antiparallel β-sandwich connected on one side by an eighth strand. This folding topology can be described as an immunoglobulin-like fold, also seen in three other heme proteins: cellobiose dehydrogenase (36), ethylbenzene dehydrogenase (37), and cytochrome f (38) (Fig. 2 and supplemental Fig. 2). In these three proteins, the heme is located in a topologically equivalent position, packing against the face of one of the β-sheets and embraced by additional elements of secondary structure as well as substantial loop regions. By contrast, IsdC has a simpler architecture with a distinct heme site, formed at the edge of the hydrophobic core between the two sheets (Fig. 2). A prominent feature of this heme-binding region is a long β-hairpin-like structure that protrudes from one of the sheets, providing a platform onto which the ligand rests (Fig. 3). From this side, proximal to the heme, Tyr-132 reaches the five-coordinate iron, which is displaced 0.5 Å from the plane of the pyrrole nitrogens. On the distal side, a short stretch of 4–5 amino acids (48–52) in a 310-helical conformation acts as a lip that clasps onto one side of tetrapyrrole ring (Fig. 3A) extending across almost half of the porphyrin plane (Fig. 3B). Ile-48 is placed more or less centrally over the iron and pairs up with Tyr-52 that stacks, offset, over one of the pyrrole rings. More extensive interactions occur at the proximal side and by the edge of the heme. Tyr-136 is engaged with the proximal tyrosine through a hydrogen bond, which may help to stabilize the coordinate bonding to the iron. In addition, Tyr-136 is coplanar with one of the pyroles, approximately opposite Ile-48 (Fig. 3A). Other proximal residues include Val-119 and Ile-121 that cluster with the two tyrosines and offer further hydrophobic surfaces to the binding pocket.

A key set of contacts is provided by Trp-77, Ile-78, and Ile-117, which pack along two sides of the tetrapyrole; Ile-117 and Trp-77 in particular make van der Waals contacts with the vinyl

FIGURE 1. Electron density of the heme in the heme-IsdC complex. Shown is a stereo view of the electron density omit map displayed at 1σ level calculated with the coordinates of the heme-IsdC complex, missing the atoms of heme and its neighboring residues, at 1.5 Å resolution. Two key amino acids are also shown: the proximal Tyr-132, coordinating the iron, and the distal Ile-48, positioned over the porphyrin.

FIGURE 2. Comparison of heme proteins with an immunoglobulin-like fold. A, IsdC; B, cellobiose dehydrogenase (1D7B) (36); C, ethylbenzene dehydrogenase (2IVF) (37); D, cytochrome f (1CFM) (38). The heme appears to bind to a topologically equivalent site in the three enzymatic heme proteins, whereas IsdC features a novel binding pocket.
and methyl groups of the porphyrin, interlocking the heme into its pocket. The interactions associated with Trp-77 (Fig. 4) are believed to be unique to IsdC because the other staphylococcal NEAT domains (20) all lack this tryptophan but instead have a conserved tryptophan at a position adjacent to Trp-77. A structural superposition of IsdC and IsdHN1 shows that the conserved tryptophan would be unlikely to contribute the same contacts of high steric complementarity as Trp-77. It is therefore possible that the heme in IsdHNN and other NEAT domains binds in a slightly different orientation. It is also tempting to speculate that Trp-77 is responsible for the stability of the heme-IsdC complex since IsdC is thought to receive its cargo from the other NEAT domains.

In the heme-IsdC complex, the ligand is not completely buried into the protein and remains distinctively exposed to the solvent (Fig. 3B). Despite this exposure, the site above the center of the porphyrin is occupied by Ile-48, which is less than 4 Å away from the iron. The distal isoleucine therefore appears to sterically hinder the sixth coordination position of the iron, providing an explanation for our spectroscopic observations in which no binding of cyanide or azide could be detected even after prolonged incubations of protein samples with high concentrations of these strong-field ligands. Modeling of a cyanide molecule bound to the heme in IsdC shows that C9 of the distal isoleucine would only be 1.8 and 2.5 Å away from the N and C atoms, respectively. Interestingly, a similar observation was made in spectroscopy experiments on IsdA in which cyanide did not bind to the heme-loaded IsdA (21). Given that the binding of cyanide is so effectively prevented, it appears that the interactions of both Ile-48 and Tyr-52 are strong enough to maintain a stable molecular “seal” across the surface of the heme. Different rotameric conformations do not appear to be sufficient to relieve steric hindrance, and a more substantial displacement involving backbone atoms may be required to “peel” the 310-helical lip off the top of the porphyrin.

