The heme-sensitive regulator SbnI has a bifunctional role in staphyloferrin B production by *Staphylococcus aureus*

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*Staphylococcus aureus* infection relies on iron acquisition from its host. *S. aureus* takes up iron through heme uptake by the iron-responsive surface determinant (Isd) system and by the production of iron-scavenging siderophores. Staphyloferrin B (SB) is a siderophore produced by the 9-gene *sbn* gene cluster for SB biosynthesis and efflux. Recently, the ninth gene product, SbnI, was determined to be a free L-serine kinase that produces O-phospho-L-serine (OPS), a substrate for SB biosynthesis. Previous studies have also characterized SbnI as a DNA-binding regulatory protein that senses heme to control *sbn* gene expression for SB synthesis. Here, we present crystal structures at 1.9–2.1 Å resolution of a SbnI homolog from *Staphylococcus pseudointermedius* (SpSbnI) in both apo form and in complex with ADP, a product of the kinase reaction; the latter confirmed the active-site location. The structures revealed that SpSbnI forms a dimer through C-terminal domain swapping and a dimer of dimers through intermolecular disulfide formation. Heme binding had only a modest effect on SbnI enzymatic activity, suggesting that its two functions are independent and structurally distinct. We identified a heme-binding site and observed catalytic heme transfer between a heme-degrading protein of the Isd system, IsdI, and SbnI. These findings support the notion that SbnI has a bifunctional role contributing precursor OPS to SB synthesis and directly sensing heme to control expression of the *sbn* locus. We propose that heme transfer from IsdI to SbnI enables *S. aureus* to control iron source preference according to the sources available in the environment.

*Staphylococcus aureus* is a prominent cause of infectious disease in humans (1, 2). Frequently associated with minor skin and soft tissue infections, *S. aureus* can also cause more serious conditions like infectious pneumonia, infectious endocarditis, and sepsis (3). *S. aureus* has proven to be capable of infecting a variety of host niches, including skin, soft tissue, respiratory, bone, joint, and endovascular tissue (4). Despite the differences in these tissue tropisms, one key environmental condition shared among them is iron limitation. Iron is an essential element required by *S. aureus*, and most bacterial pathogens, to establish disease. The host protects itself against infection by restricting the amount of nutrient metal available to invading microbes as a type of innate, nutritional immunity. Host proteins tightly sequester iron not only as a means to limit toxicity to the host, but also to restrict the bioavailability of free iron to pathogens (5). Additionally, the host hypoferremic response to infection further enhances iron-withholding defenses (5).

As iron is essential for bacterial growth and pathogenesis, iron deprivation triggers gene expression and metabolic changes to elevate iron scavenging. In *S. aureus*, the iron-binding protein Fur (ferric uptake regulator) mediates this response through derepression of several iron acquisition systems and by modulating the expression of different virulence factors in response to iron starvation (6, 7). Fur also coordinates a metabolic rearrangement, termed the iron-sparing response, to decrease the iron demands of the cell by reducing expression of nonessential iron-containing pathways (8–10). Concomitant up-regulation of glycolytic and fermentative pathways allows for suppression of the tricarboxylic acid (TCA) cycle, which relies on many iron-containing enzymes (8).

*S. aureus* infection depends on its ability to circumvent nutritional immunity and exploit a variety of host iron sources (11, 12). *S. aureus* can target both iron directly bound to host proteins and iron found in the form of heme to satisfy nutritional needs. Heme is proposed to be the preferred iron source for *S. aureus* during the initiation of infection (13). Heme uptake is primarily achieved using the iron-responsive surface determinant (Isd) system. This 9-gene system allows for high affinity binding of hemoglobin at the cell surface from which...
heme is rapidly extracted and relayed into the cell cytoplasm (14–16). Internalized heme is either incorporated into bacterial membrane heme-binding proteins for use as a cofactor or degraded by Isd heme-degrading enzymes IsdG or IsdI to liberate nutrient iron (17).

A second iron uptake strategy employed by *S. aureus* during infection is the biosynthesis and secretion of siderophores (18). Siderophores are low molecular weight molecules that can chelate ferric iron from host iron-binding proteins like lactoferrin and transferrin and courier it back to the bacterial cell (19). *Staphyloferrin A* (SA) and *staphyloferrin B* (SB) are two characterized iron-chelating siderophores produced by *S. aureus* and under the control of Fur (20, 21). SA production and efflux is mediated by the proteins encoded in the *sfaABCD* locus and is reliant on the TCA cycle for precursor molecules (22). Suppression of the TCA cycle in favor of glycolytic and fermentative pathways hinders SA production and it is not the primary siderophore expressed during bacteremia (22). Conversely, SB biosynthetic genes, *sbnABCDEFGHI*, encode not only the biosynthetic machinery for assembly and efflux of SB, but also the enzymes necessary to generate precursors from metabolites in central metabolism (21, 23, 24). Thus, production of SB occurs regardless of TCA cycle repression during times of iron-restriction (22). SB is believed to be the primary siderophore expressed during invasive infection, whereas SA is speculated to serve more of a housekeeping role in iron acquisition conducive to commensalistic colonization (12). Some of the most strongly up-regulated genes in the iron-restricted host include *sirABC* encoding the SB surface receptor and cognate ABC transporter, *sirABC* (10, 25, 26). These findings have spurred interest in understanding the biochemistry of SB biosynthesis and the regulatory mechanisms in place for SB production.

Deletion of *sbnl* results in loss in expression of *sbnD-H* and DNA binding by Sbnl is inhibited by heme (27). A model is proposed by which Sbnl is required for transcription of the full SB biosynthetic operon and senses intracellular heme to reduce SB synthesis in favor of heme acquisition. Structural study of a C-terminal–truncated construct of *S. aureus* Sbnl (*Sbnl*1–240) revealed homology to a free serine kinase, SerK from *Thermo- coccus kodakarenensis*, and biochemical assays showed Sbnl catalyzes phosphotransfer from ATP to free L-serine to generate O-phospho-L-serine (OPS) (28). OPS is a substrate of SbnA, which, together with SbnB, produces L-2,3-diaminopropionic acid and α-ketoglutarate, precursors for SB biosynthesis (24, 29). Sbnl-generated OPS was demonstrated to be sufficient to support SB biosynthesis *in vivo* (28).

Herein we present insight into the dual function of Sbnl in SB biosynthesis by studying heme-binding and its effect on L-serine kinase activity. *Sbnl*1–240 from *S. aureus* has decreased kinase activity and is monomeric, whereas full-length Sbnl is dimeric. As crystals of full-length Sbnl were not obtained, we crystallized the homolog from *Staphylococcus pseudinterme- dius* (*SpSbnl*). *SpSbnl* formed a dimer through C-terminal domain swapping and a dimer of dimers through the formation of intermolecular disulfides. A co-crystal structure with ADP confirms the location of the L-serine kinase active site. Using site-directed mutagenesis and spectroscopic analyses, we propose Sbnl binds heme in a low-spin, hexacoordinate manner with Cys-His ligation. Also, we demonstrated that Sbnl can obtain heme from IsdI, consistent with a role as a heme-sensing protein. This provides a basis for how successful Isd-mediated heme uptake could be sensed by Sbnl, leading to decreased SB production as a way for *S. aureus* to control iron source preference.

