Mapping Ultra-weak Protein-Protein Interactions between Heme Transporters of Staphylococcus aureus

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Background: Isd proteins convey heme molecules across the bacterial cell wall by means of sequential and transient protein-protein complexes.

Results: Photo-cross-linking experiments revealed the contact regions between the IsdC transporter and other Isd proteins.

Conclusion: Transient interactions are governed by distinct structural elements around the heme-binding pocket of IsdC.

Significance: Targeting this epitope could lead to successful therapeutic strategies against Staphylococcus aureus.

Iron is an essential nutrient for the proliferation of Staphylococcus aureus during bacterial infections. The iron-regulated surface determinant (Isd) system of S. aureus transports and metabolizes iron porphyrin (heme) captured from the host organism. Transportation of heme across the thick cell wall of this bacterium requires multiple relay points. The mechanism by which heme is physically transferred between Isd transporters is largely unknown because of the transient nature of the interactions involved. Herein, we show that the IsdC transporter not only passes heme ligand to another class of Isd transporter, as previously known, but can also perform self-transfer reactions. IsdA shows a similar ability. A genetically encoded photoreactive probe was used to survey the regions of IsdC involved in self-dimerization. We propose an updated model that explicitly considers self-transfer reactions to explain heme delivery across the cell wall. An analogous photo-cross-linking strategy was employed to map transient interactions between IsdC and IsdE transporters. These experiments identified a key structural element involved in the rapid and specific transfer of heme from IsdC to IsdE. The resulting structural model was validated with a chimeric version of the homologous transporter IsdA. Overall, our results show that the ultra-weak interactions between Isd transporters are governed by bona fide protein structural motifs.

Staphylococcus aureus is a bacterium that causes life-threatening infections (1–4). In recent years, the number of strains of S. aureus resistant to antibiotics of last resort ("superbugs") has increased at an alarming pace (5–7). It is critical to develop new drugs to reduce the risks posed by this bacterium to public health.

The so-called Isd (iron-regulated surface determinant) system is an attractive target to battle S. aureus. Inactivation of the Isd system would interfere with the uptake of nutritional iron necessary for the proliferation of this bacterium during infection (8–10). The Isd system comprises 12 proteins that seques-ter heme molecules from the host organism to extract the iron atom contained in it (supplemental Fig. S1) (11–13).

Iron acquisition is initiated with the capture of heme from hemoglobin by the extracellular receptors IsdH and IsdB. Heme molecules are subsequently transferred to the intermediate transporters IsdA and IsdC, both of which are anchored to the cell wall via specific sorting motifs (11, 14). IsdA and IsdC must relay their cargo throughout the thick cell wall of the bacterium to membrane-anchored IsdE. IsdE, together with IsdF (and possibly other proteins), catalyzes the passage of heme across the plasma membrane. Finally, the cytoplasmic enzymes IsdG and IsdI release the iron atom from the porphyrin ring.

Structural studies have shown that the IsdA and IsdC transporters, as well as the C-terminal domains of IsdH and IsdB, share a common heme-binding domain known as NEAT (near transporter) (supplemental Movie 1) (15–21). This domain is characterized by a rather hydrophobic pocket and displays a conserved tyrosine residue that coordinates the metal atom of the porphyrin ring. NEAT domains have been identified also in other bacterial pathogens such as Bacillus anthracis and Streptococcus pyogenes, thus expanding the therapeutic potential of this class of proteins (22, 23).

On the other hand, the heme transporter IsdE does not possess a NEAT domain (24). Instead, IsdE belongs to class III of the periplasmic heme-binding proteins, which are characterized by a distinctive bilobular architecture (25, 26). The crystal structure of IsdE with heme bound shows that the iron atom is coordinated by the axial ligands His-229 and Met-78 of the protein, although only His-229 is essential for heme uptake in vivo (24).

A key aspect of heme relay and transport in the Isd system involves the recognition mechanism between NEAT domains. A recent NMR study has proposed that heme is transferred from IsdA to IsdC via an ultra-weak affinity “handclasp” complex (27). The term ultra-weak affinity indicates transient interactions with binding constants in the millimolar range (27). According to the handclasp model, NEAT domains juxtapose their $\beta_10$-helices and $\beta_7/\beta_8$ strands during heme transfer. This
MAP 1. Summary of the current model for heme relay between IsdA and IsdC. The scheme illustrates the proposed reaction pathway leading to the delivery of heme from IsdA to IsdC. The heme relay process is initiated when heme is transferred from IsdA to IsdC. Subsequently, heme is transferred from IsdC to IsdE. Both of these heme transfers are slow and may be rate-limiting steps in the overall heme relay process. The model also predicts that the transfer of heme from IsdA to IsdC is faster than the transfer of heme from IsdC to IsdE.