The heme protein contact area is limited (380 Å²) relative to other heme transport proteins such as HemS (480 Å²) (10), HasA (468 Å²) (2), and hemopexin (479 Å²) (39). Accordingly, the heme is less buried into the protein, with 34% of the heme surface remaining accessible to the solvent in IsdC, as opposed to 19% in HemS and 25% in both HasA and hemopexin. The trend is that in comparison with catalytic heme proteins, the ligand remains significantly exposed to the solvent in heme carriers (Table 2). This is likely to be related to their function, which is to unload the cargo just as much as to sequester it. As
part of the mechanism of binding and release, we envisage that a conformational change, limited to the heme pocket, is probable, with local shifts of side chains, and likely to involve a structural rearrangement of the $3_{10}$-helical lip (residues 48–52), distal to the heme. In the apo structure of IsdH$^{N1}$ (20), the NMR data indicate that the peptide segment corresponding to this structural feature is the most flexible and mobile part of the molecule. It is therefore conceivable that the helical lip plays a critical role in the association and dissociation of heme, whereby the tight $3_{10}$-helix unwinds, thus switching from a fixed, locked conformation in the ligand-loaded state to an open, more mobile structure in the unbound state. This mechanism may well be a universal feature of all NEAT domains since IsdC, IsdA, IsdB$^{N2}$, and IsdH$^{N3}$ all have very similar sequences in the lip region and, despite the weaker conservation, a relationship can also be recognized in IsdB$^{N1}$, IsdH$^{N1}$, and IsdH$^{N2}$.

Homology modeling, based on the heme-IsdC structure, was used to evaluate how the NEAT domain of IsdA binds heme (see “Experimental Procedures” and supplemental Fig. 1B); although it appears that the two proximal tyrosine residues are conserved, changes in the pocket of IsdA suggest a different binding orientation for the ligand. The model also shows that in IsdA, a histidine replaces Ile-48 of IsdC, and it is thus positioned above the heme (supplemental Fig. 3), in line with spectroscopic measurements in which it was observed that upon reduction of the iron, there is a swap from a coordinating tyrosine to a histidine (21). The model also shows that the histidine is unlikely to relieve the hindrance on the sixth coordination site of the iron unless significant main chain rearrangements take place, and this explains the observations made on the lack of cyanide binding (21). The UV-visible spectrum of IsdC (supplemental Fig. 4), with absorption maxima at 400, 500, 535, and 630 nm, is similar to that of IsdA (21), which shows major peaks at 406, 505, 540, and 627 nm. This suggests that the two proteins share an equivalent iron coordination environment. The absorption maxima observed for IsdC and IsdA are also common to native UV-visible spectra of bovine liver catalase (40) and coral allene oxide synthase (41). These data, together with measurements using other spectroscopic techniques (i.e. magnetic circular dichroism and EPR), indicated that in these proteins, the iron is liganded by a tyrosine and has a high spin, five-coordinate geometry. This is in agreement with both the crystal structure of IsdC and the homology model of IsdA.

Since ligand loading and unloading, as well as ligand transfer, are the main molecular tasks of Isd-NEAT proteins, correlating structural with functional features should provide insights into the passage of heme from cell surface proteins to IsdC in Staphylococcus as well as other species with IsdC homologues (supplemental Fig. 1C) or with molecules containing NEAT domains. The structure of the heme-IsdC complex offers a template to help in the understanding of these novel heme-binding modules.

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Addendum—It is noted that at the time of review of this manuscript, the structure determination of the NEAT domain of IsdA in complex with heme was reported (42). Our prediction of the binding of IsdA is in line with the heme protein association revealed by this new structure.

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