**Results**

**Structure of SpSbnl and SpSbnl bound to ADP**

*S. pseudintermedius* is closely related to *S. aureus* and is a pathogen of companion animals such as dogs. SpSbnl shares 60% amino acid sequence identity with *S. aureus* Sbnl. SpSbnl was crystallized and the structure was solved to 2.1 Å resolution using the *S. aureus* *Sbnl*1–240 structure (PDB code 5UJE) for phasing by molecular replacement. Data collection and refinement statistics are summarized in Table S1. The protein crystallized with a dimer in the asymmetric unit. The structure of SpSbnl is very similar to the C-terminal–truncated structure of *S. aureus*, *Sbnl*1–240. SpSbnl promomers overlay with *Sbnl*1–240 with a root mean square deviation of 1.4 Å across 240 Cα atoms for protomer A and 1.9 Å across 240 Cα atoms for protomer B. The SpSbnl promomer consists of three domains. Domain I consists of residues Met1–Gln83 and Ile205–Ala240 and domain II contains Tyr84–Asn208 (Fig. 1a). These domains are consistent with *Sbnl*1–240 domains I and II. Domain I contains a conserved core a ParB/Srx-fold that is consistent with the serine kinase active site architecture of *Sbnl*1–240. Domain II has a similar overall-fold as in *Sbnl*1–240. Domain III encompasses the C-terminal 14 amino acids absent from the *Sbnl*1–240 structure, Met241–Glu254, and forms the dimer interface (Fig. 1a). This interface involves C-terminal domain swapping between the two promomers (Fig. 1b). Domain III assembles into two mixed 3-strand β-sheets with one β-strand originating from the other
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Figure 2. SpSbnI disulfide bonds and conservation with S. aureus SbnI. a, intermolecular and b, intramolecular disulfide bonds of SpSbnI. F, F, electron density omit maps are contoured at 3σ. SpSbnI residues are drawn as sticks with separate protomers colored teal or pink. Oxygen, nitrogen, and sulfur atoms colored red, blue, and yellow, respectively. c, Superimposition of SbnI1–240 (blue, residue labels in bold font) and SpSbnI (teal and pink) homologous strands at the dimer of dimers interface seen in the SpSbnI crystal structure. d, a superimposition of SbnI1–240 (blue, residue labels in bold font) and SpSbnI (teal), where SpSbnI forms an intramolecular bond between Cys99 and Cys156. Cysteine residues are represented as sticks with carbon colored teal, blue, or light pink and sulfur atoms colored yellow.

A co-crystal structure of SpSbnI with ADP was solved to 1.9 Å resolution. Data collection and refinement statistics are summarized in Table S1. As observed for apo-SpSbnI, the co-crystal structure was in space group $P2_12_12_2$ and found in the dimer of dimer conformation; however, the crystals are not isomorphous. The dimers linked by disulfide bonds were within the asymmetric unit, and protomers related by crystallographic symmetry formed the dimer interface by C-terminal domain swapping (Fig. 3, a and b). Although the crystallization solution contained both products, ADP and OPS, only clear electron density was seen for the diphosphate of ADP and a modeled magnesium ion for protomer A (Fig. 3c) and the diphosphate, sugar, and magnesium ion for protomer B (Fig. 3d). The first free serine kinase (SerK) characterized is from the archaea *T. kodakarensis*, which catalyzes phosphotransfer from ADP to $\lambda$-serine to generate OPS (32, 33). A crystal structure of a SerK ternary complex with AMP and OPS (PDB ID 5X0E) was superimposed with SpSbnI protomer A with ADP modeled and a magnesium ion based on electron density present in the crystal structure. The coordinates of OPS from the SerK-bound structure were merged with SpSbnI-ADP coordinates to generate a model with both products, ADP and OPS, bound to SpSbnI (Fig. S1). The C-terminal domain partially encloses the $\lambda$-serine kinase active site in SerK and this feature may be important for catalysis and partially explain the lower catalytic efficiency of SbnI1–240 compared with full-length SbnI (28).

Conservation and molecular surface electrostatics of the SpSbnI structure

ConSurf was used to identify conserved regions at the SpSbnI molecular surface (Fig. 4A). The groove that separates domain I from domain III is highly conserved and partially formed from the adjacent protomer. As SpSbnI is a homodimer, there are two conserved grooves per dimer located on opposite faces. Conserved, solvent-exposed residues in this groove include His19, Glu20, Glu23, Arg26, Asp58–Arg62, Trp89, Gly199–Arg202, Gly207–Asn211, Arg243–Tyr243, Gly246, Tyr249, and Glu252. These residues are highlighted in a multiple sequence alignment of staphylococcal SbnI homologs (Fig. S2). Importantly, these include residues identified as being involved with substrate binding and catalysis in the serine kinase active site (28). However, the serine kinase active site only accounts for a small portion of the conserved surface area suggesting the groove may support another function. Highly conserved residues in domain III form the dimerization interface (Fig. 4A). Absolutely conserved sequence motifs among staphylococcal SbnI homologs that form the C-terminal dimer interface include FNIXGRCLNL (residues 204–213) and RCYXEK(V/I)YL(V/I)E (residues 242–252); residue numbering from SpSbnI (Fig. S2).

The APBS plugin in PyMOL were used to generate an electrostatic surface map for the SpSbnI structure (Fig. 4B). The surface of SpSbnI has two regions of notable charged surface. The first is the outward facing end of domain II has a patch of negative charge. Conserved, negatively charged amino acids in this region include Glu103, Glu112, Glu117, and Glu29. The second surface is between domain I and domain III and forms a region of positive charge, the same conserved groove identified...
with ConSurf. Conserved, positively charged residues in this area include His\textsuperscript{19}, Arg\textsuperscript{26}, Arg\textsuperscript{40}, His\textsuperscript{41}, His\textsuperscript{61}, Arg\textsuperscript{62}, Arg\textsuperscript{202}, Arg\textsuperscript{208}, and Arg\textsuperscript{242}.

**Heme binding by SbnI modestly reduces $\alpha$-serine kinase activity**

To determine whether heme binding to SbnI impacted serine kinase activity, steady-state enzyme kinetics of heme-bound SbnI were measured using a pyruvate kinase/lactate dehydrogenase (PK/LDH) assay for detecting ADP (Fig. S3, a and b). Heme-bound SbnI was purified by gel filtration to avoid confounding effects of excess heme in solution. The apparent steady-state kinetic parameters of SbnI:heme with ATP and $\alpha$-serine are summarized in Table 1. Notably, the specificity constant ($k_{cat}/K_m$) measured for apo-SbnI differed by less than a factor of two from the value for heme-bound SbnI, which

**Figure 3. Structure of SpSbnI bound to ADP.** a, SpSbnI crystallographic dimer formed by intermolecular disulfide bonds. Protomer A is colored light blue and protomer B is yellow. b, SpSbn dimer of dimers formed by C-terminal domain swapping. c and d, F$\_o$–F$\_c$ electron density omit maps (contoured to 3$\sigma$) of active sites for protomer A and B. SpSbn residues are drawn as sticks with separate protomers colored light blue or yellow. ADP carbon is colored gray, water molecules are red, and magnesium ions are green. Oxygen, nitrogen, and phosphorus atoms colored red, blue, and orange, respectively.

**Figure 4. Amino acid conservation and surface electrostatics of SpSbnI.** a, conservation pattern of the SpSbnI protomer and dimer generated using ConSurf. The color-coding bar shows the coloring scheme; conserved amino acids are colored bordeaux, residues of average conservation are white, and variable amino acids are turquoise. b, electrostatic potential mapped on the SpSbnI protomer and dimer molecular surface; a blue color indicates regions of positive potential ($> + 5$ kT/e), whereas red represents negative potential ($< -5$ kT/e) values.
**SbnI structure and heme binding**

**Table 1**

|                 | $K_{m}$  | $k_{cat}$ | $k_{cat}/K_{m}$ |
|-----------------|----------|-----------|-----------------|
|                 | ms       | min$^{-1}$| ms$^{-1}$min$^{-1}$ |
| ATP             |          |           |                 |
| SbnI$^a$        | 0.6 ± 0.1| 3.9 ± 0.1 | 6.8 ± 1.0       |
| SbnI + heme     | 0.75 ± 0.15 | 3.2 ± 0.2 | 4.3 ± 0.9       |
| L-Serine        |          |           |                 |
| SbnI$^a$        | 340 ± 40 | 14.3 ± 0.8| 0.04 ± 0.01     |
| SbnI + heme     | 940 ± 30 | 28 ± 6   | 0.03 ± 0.01     |

| $^a$ Kinetic values obtained from Ref. 28. |

suggests that the presence of heme does not drastically alter L-serine kinase activity.