Figure 1. Experimental procedures: (A) Biochemical analysis of IsdA and IsdC. SDS-PAGE analysis of IsdA and IsdC. (B) Photo-cross-linking assay. The photo-cross-linking assay was used to determine the molecular weight of the IsdA and IsdC complexes. (C) Immunoblot analysis of IsdA and IsdC. Immunoblot analysis of IsdA and IsdC using anti-IsdA and anti-IsdC antibodies. (D) Heme transfer assay. The heme transfer assay was used to determine the efficiency of heme transfer between IsdA and IsdC.

Figure 2. Kinetic analysis of heme transfer between IsdA and IsdC. The kinetic analysis of heme transfer between IsdA and IsdC was performed using a stopped-flow apparatus. The rate of heme transfer was measured by following the change in absorbance at 405 nm. The data were fitted to a single exponential function to obtain the rate constant.

Table 1. List of Materials used in the experiments. The materials used in the experiments are listed in the table. The materials include proteins, buffers, and reagents.

Table 2. Summary of the reaction conditions used in the experiments. The reaction conditions used in the experiments are summarized in the table. The conditions include temperature, pH, and buffer concentration.

Table 3. Results of the experiments. The results of the experiments are presented in the table. The results include the measured rates and efficiencies of heme transfer.

Table 4. Kinetic parameters of heme transfer. The kinetic parameters of heme transfer are presented in the table. The parameters include the rate constant, the dissociation constant, and the association constant.

4 The abbreviations used are: pBPA (X), p-benzoylphenylalanine; IMAC, immobilized metal ion affinity chromatography.
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RESULTS

Self-transfer Reactions—We examined the possibility of self-transfer reactions in IsdC (Fig. 1). Exchange of heme between untagged IsdC loaded with heme (holo-form) and the His_{6}tagged form of IsdC without ligand bound (apo-form) was monitored by UV-visible spectroscopy (Fig. 1A).

The UV-visible spectrum of holo-IsdC was characterized by two absorption peaks centered at 280 and 404 nm (Soret band) that corresponded to protein and heme, respectively (Fig. 1B). As expected, His_{6}-apo-IsdC did not exhibit the characteristic Soret band of heme (Fig. 1C). Incubation of holoprotein with apoprotein for 10 min, followed by a short separation by IMAC, led to a reduction in the amount of heme attached to holo-IsdC. In relative terms, the A_{Soret}/A_{280} ratio decreased from 2.1 to 1.3. A control experiment showed no spontaneous discharge of heme during the IMAC separation step (supplemental Fig. S2).

The heme molecules lost from holo-IsdC were therefore transferred to His_{6}-apo-IsdC (Fig. 1C). After the incubation step, a strong Soret band (not present before incubation) appeared in samples of His_{6}-apo-IsdC. The A_{Soret}/A_{280} ratio increased from 0.07 to 0.73. The fraction of heme transferred to the apo-form during the 10-min incubation step was higher than that caused by dissociative transport (29, 38–40). These results are consistent with a heme transfer mechanism involving activated protein complexes.

Similarly, incubation of holo-IsdA with His_{6}-apo-IsdA was conducive to a net transfer of heme from its holo- to its apo-form (Fig. 1, D and E). The A_{Soret}/A_{280} ratio in samples of holo-IsdA decreased from 2.3 to 0.84, whereas that in samples of apo-IsdA increased to 0.47. Overall, these two experiments demonstrated self-transfer reactions in IsdC and IsdA transporters.

Detection of IsdC-IsdC Complexes—IsdC modified with the photoreactive residue pBPA was expressed in E. coli cells. This non-natural residue reacts with C–H bonds of proteins (except vinylic or aromatic C–H bonds) under UV illumination conditions, forming carbon–carbon covalent bonds (supplemental Fig. S3) (41–43).

Transient IsdC-IsdC interactions were monitored by immunoblotting following a period of 30-min irradiation with UV light at 365 nm (Fig. 2). In these experiments, it was assumed that the appearance of cross-linked dimers demonstrates proximity between pBPA and a neighboring residue of the partner protein (41, 42, 44). It follows that the mutated residue should be located in the interaction site of the unmodified (wild-type) version of the protein (35, 45–49).

UV irradiation of mutein I48X led to the appearance of a weak band at ~45 kDa corresponding to the photo-cross-linked homodimer (Fig. 2A). This band was absent in samples not irradiated. The intensity of this band was significantly lower than that of the monomeric form, suggesting a low photocross-linking yield. This is a consequence of the transient nature of IsdC-IsdC encounter complexes.

We note that this experiment was carried out with the apo-form of IsdC. The presence of heme during the irradiation step considerably degraded the samples, rendering them unsuitable for analysis by Western blotting (data not shown). The use of the apo-form of IsdC as a surrogate of the holo-form in the photo-cross-linking assay assumes that the structure of the apoprotein is largely maintained in the heme-free form. This proposition is supported by a previous NMR study concluding that the structure of apo-IsdC in solution is largely preserved (17). Similar observations were reported in the crystal structures of the NEAT transporters IsdH and IsdA in their holo- and apo-forms (16, 18).

The interaction surface of the IsdC-IsdC dimer was mapped with 17 single muteins of pBPA at positions 40, 48, 52, 60, 71, 77, 82, 87, 96, 112, 116, 120, 124, 128, 130, 135, and 141 (Table 1). These mutations were selected with the PISA server (50),

where $A$ is absorbance, $k_{obs}$ is the observed rate constant, and $t$ is time.

Preparation of Samples for MS/MS—Photo-cross-linked dimers composed of mutein F124X (where X denotes a different residue at position 124) were separated by SDS-PAGE. The band containing the photo-cross-linked product was digested overnight with trypsin (Promega) at 37 °C. Peptides were desalted with ZipTip C18 microcolumns (Millipore) and concentrated to 20 μl prior to analysis by nano-LC-MS/MS.

Characterization by MS/MS—Peptide analyses were performed using a linear ion trap/orbitrap mass spectrometer (LTQ Velos Orbitrap, Thermo Scientific) coupled to a nano-flow DiNa-2A LC system (KYA TECH Corp., Tokyo, Japan). Peptides were injected into a 75-μm reversed-phase C18 column at a flow rate of 5 μl/min and eluted with a linear gradient of 98% solvent A (2% acetonitrile and 0.1% formic acid in H₂O) to 50% solvent B (80% acetonitrile and 0.1% formic acid in H₂O) at 300 nl/min. The separated peptides were sequentially sprayed from a nanoelectrospray ion source (KYA TECH Corp.) and analyzed by collision-induced dissociation, which automatically between MS and MS/MS acquisition. Full-scan mass spectra, containing a target value of 500,000. The 20 most intense ions at a threshold above 2000 were fragmented in the orbitrap at a resolution of 7500 at m/z 400. The orbitrap analyzer was operated with the “lock mass” option to perform highly accurate detection (36).

Analysis of MS Data—Identification of photo-cross-linked peptides was performed as reported (37). Briefly, we built a database with virtual protein sequences in which the pBPA-containing peptide derived from IsdC was concatenated with peptides of IsdE digested in silico. We included the NCBI Reference Sequence human protein database for internal consistency. Peptide identification was performed with Mascot Version 2.3.02 (Matrix Science, Tokyo, Japan).
which identified solvent-exposed residues in the crystal structure of IsdC (15).

Overall, photo-cross-linked products were observed in eight different muteins (Fig. 2B and Table 1). The intensity of the photo-cross-linked bands varied among the eight dimerizing muteins, which suggested an additional level of specificity during IsdC self-interaction. Importantly, all of the mutations producing dimers were located in the vicinity of the heme-binding pocket (Fig. 2C). For example, positions 48 and 52 belong to the 310-helix, whereas Lys-116, Lys-124, Lys-128, and Tyr-120 belong to the strand. Mutations far from the heme-binding pocket did not yield the photo-cross-linked dimer (Fig. 2C).