**Heme coordination structure of S. aureus SbnI and SpSbnI**

Heme binding was evaluated based on the UV-visible spectra of SbnI or SpSbnI incubated with equimolar heme. When SbnI was incubated with excess heme and purified by gel filtration, quantification of heme and total protein using the pyridine hemochromogen and bicinchoninic acid assays found SbnI monomer binds heme at a ratio of ~1:1.

The ferric spectra of heme-bound SbnI and SpSbnI were characterized using UV-visible spectroscopy to examine the heme iron electronic state and coordination structure. SbnI-Fe(III) heme spectrum was characteristic of low-spin, 6-coordinate ferric heme that undergo a redox-mediated ligand switch upon reduction of the low-spin 6-coordinate state when the heme iron is reduced to the ferrous form.

The spectral features that accompany this redox-mediated ligand switch are largely consistent with those observed for SbnI. The Soret peak for Fe(II) heme centers that are coordinated by two neutral donors is usually in the 420–430-nm range, accompanied by intense, well-resolved, asymmetric visible region peaks ($\alpha > \beta$) with the $\alpha$ band between 550 and 560 nm (37, 41). By contrast, the 5-coordinate, high-spin Fe(II) state with a sole cysteine ligand exhibits distinct spectral features with a Soret peak at 410 nm and a band in the visible region at 540 nm (37). Reduction of the SbnI-heme complex also resulted in a weak charge transfer band at 650 nm (Fig. 5b, which is usually not observed with ferrous low spin species. Rather, a 650-nm band, concomitant bands in the 500–600 nm range, and a broad, blue-shifted Soret peak at 390–400 nm are observed for high-spin ferric heme complexes (37, 42). Alternatively, low-spin, ferric heme-thiolate complexes are observed to have bands at 650 and 750 nm (37). Nonetheless, overall the spectra upon reduction are consistent with the replacement of cysteine ligand by a nearby neutral donor, like a histidine or methionine, to maintain the low-spin, 6-coordinate state of the Fe(II) heme.

Incubation of heme proteins with CO is commonly used to probe heme centers. When heme-bound SbnI was exposed to CO and subsequently reduced with dithionite, a shift in the Soret peak to 421 nm, and $\alpha/\beta$ bands to 570 and 540 nm, respectively, indicate a change occurred in the electronic environment of the heme and that CO was binding directly to the heme iron (Fig. 5c). SpSbnI displayed similar spectral changes with a shift in the Soret peak to 422 nm and $\alpha$ and $\beta$ bands to 570 and 540 nm, respectively (Fig. 5i). When a cysteine ligand is replaced either by a redox-mediated ligand switch, or by CO directly, the CO-adducts typically display a Soret peak at 420 nm and approximately equivalent $\alpha$ and $\beta$ peaks around 560 and 540 nm (37). The spectra of SbnI and SpSbnI in the presence of CO displayed features that are characteristic of 6-coordinate, low-spin heme, similar to CO adducts of hemoglobin, myoglobin, and other proteins that maintain an axial His-Fe(II) ligation (43–45). Reaction of CO with the Fe(II) heme-thiolate of cytochrome P450 results in a Soret peak at 450 nm that is attributed to CO binding trans to the cysteine (thiolate) (46, 47).

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Magnetic CD (MCD) spectra of heme-bound SbnI was collected to probe SbnI heme iron coordination and ligation. Fig. 5, d and e, shows the MCD absorption data acquired for the heme-bound species of 10 $\mu$m SbnI. The samples were measured under three conditions: (d) apo-SbnI + 0.8 eq of heme added; (e) apo-SbnI + 0.8 eq of heme + dithiothreitol (DTT) reducing agent to reduce the Fe(III) to Fe(II); and (f)
apo-SbnI + 0.8 eq of heme + CO + DTT (to ensure the Fe(III) was reduced to Fe(II)). The MCD spectra were measured with less than 1 eq of added heme to ensure that all of the heme added was bound to the protein, resulting in cleaner spectra. Consistent with a maximum centered around 420 nm for SbnI-bound heme (Fig. 5a), MCD spectra (Fig. 5d) has a trough centered at ~586 nm and some charge transfer features in the 575 to 500 nm region. The MCD fingerprint identifies the heme in these MCD spectra as a 6-coordinate ferric heme with imidazole N from His occupying the 5th position and most likely a cysteine or possibly another amine (from buffer or ammonium hydroxide) in the 6th position. Vetter et al. (48) collected MCD spectral data on the H60C mutant of the heme-binding protein nitrophorin 1, providing a “fingerprint” of cysteine-coordinated heme in a similar system. These model spectra show remarkable similarity to the data shown here. We, therefore, assign the ligation of the ferric heme species as 6-coordinate iron, with histidine and cysteine completing the coordination sphere. Preparation of reduced heme (Fig. 5, b and e) produces a 5-coordinate ferrous fingerprint that also indicates histidine in the 5th position. Finally, there is no significant change to the MCD spectral data after reaction with CO.

**SbnI heme affinity and heme off-rate**

Aggregation of recombinant SpSbnI prevented determination of heme-binding affinity and binding kinetics. Full-length, recombinant *S. aureus* SbnI was, however, sufficiently soluble for these studies. Intrinsic tryptophan fluorescence of SbnI (250 nM) was used to measure heme-binding affinity as heme binding causes fluorescence quenching and a $K_D$ value of 125 ± 105 nM was determined (Fig. S4, a and b). Attempts to measure heme-affinity by fluorescence quenching using lower protein concentration (50 nM) did not yield reproducible results likely due to instrument sensitivity and potentially the SbnI monomer-dimer equilibrium. Recently published $K_D$ values for heme-binding by *S. aureus* IsdI and IsdG are 12.9 and 1.4 nM, respectively, and are 2–3 orders of magnitude lower than previously reported (49). These higher heme affinities of IsdI and IsdG were measured using intrinsic fluorescence quenching of 60–80 nM protein samples by heme (49). Because $K_D$ values well below the protein concentration are poorly measured by fluorescence quenching, our value for the SbnI heme affinity likely represents an upper estimate of heme affinity rather than the actual $K_D$.

The SbnI heme off-rate was determined as the rate of heme transfer to the nonphysiological heme acceptor apomyoglobin.
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Heme binding by apomyoglobin was followed by stopped-flow spectroscopy at 408 nm, the Soret maximum for holomyoglobin. The rate of heme transfer to apomyoglobin was independent of apomyoglobin concentration (data not shown). The transfer rate data were best fit by a triple exponential curve, as judged by the residual plot. The rate of heme dissociation from SbnI was measured to have an average $k_1$ of $0.044 \pm 0.003 \text{ s}^{-1}$, $k_2$ of $0.015 \pm 0.001 \text{ s}^{-1}$, and $k_3$ of $0.003 \pm 0.001 \text{ s}^{-1}$. Each accounted for 31, 34, and 35% of the absorption change, respectively (Fig. S5c).

**Oligomeric state of heme-bound SbnI**

Apo-SbnI was previously shown to be dimeric and the SbnI1–240 C-terminal–truncated construct to be monomeric under reducing conditions (28). Here we investigated the effect of heme-binding on SbnI oligomerization using dynamic light scattering (DLS). The calculated molecular mass of monomeric SbnI is 30 kDa, whereas apo-SbnI was measured to be 61 ± 6 kDa with an average of 24 ± 7% polydispersity using DLS (28) and is presumed to form a dimer in solution. The average molecular mass of SbnI reconstituted with heme was determined as 43 ± 10 kDa with 29 ± 5% polydispersity, suggesting that heme may shift the monomer-dimer equilibrium toward its monomeric form (Fig. S5). Alternatively, the heme-bound SbnI dimer may have smaller hydrodynamic radius than apo-SbnI due to a conformational change. An estimate for SpSbnI oligomerization was not possible due to the propensity of the protein to aggregate and inability to obtain a consistent qualitative analysis by DLS.