Detection of Transient IsdC-IsdE Complex—Analogous photo-cross-linking experiments were carried out to examine the transient interactions between IsdC and IsdE (27). Irradiation of apo-IsdC mutein F130X with wild-type apo-IsdE produced a new band with an apparent molecular mass of 55 kDa (Fig. 3). The position of the band was consistent with the molecular mass of the IsdC-IsdE dimer (≈10 kDa heavier than the IsdC-IsdC self-dimer shown in Fig. 2A). This observation was recapitulated with the IsdE mutein Y115X, with replacement of a residue farther from the heme-binding site, did not result in a photo-cross-linked product (Fig. 3B).

We carried out a more complete mapping of the interaction surface of IsdC with 17 amber mutants (Fig. 3C and Table 1). Eight single muteins containing pBPA at positions 52, 77, 96, 120, 124, 128, 130, and 135 gave rise to the characteristic band of the photo-cross-linked dimer. In contrast, we could not detect dimerization in muteins at positions 40, 48, 60, 71, 82, 87, 112, 116, and 141. When mapped on the crystal structure of IsdC, the mutated residues were closely clustered around the heme-binding pocket (Fig. 3D and supplemental Fig. S5). Compared with the self-dimerization of IsdC (Fig. 2C), the transient dimerization between IsdC and IsdE showed a more obvious segregation of the residues involved in the interactions.

The SDS-PAGE band of the photo-cross-linked dimer obtained by mixing IsdC mutein K124X and wild-type IsdE was digested in-gel with trypsin protease and analyzed by nano-LC-MS/MS. A search of photo-cross-linked peptides in the mass spectra with the program Mascot (37) revealed two peaks consistent with a photo-cross-linked peptide (supplemental Fig. S6). These two peaks were absent in samples not irradiated with UV light (supplemental Fig. S6). Although the relative abun-
dance of these peaks was small, we could confirm their identity by collision-induced dissociation (supplemental Fig. S7). The sequence of this peptide comprised residues 117–128 of IsdC (except for pBPA at position 124) and residues 50–62 of IsdE (supplemental Table S2).

Role of $\beta_{7}/\beta_{8}$ Strand of IsdC in Heme Transfer to IsdE—The above results suggested that the $\beta_{7}/\beta_{8}$ strand of IsdC is essential for self-association and transient interaction with IsdE. Interestingly, the $\beta_{7}/\beta_{8}$ strand of IsdC is six residues longer than that of IsdA (Fig. 4, A and B). Because IsdA cannot transfer heme efficiently to IsdE (29, 30), we examined the hypothesis that the length and identity of this strand are critical factors affecting the transfer of heme to IsdE. Thus, the sequence of the $\beta_{7}/\beta_{8}$ strand of IsdC was grafted into IsdA by site-directed mutagenesis. We called this construct IsdA long loop.

Heme transfer assays showed a dramatic increase in the ability of IsdA long loop to relay heme molecules to IsdE compared with wild-type IsdA (Fig. 4, C and D). When incubated with apo-IsdE, the $\lambda_{max}$ of the Soret band shifted from 404 to 412 nm in samples of holo-IsdA long loop but not in samples of wild-type holo-IsdA (Fig. 4, C and D). In fact, after a 60-min treat-

### TABLE 1
Summary of photo-cross-linking experiments

| Mutation | Secondary structure element | Cross-linking intensity | IsdC-IsdC | IsdC-IsdE |
|----------|-----------------------------|------------------------|-----------|-----------|
| K40X     | $\beta_1$                   | -                      | -         | -         |
| I48X     | $\beta_1$-$\beta_2$-Helix   | +                      | +         | -         |
| Y52X     | $\beta_2$-$\beta_3$-Helix   | +                      | +         | -         |
| I60X     | $\beta_3$                   | -                      | -         | -         |
| T71X     | $\beta_4$                   | -                      | -         | -         |
| W77X     | $\beta_3/\beta_4$ loop     | +                      | +         | -         |
| S82X     | $\beta_4$                   | -                      | -         | -         |
| K87X     | $\beta_5$                   | -                      | -         | -         |
| A96X     | $\beta_5/\beta_6$ loop     | ++                     | +         | -         |
| K112X    | $\beta_7$                   | +                      | +         | -         |
| K116X    | $\beta_7$                   | -                      | -         | -         |
| Y120X    | $\beta_7$                   | -                      | -         | -         |
| K124X    | $\beta_7/\beta_8$ loop     | +                      | +         | -         |
| K128X    | $\beta_7/\beta_8$ loop     | +                      | +         | -         |
| F130X    | $\beta_8$                   | -                      | -         | -         |
| H135X    | $\beta_8$                   | -                      | -         | -         |
| K141X    | $\beta_8$                   | -                      | -         | -         |

* The letter X indicates photoreactive pBPA. This residue is encoded by the amber codon in the pEVOL system used to produce pBPA-labeled protein (34).

* Intensity was qualitatively evaluated based on visual inspection of the photo-cross-linked bands in the Western blot. --, no reaction; +, visible band; ++, intense band.

### FIGURE 2.
**Self-dimerization of IsdC detected by photo-cross-linking.** A, identification of the photo-cross-linked self-dimer of IsdC mutein I48X by immunoblotting with an anti-His probe. Samples were irradiated with UV light (365 nm) for 30 min. The arrow indicates the position of the photo-cross-linked product in the Western blot. B, interaction analysis with 17 different muteins. Only the 40–50-kDa region of the Western blot is shown. C, interaction map. Mutated residues leading to photo-cross-linked products are depicted in green. Residues in orange indicate no dimerization reaction. Heme is shown in dark blue. The magenta sphere corresponds to iron. The majority of residues involved in dimerization are clustered around the distal $\beta_{3}$-helix and the $\beta_{7}/\beta_{8}$ strand. Molecular graphics images were produced with the UCSF Chimera package (54) using the coordinates of holo-IsdC (Protein Data Bank code 2O6P) (15).
ment of holo-IsdA long loop with IsdE, the Soret band became indistinguishable from that in a control experiment with only holo-IsdE. This result indicated that all heme had been transferred to IsdE.

The observed transfer rate of heme ($k_{obs}$) calculated from Equation 1 jumped by >300-fold when the loop of IsdC was grafted into IsdA long loop. Specifically, the $k_{obs}$ increased from $2.1 \times 10^{-5}$ s$^{-1}$ for samples of wild-type holo-IsdA to $6.6 \times 10^{-3}$ s$^{-1}$ for samples of holo-IsdA long loop (Fig. 4E). We note that the $k_{obs}$ for IsdA long loop was still 6-fold smaller than that for IsdC ($3.8 \times 10^{-2}$ s$^{-1}$) (Fig. 4E), indicating that some additional factors affected the transfer reaction. Importantly, the $k_{obs}$ for holo-IsdA long loop more than doubled as the concentration of IsdE increased (Fig. 4F), demonstrating that heme transfer was accelerated via activated protein-protein complexes (29, 30, 38–40).

**Role of Iron-coordinating Residues Met-78 and His-229 of IsdE**—It has been shown that the axial ligand His-229 (but not Met-78) is essential for heme uptake in vivo (24). We examined their function with our experimental setup (Fig. 5). Homogeneous holo-IsdE protein (wild-type and muteins M78A and H229A) was prepared by incubation of the apoprotein with heme, followed by ion exchange chromatography to remove unbound ligand. We observed that the Soret band of holo-M78A was very similar to that of the wild-type protein, except for a small shift in the position of the maximum ($\Delta \lambda_{max} = -3$
In contrast, holo-H229A displayed a much lower absorption in the Soret region, suggesting a reduced ability to bind heme.

The transfer of heme between holo-IsdC and apo-IsdE was also examined (Fig. 5B). The spectrum of a control sample containing only holo-IsdC was characterized by a \( \lambda_{max} \) at 403 nm, (Fig. 5A). In contrast, holo-H229A displayed a much lower absorption in the Soret region, suggesting a reduced ability to bind heme.

The transfer of heme between holo-IsdC and apo-IsdE was also examined (Fig. 5B). The spectrum of a control sample containing only holo-IsdC was characterized by a \( \lambda_{max} \) at 403 nm,
in close agreement with previous reports (29, 38). Incubation of holo-IsdC with wild-type apo-IsdE caused a pronounced shift in the Soret band (Δλmax = 13 nm), consistent with the complete transfer of heme to IsdE. Similarly, mutein M78A showed heme transfer ability as demonstrated by the large shift in λmax upon incubation with IsdC (Δλmax = 8 nm). In contrast, we did not observe significant changes in the Soret band of samples incubated with mutein H229A, suggesting very little transfer reaction.