**Site-directed mutagenesis to probe SbnI heme-binding mode**

Spectra of full-length SbnI bound to heme has a Soret peak at 423 nm, a shoulder at 350 nm, and well-resolved $\alpha$ and $\beta$ bands at 574 and 543 nm, respectively (Fig. S6a). Comparatively, the C-terminal–truncated monomeric construct, SbnI1–240, has altered heme binding as determined by a decrease in the Soret peak and $\alpha$ and $\beta$ bands compared with full-length SbnI spectra (Fig. S6b), suggesting there is a change in the amino acid environment around the heme. The C-terminal 14 amino acids excluded from the SbnI1–240 construct may be important for interaction with heme or dimerization of the protein may be necessary for heme binding, or both. Within these 14 amino acids, a conserved cysteine was identified as a putative heme iron coordinating residue. A site-directed mutant was made by replacing Cys$^{244}$ with alanine (SbnI C244A). This variant was found to have altered heme binding based on the decrease in Soret peak and diminished $\alpha$ and $\beta$ bands in electronic spectra measured after incubation with heme (Fig. S6c). Additionally, SbnI uniquely contains an N-terminal HXHXXH motif (amino acids 3–7). A site-directed mutant of His$^3$ to an alanine (SbnI H3A) was made. This variant was also found to have altered heme binding based on the decrease in Soret peak and diminished $\alpha$ and $\beta$ bands in electronic spectra measured after incubation with heme (Fig. S6d). A SbnI variant with both His$^3$ and Cys$^{244}$ mutated to alanine (SbnI H3A/C244A) was generated and found to have a cumulative effect on decreasing the Soret peak intensity relative to the ~360 nm shoulder, compared with single amino acid substitutions of His$^3$ and Cys$^{244}$, but did not completely abolish heme binding (Fig. S6e).

The crystal structures of SbnI1–240 and SpSbnI were examined for alternative surface-exposed cysteines that could be involved in heme binding. *S. aureus* SbnI Cys$^{168}$ is predicted to participate in formation of the dimer of dimers by intermolecular disulfide bond formation (Fig. 2c). A site-directed mutant of this residue, SbnI C168A, bound heme with UV-visible spectra resembling WT SbnI (Fig. S6g). Other surface-exposed cysteines in *S. aureus* SbnI include Cys$^{155}$ and Cys$^{156}$. SpSbnI has a single Cys in this region, Cys$^{156}$, but it was found to form an intramolecular disulfide bond with Cys$^{204}$ in the structure (Fig. 2). Mutagenesis of SbnI to generate SbnI C155A had no change in the heme-bound spectra compared with WT SbnI indicating this residue is not involved in heme binding (Fig. S6f). *S. aureus* has two other Cys residues, Cys$^{124}$ and Cys$^{209}$, that were not tested for a role in heme binding. Cys$^{124}$ is located in domain II and Cys$^{204}$ is buried in the dimer interface formed by C-terminal domain swapping. SpSbnI heme-binding was found to have spectral features similar to SbnI but less hyperchromatic. SpSbnI has a Soret peak at 420 nm, a shoulder at 365 nm, and a broad band in the $\alpha/\beta$ region at 542 nm (Fig. S6h).

**Catalytic heme transfer from IsdI to SbnI**

The Isd system is the primary means of heme uptake by *S. aureus* (50, 51). Once transported into the cell, heme is degraded by either IsdI or IsdG, two paralogous enzymes. As *S. aureus* demonstrates heme iron preference for heme in vitro (13) and little, if any, free heme is present in the cytoplasm, we hypothesized that IsdI serves as a heme source for SbnI. This hypothesis was tested by assaying for heme transfer from IsdI to SbnI and comparing to rate of heme transfer to the heme off-rate from IsdI.

IsdI heme transfer to apo-SbnI was measured using UV-visible spectroscopy. apo-SbnI was added to holo-IsdI and within the time of mixing and reading the spectra, the Soret peak shifted from 412 to 417 nm, and a shoulder around 360 nm, and $\alpha$ and $\beta$ bands at 573 and 541 nm, respectively, appeared (Fig. S7a). Further incubation of the mixture resulted in the Soret peak shifting to 421 nm, characteristic of holo-SbnI (Fig. S7a). The rapid spectral shift from that of holo-IsdI to holo-SbnI indicates that heme is transferred from IsdI to SbnI. Little to no spectral shift was observed upon addition of SbnI H3A, SbnI C244, SbnI H3A/C244A, or SbnI1–240 to holo-IsdI suggesting these alterations impaired heme transfer (Fig. S7, b–d and g). IsdI heme transfer to SbnI C155A and SbnI C168A resemble WT transfer (Fig. S7, e and f).

Heme transfer from IsdI to SbnI variants was also assayed using immobilized IsdI followed by UV-visible spectroscopy. The $A_{280}/A_{415}$ ratio of IsdI eluted after incubated with buffer was treated as a no transfer control. A comparable proportion of heme was transferred from IsdI to SbnI C168A and SbnI C155A as to WT SbnI (Fig. 6). SbnI H3A, SbnI C244A, SbnI H3A/C244A, and SbnI1–240 were impaired in accepting heme from IsdI (Fig. 6). Spectra of the flow-through and eluent fractions collected from the pulldown assay is representative of the results from three replicates (Fig. S7). As analyzed by SDS-PAGE, the flow-through and eluent contained proteins with
SbnI structure and heme binding

**Figure 6. Fraction of heme transferred from holo-IsdI to SbnI and SbnI variants.** Quantification of IsdI heme transfer to SbnI variants was determined using a pulldown assay where strep-tagged holo-IsdI was bound to streptactin resin. SbnI variant or buffer (as no transfer control) was added, incubated for 1 min, separated by centrifugation, and the supernatant was removed. The fraction of IsdI heme transferred was calculated as the amount of holo-IsdI eluted when incubated with buffer compared with incubation with the SbnI variant. The amount of holo-IsdI was calculated based on the A_{412} to A_{280} ratio of the IsdI eluent. No heme transfer is defined as the A_{412}/A_{280} of holo-IsdI incubated with buffer. Statistics are calculated based on multiple comparisons with WT SbnI. **, p value < 0.0021; ***, p value < 0.0002. Corresponding molecular weights to SbnI and IsdI, respectively. The absence of SbnI detected in the eluent suggests against formation of a stable complex (data not shown).

The rate of heme transfer from holo-IsdI to apo-SbnI was measured using stopped-flow spectroscopy. The heme transfer rates were determined by following the absorbance change at 426 nm, the wavelength of maximal difference in the visible region between holo-IsdI and holo-SbnI. Data were best fit by a triple exponential curve, as judged by the randomness of the residual plots. Heme transfer kinetic constants are k_1 of 1.55 ± 0.08 s^{-1}, k_2 of 0.16 ± 0.01 s^{-1}, and k_3 of 0.05 ± 0.01 s^{-1}, which account for 14, 78, and 8% of the absorption change, respectively (Fig. S8a). IsdI and SbnI are both dimers that bind one heme in each protomer, so the overall rate of heme transfer reflected the rate of transfer of two heme molecules from one IsdI dimer to one or two SbnI dimers because SbnI was in excess. The observation of absorption changes with varying rates is consistent with the presence of multiple heme-bound species; however, assignment of these rates to specific steps in heme transfer is not yet possible.