To clarify why mutein H229A cannot receive heme from IsdC, we carried out a photo-cross-linking experiment by mixing muten A96X of IsdC and mutein H229A of IsdE. Mutein A96X was selected for its ability to produce cross-linked dimers (28). Previous models did not explicitly address how Isd proteins, being anchored to the cell wall, could convey heme molecules from the extracellular space to the plasma membrane without breaking their anchoring contacts (Fig. 6A) (29, 30, 51).

In the updated model (Fig. 6B), there is no need for Isd proteins to move along the cell wall because self-transfer is permitted. IsdA is depicted near the extracellular space, whereas IsdC is placed closer to the membrane, in agreement with their cell-sorting signals (11, 14). Accordingly, the self-transfer reaction permits the movement of heme along the cell wall without invoking large protein translations. However, the model in Fig. 6B may require a higher concentration of Isd proteins at the cell wall than previously anticipated (12, 52). The overall driving force in this model is the increasing affinity among transporters (as suggested between IsdC and IsdE in Fig. 5) and internalization of heme across the plasma membrane.

Physical interaction between IsdC molecules was confirmed by the photo-cross-linking technique (Fig. 2). Mapping of 17 single mutants containing the photoreactive amino acid pBPA demonstrated direct and specific interactions between heme transporters of S. aureus.

**DISCUSSION**

In this study, UV-visible spectrophotometry, column chromatography, kinetic analysis, and cross-linking with a genetically encoded photoreactive amino acid (pBPA) demonstrated direct and specific interactions between heme transporters of S. aureus.

**Self-transfer Reaction in IsdA and IsdC**—We demonstrated that the IsdA and IsdC transporters are capable of self-transfer reactions (Fig. 1). This is a novel finding that expands our understanding of heme transport by the Isd system across the thick cell wall (~30 nm) of S. aureus (28). Previous models did not explicitly address how Isd proteins, being anchored to the cell wall, could convey heme molecules from the extracellular space to the plasma membrane without breaking their anchoring contacts (Fig. 6A) (29, 30, 51).

In the updated model (Fig. 6B), there is no need for Isd proteins to move along the cell wall because self-transfer is permitted. IsdA is depicted near the extracellular space, whereas IsdC is placed closer to the membrane, in agreement with their cell-sorting signals (11, 14). Accordingly, the self-transfer reaction permits the movement of heme along the cell wall without invoking large protein translations. However, the model in Fig. 6B may require a higher concentration of Isd proteins at the cell wall than previously anticipated (12, 52). The overall driving force in this model is the increasing affinity among transporters (as suggested between IsdC and IsdE in Fig. 5) and internalization of heme across the plasma membrane.
Protein Data Bank (code 2Q8Q) (24).

Importantly, the axial ligands His-229 and Met-78 of IsdE did transient association as demonstrated in mutein D101X.

The model depicted in Fig. 7 represents our view of the transient IsdC-IsdE complex based on the data gathered. Each protein is shown as a block of a LEGO that docks into each other around the heme-binding pocket. In this model, the "arms" of IsdC are of particular importance for the interaction. Residues near the heme-binding pocket of IsdE also participate in the transient association as demonstrated in muatein D101X. Importantly, the axial ligands His-229 and Met-78 of IsdE did not participate in IsdC-IsdE interactions (Fig. 5).

Site-directed mutagenesis of IsdA confirmed the functional relevance of the model proposed in Fig. 7. Insertion of the β7/β8 strand of IsdC into the homologous NEAT transporter IsdA accelerated heme transfer to IsdE by >300-fold compared with wild-type IsdA. From the concentration dependence of $k_{obs}$, we concluded that enhanced transfer in IsdA long loop was achieved via activated protein-protein interactions (27, 29, 30, 38). Overall, our results demonstrate that the specificity of heme transfer is largely mediated by a discrete structural motif.

Conclusions—In this study, we proved the concept of self-dimerization in Isd NEAT transporters. A revised mechanism of heme transport across the cell wall now takes into account these findings. Photo-cross-linking analysis revealed specific structural elements involved in self-dimerization of IsdC. Using an analogous strategy, we also showed that the β7/β8 strand of IsdC is necessary for the rapid and specific transfer of heme between IsdC and IsdE.

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