To determine whether the observed transfer from IsdI to SbnI was the result of an active transfer or heme release from IsdI followed by SbnI binding, the heme transfer rates were compared with the heme off-rate for IsdI. The heme transfer rates from IsdI were independent of the concentration of apomyoglobin consistent with the observed rate being the off-rate. The transfer data were best fit by a double exponential curve, as judged by the randomness of the residual plot. The rate constants of heme dissociation from IsdI were: k_{fast} of 0.13 ± 0.01 s^{-1} and k_{slow} of 0.04 ± 0.01 s^{-1}, which account for 93 and 7% of the absorption change, respectively (Fig. S8b). Importantly, the rate of IsdI heme transfer to SbnI was ~10-fold greater than the rate of passive heme dissociation from holo-IsdI indicative of the formation of an IsdI-SbnI complex to facilitate heme transfer. To determine whether the source of the heme bound to SbnI affected kinase activity, apo-SbnI was assayed in the presence of IsdI-heme (Fig. S3c) under same conditions as holo-SbnI (Fig. S3, a and b). The presence of IsdI-heme slightly reduced SbnI l-serine kinase activity similar to that observed for heme-bound SbnI.

In the presence of molecular oxygen and a reductant, IsdI functions as a heme-degrading enzyme (52, 53). IruO was identified as a flavin mononucleotide containing oxidoreductase that transfers electrons from NADPH to IsdI (54). In a bacterial cell, heme transfer from IsdI to SbnI would compete with heme degradation. To test if IsdI preferentially degraded heme or transferred it to SbnI, IsdI heme transfer to SbnI was assayed in the presence of IruO and NADPH. IsdI heme degradation can be assessed by a decrease in the Soret peak at 412 nm. Instead, IsdI was found to transfer heme to SbnI in the presence of IruO and NADPH as indicated by the shift of the Soret peak from 412 to 424 nm and the appearance of spectra characteristic of SbnI bound to heme (Fig. S9a). However, a gradual decrease in the Soret peak height suggested heme was still degraded with a half-life of ~17 min. The residual activity could be due to heme transfer equilibrium between SbnI and IsdI. Control reactions were monitored for IsdI degradation of heme in the presence of IruO and NADPH, IsdI heme transfer to SbnI in the presence of IruO only, no degradation of heme by IsdI in the presence of IruO only, and that no heme degradation by SbnI took place in the presence of IruO and NADPH (Fig. S9, b—e).

**Discussion**

Many bacteria rely of siderophore systems to capture extracellular iron to fulfill nutritional iron needs. In *S. aureus*, iron deprivation results in derepression of staphyloferrin production through the global regulatory protein Fur. SbnI establishes a key connection between staphyloferrin production and heme uptake and thus functions at the interface between two iron acquisition systems important for *S. aureus* pathogenesis. SbnI has two distinct roles in SB biosynthesis: first, to serve as a heme-dependent regulator of the sbn locus (27) and, second, to produce OPS, a precursor for SB production (28). Structural study of full-length SpSbnI adds to our understanding of the function of SbnI as a heme-sensitive regulator of SB production. SpSbnI is dimeric under reducing conditions and provides a rationale for the observed defects in oligomerization, l-serine kinase activity, and heme-binding observed in the *S. aureus* C-terminal truncated SpSbnI^{1–240} (28). Heme-binding has only a modest effect on SbnI enzymatic activity suggesting that these two functions are independent of each other and that the heme-binding site is structurally distinct from the active site for serine kinase activity. Thus, SbnI mediates *S. aureus* iron source preference switch between heme and siderophore acquired iron by contributing precursor OPS to initiate SB synthesis when heme iron is not available and directly sensing heme to shut off SB synthesis when heme iron is present.

Site-directed mutagenesis and biochemical methods were used to probe the heme-binding site and heme-binding function of SbnI. Features of the UV-visible and MCD spectra of SbnI bound to Fe(III) heme were consistent with the spectral characteristics of hemoproteins that bind heme iron hexacoordinate and low-spin with a cysteine (thiolate) ligand opposite a reference switch between heme and siderophore acquired iron by contributing precursor OPS to initiate SB synthesis when heme iron is not available and directly sensing heme to shut off SB synthesis when heme iron is present.
Sbnl structure and heme binding

Sbnl variants with substitutions at His\(^3\) and Cys\(^{244}\) were altered and the variants were impaired in heme transfer from IsdI support that these residues serve as the axial heme iron-coordinating ligands. In contrast, mutagenesis of other conserved cysteine residues, Cys\(^{155}\) and Cys\(^{68}\), had no apparent effect on heme binding or transfer. The crystal structures of Sbnl and SpSbnl do not provide a direct model for heme binding with hexacoordinated Cys/His ligation. Heme-binding likely is associated with a conformational change. Moreover, this conformational change may be a functional requirement for the heme-dependent regulation of SB production.

Notably, the single and double H3A and C244A mutations of Sbnl diminished the Soret peak intensity, relative to the \(\sim 360\) nm shoulder, similar to observations for other heme-binding proteins. For example, PhuS is a heme-trafficking protein in Pseudomonas aeruginosa that delivers heme to the heme oxygenase, HemO (55). Mutation of both PhuS His ligands to the heme iron did not eliminate heme-binding even though both His ligands are required for protein–protein interaction with HemO and subsequent heme transfer, highlighting the flexibility of the heme environment (56). Similarly, S. aureus ChdC site-directed mutants of eight distinct residues in the substrate-binding site all bound coproheme with >80% occupancy and had measured \(K_D\) values within an order of magnitude of WT (57). However, mutants that had a functional defect in catalytic competence were identified (57). Single amino acid substitutions to alanine may be insufficient to abrogate heme-binding for proteins with a high-affinity for heme such as Sbnl.

UV-visible absorption spectra suggest that SpSbnl also binds heme iron in a low-spin, hexacoordinate manner with ligation by a cysteine (thiolate). The second axial heme ligand is unknown as SpSbnl has a glutamine at position 3. His\(^3\) is only conserved among Sbnl homologs from the closely related and recently distinguished staphyloccocal species, Staphylococcus argentes (formerly S. aureus clonal complex 75) and Staphylococcus weitei (58, 59). Possibly, His\(^3\) in these homologs has a role in heme transfer from IsdI and another, yet to be determined, residue as a solvent molecule serves as the sixth ligand in all Sbnl homologs. This hypothesis does not fully explain the UV-visible and MCD spectra and a more likely explanation is heme coordination in SpSbnl is similar but distinct from Sbnl.

This likely difference in heme coordination in Sbnl homologs complicates connecting the spectroscopic data with the SpSbnl and Sbnl\(^{1–240}\) crystal structures. Collectively, the data are consistent with heme binding to the conserved groove adjacent to the serine kinase active site in both enzymes even if the specific residues involved differ. Together, the data are a bridge to understanding how Sbnl accomplishes its bifunctional role in staphyloferrin B biosynthesis.

The observation that SpSbnl crystallized as a dimer of dimers through intermolecular disulfide bond formation suggests the redox status of the cell may impact Sbnl oligomerization state and function. The role of reversible disulfide bond formation in Sbnl and the effect of oxidative stress on Sbnl-dependent regulation of \(sbn\) gene transcription and kinase activity offer new areas for exploration of function. As \(S. aureus\) encounters a gradient of iron concentrations during infection, it must also contend with oxidative stress imposed by the host as an immune defense (60). The detrimental effects of oxidative stress and excess iron may warrant redox-sensitive regulation of siderophore biosynthesis to avoid iron accumulation. Disulfide bond formation and oligomeric state is a mechanism used by bacterial transcription factors to sense oxidative stress (61, 62). An example is AgrA in \(S. aureus\) that senses oxidative stress using an intramolecular disulfide that leads to decreased affinity for DNA (62). Given the close relationship between oxidative stress and intracellular iron status in \(S. aureus\) (63–65), further studies to discern whether Sbnl can integrate heme status and the redox state of the cell to affect SB biosynthesis will contribute to our understanding of the regulatory potential of Sbnl.

Sbnl is a heme-sensing protein that can obtain heme from IsdI. The \(K_D\) of IsdI for heme is 13 nM (49) and is consistent with its function as a heme-degrading protein for maintenance of cytosolic heme homeostasis around 20–40 nm, based on the labile heme concentration in eukaryotic cells (66, 67). Heme transfer kinetics imply heme is physically transferred from IsdI to Sbnl through a protein–protein interaction. Moreover, IsdI heme transfer to Sbnl was found to occur in the presence of the physiological reductase for IsdI heme degradation, IruO. Heme acquisition provides an advantage during pathogenesis by supporting aerobic respiration and catalase activity. IsdI heme transfer to Sbnl may function as a signal of active heme uptake by the Isd system leading to decreased SB production; however, further experimentation in vivo is required to strengthen this hypothesis. As \(S. aureus\) is likely to encounter sudden, drastic changes in extracellular heme concentrations such as upon hemolysis, heme may function as an important environmental signal that is immediately sensed upon uptake to impart changes on gene transcription and facilitate niche adaptation. The ultimate fate of cytosolic heme is likely dependent on the intracellular and extracellular availability of iron and heme (17).

Sbnl is proposed to serve as a regulator of iron source preference in \(S. aureus\) through direct interaction with IsdI and indirectly with IruO. Low-spin, hexacoordinate heme iron with thiolate ligands are frequently components of signaling pathways as the labile nature of the Cys ligand render these proteins well-suited for small molecule sensing and transport (37). These results support the existence of a regulatory network between Sbnl, IsdI, and IruO, to optimize iron uptake strategies and creates a model for how intracellular heme homeostasis is maintained. The regulatory role of Sbnl provides rationale for how \(S. aureus\) demonstrates heme iron preference as exogenous heme uptake would inhibit positive regulation of the \(sbn\) gene cluster, thereby limiting the production of SB. When infection is in a heme-rich environment, \(S. aureus\) may preferentially devote energy toward heme uptake rather than the metabolically taxing process of siderophore biosynthesis. This close relationship between nutrient supply and gene expression is a form of host sensing for \(S. aureus\) adaptation, survival, and ultimately to advance pathogenesis and infection.
Experimental procedures

Cloning, expression, and purification of SbnI and SbnI variants

Constructs with an N-terminal His₆ tag and thrombin-cleavage site in pET28a vectors were used for recombinant expression of S. aureus full-length SbnI (residues 1–254) and C-terminal-truncated construct SbnI¹–240 (residues 1–240), with the first codon mutated from the native TTG to a common start codon, ATG, as previously described (28). Bacterial strains and plasmids used in this study are listed in Table S2. The S. aureus SbnI nucleotide sequence can be accessed in the GenBank database under accession code NC_009641.1 (90178–90942) (gene locus NWMN_RS00380) and the amino acid sequence can be accessed through NCBI Protein Database under accession code NP_061787.1.

S. aureus SbnI variants: SbnI H3A, SbnI E20A, SbnI D58A, SbnI C155A, SbnI C168A, and SbnI C244A were produced using a single primer mutagenesis method (68). Mutagenesis primers used in this study are summarized in Table S3. A double site-directed mutant, SbnI H3A/C244A, was generated by performing a subsequent round of mutagenesis on the pET28a-sbnI-H3A plasmid with the sbnI C244A mutagenesis primer. All clones were introduced into E. coli BL21 (DE3) and confirmed by DNA sequencing. E. coli strains.

Recombinant full-length SbnI, SbnI¹–240, SbnI H3A, SbnI E20A, SbnI D58A, SbnI C155A, SbnI C168A, and SbnI C244A and SbnI H3A/C244A constructs were overexpressed in E. coli BL21 (DE3) cells. Cultures were grown in 2×YT media supplemented with 25 μg/ml of kanamycin at 30 °C to an A₆₀₀ of 0.7–0.9. Cultures were then induced with 0.5 mM IPTG and grown for an additional 18 h at 20 °C. Cells were pelleted by centrifugation at 4400 × g for 7 min at 4 °C and resuspended in buffer containing 50 mM HEPES (pH 7.4), 300 mM NaCl, 5% (v/v) glycerol, and 2 mM TCEP and then cleaved with thrombin at a 1:500 ratio by weight of His₆ protein to remove the His₆ tag over 18 h at 4 °C. Subsequently, recombinant protein was purified by anion exchange chromatography using a Source 15Q column (GE Healthcare). Purified protein was obtained by elution with a NaCl gradient and further dialyzed into 50 mM HEPES (pH 7.4), 300 mM NaCl, 5% (v/v) glycerol, 2 mM glutathione (GSH). The sample was concentrated to ~20 mg/ml, flash frozen, and stored at ~80 °C.

Cloning, expression, and purification of IsdI

An isdI construct with N-terminal Strep-tag was generated in pET52b using megaprimer-based whole-plasmid synthesis PCR cloning strategy (69). Template DNA for isdI was subcloned from a previously made construct in pET15b containing S. aureus strain Newman DNA sequence (52). The clone was introduced into E. coli BL21 (DE3) and confirmed by DNA sequencing.

IsdI containing strep tag was expressed in E. coli BL21 (DE3). Cultures were grown in 2×YT media supplemented with 100 μg/ml of ampicillin at 30 °C to an A₆₀₀ of 0.7–0.9. Cultures were then induced with 0.5 mM IPTG and grown for an additional 18 h at 25 °C. Cells were pelleted by centrifugation at 4400 × g for 7 min at 4 °C and resuspended in buffer containing 100 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM EDTA on ice. 5 mg of DNase was added to cell suspension prior to lysis at 4 °C using an EmulsiFlex-C5 homogenizer (Avestin). Insoluble material was removed by centrifugation at 39,000 × g for 1 h and recombinant protein was purified from soluble lysate using a HisTrap nickel affinity column (GE Healthcare) by elution with an imidazole gradient. Protein was dialyzed against 50 mM HEPES, 100 mM NaCl, 5% (v/v) glycerol, and 2 mM TCEP and then cleaved with thrombin at a 1:500 ratio by weight of His₆ protein to remove the His₆ tag over 18 h at 4 °C. Subsequently, recombinant protein was purified by anion exchange chromatography using a Source 15Q column (GE Healthcare). Purified protein was obtained by elution with a NaCl gradient and further dialyzed into 50 mM HEPES (pH 7.4), 300 mM NaCl, 5% (v/v) glycerol, 2 mM glutathione (GSH). The sample was concentrated to ~20 mg/ml, flash frozen, and stored at ~80 °C.

Cloning, expression, and purification of S. pseudintermedius SbnI

Full-length S. pseudintermedius SbnI homolog, termed SpSbnI for this study (residues 1–254), nucleotide sequence can be accessed in the GenBank database under GenBank accession NC_017568.1 (218050–2180814) (gene locus SPSE_RS10030) and the amino acid sequence can be accessed through the NCBI Protein Database under NCBI Accession WP_015728696.1. Briefly, the SpSbnI construct was cloned from S. pseudintermedius strain ED99 chromosomal DNA using a megaprimer-based whole-plasmid synthesis PCR cloning strategy (69). The clone was introduced into E. coli BL21 (DE3) and confirmed by DNA sequencing.

Recombinant SpSbnI constructs was overexpressed in E. coli BL21 (DE3) cells. Cultures were grown in 2×YT media supplemented with 25 μg/ml of kanamycin at 30 °C to an A₆₀₀ of 0.7–0.9. Cultures were then induced with 0.5 mM IPTG and grown for an additional 18 h at 20 °C. Cells were pelleted by centrifugation at 4400 × g for 7 min at 4 °C and resuspended in buffer containing 50 mM HEPES (pH 7.4), 300 mM NaCl, 5% (v/v) glycerol, 2 mM TCEP, and 10 mM imidazole on ice. 5 mg of DNase was added to cell suspension prior to lysis at 4 °C using an EmulsiFlex-C5 homogenizer (Avestin). Insoluble material was removed by centrifugation at 39,000 × g for 1 h and recombinant protein was purified from soluble lysate using a HisTrap nickel affinity column (GE Healthcare) by elution with an imidazole gradient. Protein was dialyzed against 50 mM HEPES (pH 7.4), 100 mM NaCl, 5% (v/v) glycerol, and 2 mM TCEP and then cleaved with thrombin at a 1:500 ratio by weight of His₆ protein to remove the His₆ tag over 18 h at 4 °C. Subsequently, recombinant protein was purified by anion exchange chromatography using a Source 15Q column (GE Healthcare). Purified protein was obtained by elution with a NaCl gradient and further dialyzed into 50 mM HEPES (pH 7.4), 300 mM NaCl, 5% (v/v) glycerol, 2 mM glutathione (GSH). The sample was concentrated to ~20 mg/ml, flash frozen, and stored at ~80 °C.
Sbnl structure and heme binding

was dialyzed against 50 mM HEPES (pH 7.4), 300 mM NaCl, and 5% (v/v) glycerol. The sample was concentrated to ~20 mg/ml, flash frozen, and stored at −80 °C.

Issl containing His6 tag was expressed in E. coli BL21 (ADE3) cells from the plasmid pET15b, purified by His tag affinity chromatography, and digested with the tobacco etch virus protease to remove His6 tag as previously described (52). Protein was dialyzed against 50 mM HEPES (pH 7.4), 300 mM NaCl, and 5% (v/v) glycerol. The sample was concentrated to ~20 mg/ml, flash frozen, and stored at −80 °C.

Expression and purification of IruO

IruO containing His6 tag was expressed in E. coli BL21 (ADE3) cells from the plasmid pET28a, purified by His tag affinity chromatography, and digested with thrombin to remove His6 tag and further purified by anion exchange chromatography as previously described (54). Protein was dialyzed against 50 mM HEPES (pH 7.4), 300 mM NaCl, and 5% (v/v) glycerol. The sample was concentrated to ~20 mg/ml, flash frozen, and stored at −80 °C.

SpSbnl structure determination

SpSbnl crystals were grown by sitting drop at 4 °C in a 1:1 mixture of ~20 mg/ml of SpSbnl in 50 mM HEPES (pH 7.4), 300 mM NaCl, 5% (v/v) glycerol, and 2 mM GSH with reservoir solution containing 4 mM sodium formate. Crystals were flash frozen in liquid nitrogen. Diffraction data were collected at the CLS on beamline 08B1-1 (70). SpSbnl was crystallized in space group P21212 with two molecules in the asymmetric unit. The structure was solved using molecular replacement with Sbnl1–240 coordinates as a search model in PhaserMR from Phenix (71). The structure was manually edited using Coot (72) and refinement was performed with phenix.refine using TLS refinement using nine TLS groups. The refined structure has all 254 residues modeled for each protomer and 187 water molecules. Although residues modeled for each protomer, three formates, and 119 water molecules. All residues were modeled but there was poor side chain electron density for residues 94–142, 153–173, and 220–229 and poor main chain electron density for residues 103–105.

SpSbnl co-crystals were obtained using a protein solution consisting of ~11 mg/ml of SpSbnl incubated with 10 mM ADP, 20 mM OPS, and 10 mM MgCl2 in 50 mM HEPES (pH 7.4), 300 mM NaCl, 5% (v/v) glycerol, and 2 mM GSH. Crystals were grown by sitting drop at 4 °C in a 1:1 mixture of the aforementioned protein solution with reservoir solution containing 0.16 M sodium acetate, 0.1 M imidazole (pH 8), and 8% (w/v) PEG 8000. Crystals were briefly soaked in reservoir buffer supplemented with 30% (v/v) ethylene glycol for cryoprotection and flash frozen in liquid nitrogen. Diffraction data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 9-2 and data were processed and integrated by AUTOXDS (73). SpSbnl co-crystal crystallized in space group P21212 with two molecules in the asymmetric unit. The structure was solved using molecular replacement with SpSbnl coordinates as a search model in PhaserMR from Phenix (71). The structure was manually edited using Coot (72) and refinement was performed with phenix.refine using TLS refinement using nine TLS groups. The refined structure has all 254 residues modeled for each protomer and 187 water molecules. Although the crystallization solution contained both products, ADP and OOPS, only clear electron density was seen for the diphosphate of ADP and a modeled magnesium ion for protomer A and the diphosphate, sugar, and magnesium ion for protomer B.

Data collection and refinement statistics for both structures are summarized in Table S1. Structure figures were generated in PyMOL (The PyMOL Molecular Graphics System, version 1.8, Schrödinger, LLC). Domain analysis was done using the Dali server for comparison of the protein structure against structures in the PDB (74).

SpSbnl conservation and molecular surface electrostatics analysis

The conservation pattern of SpSbnl monomer and dimer was generated using ConSurf (75). The multiple sequence alignment used for the analysis was generated using default ConSurf parameters and the S. pseudintermedius Sbnl amino acid sequence as the search sequence. 17 sequences were used. Electrostatic potential molecular surface map of SpSbnl was generated using default settings in the APBS plug-in, an interface to the adaptive Poisson-Boltzmann solver (APBS) (76). The results were visualized in PyMOL. The electrostatic potential was set to ±5 kT/e so a blue color indicates regions of positive potential (> +5 kT/e) and red represents negative potential (< −5kT/e) values. Superposition of SpSbnl with Sbnl1–240 or SerK (PDB ID 5X0E) was performed using the align function in PyMOL.

Heme reconstitution of proteins

Purified apo-Sbnl or Issl were incubated for 20–30 min at 4 °C with 1.2 m eq of heme solution. Heme was prepared fresh by dissolving in 0.1 M NaOH and adjusting the pH to 8.5–9. Excess and nonspecifically bound heme was removed by gel filtration chromatography on a Sephadex G-25 column (1 × 4 cm). The concentration of holo-protein was determined by quantifying heme using a pyridine hemochrome assay using ε418 extinction coefficient of 191.5 mM−1 cm−1 as previously described (34). For Sbnl, a bicinchoninic acid assay was used for protein quantification to calculate heme binding stoichiometry.

UV-visible spectroscopic analysis of oxidized, reduced, and CO-bound forms of Sbnl and SpSbnl bound to heme

Reduced holo-Sbnl and holo-SpSbnl was prepared by adding 2–3 mg of sodium hydrosulphite (dithionite) to 1 ml of 5 μM protein. The UV-visible spectrum was measured immediately. Investigation of heme-bound Sbnl and SpSbnl interaction with carbon monoxide was carried out by bubbling 950 μl of buffer in quartz cuvette with CO for 5 min, holo-Sbnl or holo-SpSbnl was added to obtain a final concentration of 5 μM holo-protein, and 2–3 mg of dithionite was added to the cuvette. The headspace of the cuvette was exchanged with CO and the cuvette was subsequently sealed, inverted to mix, and UV-visible spectra were recorded. All spectra were measured at room temperature and reactions were carried out in 50 mM HEPES (pH 7.4), 100 mM NaCl, and 5% (v/v) glycerol.
MCD spectra of heme bound to SbnI

Stock apo-SbnI (70 μM) was stored in 300 mM ammonium formate without reductant at 4 °C. Stock samples were diluted with fresh 10 mM ammonium formate buffer immediately prior to sample measurement. 1 mM heme stock was prepared by dissolving 10 mg of hemin in 500 μl of concentrated ammonium hydroxide and then diluting and neutralizing the excess base with 10 mM ammonium formate (pH 6) buffer. DTT was added to reduce the heme using a stock solution of 10 mM DTT (in deionized H2O). CO was bubbled into the reduced heme solution for 5 min to form the CO-bound species. MCD were collected on a Jasco 815 spectropolarimeter using a 1.4 T permanent magnet (acquisition = 3, Tcell ~ 298 K). Scan parameters were: step scan, range 700–300 nm; data pitch = 1 nm; bandwidth = 0.5 nm; response = 1 s. The MCD spectra were corrected for the zero field CD spectrum and zeroed at 700 nm, before a 3 point fast Fourier transform was used to smooth the raw data. The data were collected in θ (mdeg) units directly from the J815 and converted to Δε (m−1 · cm−1 · T−1) using the conversion Δε = θ/(32.98 × 1000 × B × c × l) (where B is the magnetic field, c is concentration, and l is the path length).

Fluorescence quenching of SbnI by heme

Heme binding by SbnI was measured by intrinsic tryptophan fluorescence quenching by heme. Fluorescence-detected heme titrations into SbnI were completed using 250 nM samples of SbnI in 50 mM HEPES (pH 7.4), 100 mM NaCl, and 5% (v/v) glycerol. Heme was added to the buffered protein solution in 50–250 nM increments and allowed to reach equilibrium before readings were measured. The titrations covered a heme concentration range of 50–1000 nM. Fluorescence emission spectra were acquired for a 290 nm excitation using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). Emission spectra were acquired in the 305 to 400 nm range with a step size of 1 nm and slit widths of 10 nm. The dissociation constant (Kd) was calculated from the decrease in the area under the fluorescence curve across 305 to 400 nm as a function of increasing heme concentration. The data were fit by an equation for nonlinear regression one-site binding kinetics using GraphPad Prism 7.0a.

Stopped-flow kinetic analysis of enzyme heme off-rate

All reactions were performed in 50 mM HEPES (pH 7.4), 300 mM NaCl, and 5% (v/v) glycerol at 20 °C. The rates of dissociation of heme from SbnI and IsdI were measured by single-wavelength stopped-flow spectroscopy with apomyoglobin as a heme scavenger (77). Apomyoglobin was prepared from myoglobin (Sigma) (78). Heme dissociation reactions were carried out with 2.5 μM holo-SbnI or holo-IsdI (reconstituted with heme as described previously) in one syringe and 12.5, 25, or 50 μM apomyoglobin in the second syringe. Reactions were monitored over time by recording the absorbance at 408 nm, the maximal absorbance for holomyoglobin. 1000 time points logarithmically distributed over the time frame were acquired using Pro-Data SX software. The change in absorbance was plotted versus time and fit by a triple-exponential equation for SbnI and a double-exponential equation for IsdI to determine the first-order rate constants for heme dissociation. Off-rates were calculated from five independent reactions that were averaged.

Determination of SbnI oligomeric state in solution

Samples of SbnI and SbnI with equimolar heme were analyzed by DLS using a DynaPro Plate Reader (Wyatt Technologies). Protein was diluted to 0.5 mg/ml with 50 mM HEPES (pH 7.4), 300 mM NaCl, 5% (v/v) glycerol, and 2 mM GSH and results were generated based on averaging five, 5-s acquisitions. Data were collected at room temperature. Values reported are an average of data collected.

Heme-binding by SbnI and variants

UV-visible spectra were recorded in a conventional spectrophotometer (Cary50) with the optical path length of 1 cm in a quartz cuvette. All spectra were measured at room temperature and reactions were carried out in 50 mM HEPES (pH 7.4), 100 mM NaCl, and 5% (v/v) glycerol. To examine heme binding by SbnI variants, 5 μM SbnI, SpSbnI, SbnI1–240, SbnI H3A, SbnI C155A, SbnI C168A, SbnI C244A, or SbnI H3A/C244A protein was mixed with 5 μM heme and spectra were immediately recorded. Further readings were taken as indicated and samples for the 1 or 2 h readings were incubated on ice and kept in the dark.

UV-visible spectroscopic analysis of heme-transfer reactions

Spectra were measured at room temperature and reactions were carried out in 50 mM HEPES (pH 7.4), 100 mM NaCl, and 5% (v/v) glycerol. Spectral analysis of IsdI heme transfer to SbnI, SbnI H3A, SbnI C155A, SbnI C168A, SbnI C244A, and SbnI H3A/C244A constructs was conducted by first measuring the absorbance of 5 μM holo-IsdI. Subsequently, 5 μM apo-SbnI variant was added to the cuvette, mixed, and spectra were recorded immediately.

IsdI heme transfer to SbnI in the presence of IruO was performed with IsdI reconstituted with 0.5 molar eq of heme. Reactions were assessed by first measuring the spectrum of 5 μM holo-IsdI then adding a mixture of 5 μM SbnI, 5 μM IruO, and 100 μM NADPH (final concentration) and immediately measuring the spectrum every minute for 5 or 10 min. Appropriate controls were carried out excluding SbnI, IruO, or NADPH. A control reaction using SbnI bound to equimolar heme and then adding IruO and NADPH was also measured.

Stopped-flow kinetic analysis of IsdI heme transfer to SbnI

All reactions were performed in 50 mM HEPES (pH 7.4), 300 mM NaCl, and 5% (v/v) glycerol at 20 °C. IsdI heme transfer reactions to SbnI were carried out with 2.5 μM holo-IsdI in one syringe and 12.5, 25, 50, or 100 μM apo-SbnI. The wavelength of maximal absorbance change was determined to be 426 nm based on a difference absorption spectrum between holo-IsdI and holo-SbnI. Reactions were monitored for 120 s at 426 nm. 1000 time points logarithmically distributed over the time frame were acquired using Pro-Data SX software. The change in absorbance was plotted versus time and fit by a triple exponential curve to determine the rate of heme transfer from IsdI to SbnI.
Sbnl structure and heme binding

IsdI heme transfer to Sbnl pulldown assay

Strep-tagged IsdI (150 μM of 50 μM heme) was incubated with 45 μM heme prior to binding to Strep-tactin Superflow high capacity resin (strepactin beads) (50 μl suspended volume). Samples were washed with 50 mM HEPES (pH 7.4), 100 mM NaCl, and 5% (v/v) glycerol to remove any unbound protein. Holo-IsdI bound to strep beads was subsequently incubated with excess apo-Sbnl, Sbnl H3A, Sbnl C244A, Sbnl H3A/C244A, Sbnl C168A, Sbnl C155A, Sbnl1–240, or SpSbnl (150 μM of 100 μM) or buffer as negative control for 5 min. Samples were centrifuged and supernatant (referred to as flow through) was removed and stored on ice. Samples were washed again with 50 mM HEPES (pH 7.4), 100 mM NaCl, and 5% (v/v) glycerol prior to IsdI elution form the streptactin beads using 50 mM HEPES (pH 7.4), 100 mM NaCl, 5% (v/v) glycerol, 5 mM desthiobiotin. Streptactin beads were spun down and the supernatant (referred to as eluent) was removed and stored on ice. All samples were run on SDS-polyacrylamide gel and UV-visible absorption spectra were recorded. Quantification of IsdI heme transfer to Sbnl variant was based on the percent of heme transferred from IsdI. The percentage was calculated based on the amount of holo-IsdI eluted when incubated with buffer compared with incubation with Sbnl variant. No heme transfer is equal to the A

Steady-state kinetic analysis of Sbnl serine kinase activity in the presence of heme

ATP-dependent serine kinase activity of heme-bound Sbnl was measured using a PK/LDH-coupled assay. The assay is based on a reaction in which the regeneration of hydrolyzed ATP is coupled to the oxidation of NADH (79). The rate of NADH absorbance decrease at 340 nm (A

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