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Recent advances in bacterial heme protein biochemistry

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Recent progress in genetics, fed by the burst in genome sequence data, has led to the identification of a host of novel bacterial heme proteins that are now being characterized in structural and mechanistic terms. The following short review highlights very recent work with bacterial heme proteins involved in the uptake, biosynthesis, degradation, and use of heme in respiration and sensing.

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Introduction

If iron is nature's favorite essential metal, then heme is its Swiss Army knife: a versatile, indispensable tool that, in the company of its protein sheath, can do seemingly anything. The power of heme is particularly evident in the prokaryotes, where diversity in the catalytic activities of heme proteins, as well as proteins involved in the uptake, trafficking and sensing of heme, appears to be vast. This review focuses specifically on very recent (≤ 3 years) advances in the biochemistry of bacterial heme proteins. In the interests of brevity and focus, important work with a more genetic emphasis has been omitted. Studies of proteins involved in the uptake/trafficking of heme, heme biosynthesis/degradation, respiration, and sensing are briefly reviewed.

Starting at the beginning: heme uptake and trafficking

Bacteria have highly evolved systems for the capture, uptake, import, and management of the toxicity of heme (Box 1). Genetic work over the past decade has identified many proteins with proposed roles in heme import (reviewed in [1–3]). Biochemical and structural data have followed more recently, illuminating how protein/heme binding and protein–protein interactions effect heme import into bacterial cells [4].

For pathogenic bacteria, heme (e.g. from hemoglobin following lysis of the host's red blood cells) is a major source of nutritional iron. The first step of heme acquisition for Gram-negative bacteria occurs extracellularly with excreted heme-binding proteins (hemophores). The hemophore HasA (e.g. from *Pseudomonas aeruginosa*) binds heme b with high affinity, though it does not increase the rate of heme release from met-hemoglobin [5,6]. HasA ligates heme iron at a histidine/tyrosine pair. His ligation is selectively broken upon CO binding, suggesting that the Fe–His interaction is critical for heme uptake and release [7]. Gram-positive bacteria (e.g. *Staphylococcus aureus*) generally do not excrete hemophores, instead utilizing cell-wall-anchored heme binding proteins such as IsdH (Isd = iron-regulated surface determinant). The anchored IsdH proteins contain multiple, functionally distinct NEAT (NEAR iron transporter) domains through which they bind free heme, interact directly with hemoglobin, or transfer heme to other NEAT domain proteins [8^{*}]. IsdH proteins bind multiple hemes, each via a different tyrosine residue, before undergoing multimerization with other IsdH proteins at the cell surface and co-localizing bound heme for easy transport [9,10^{*}]. Recent evidence also suggests that some Gram-positives (*Bacillus anthracis*) may also make use of secreted hemophores, for example, IsdX1 [11].

Following capture from the host/environment, heme is transferred to an outer membrane receptor or transporter. Structural evidence suggests that the surface-bound Shp protein (from the Gram-positive *Streptococcus pyogenes*) mediates heme transfer from the receptor (Shr) to the lipoprotein component of the ATP-binding cassette (ABC) transporter (HtsA) through multiple protein–protein interactions at the cell surface [12]. Shp ligates heme via two methionine residues, while HtsA uses a methionine/histidine pair. Relatively low affinity ligation is crucial for facile heme binding and release [13]. Similarly, the IsdH receptor in *S. aureus* relays heme to other surface-bound proteins, IsdA and subsequently IsdC. Kinetic studies of heme transfer from IsdA to IsdC revealed that it occurs via complexation of the proteins, driven partly by IsdC's slightly higher heme affinity [14] and by free energy released by the protein–protein interaction. IsdC then mediates transfer of heme across the thick proteoglycan layer to the membrane translocation system, IsdDEF (in particular, IsdE), serving as the central conduit for transfer from the cell surface to the lipid bilayer [15^{*},16,17]. In Gram-negative bacteria, transfer of heme from the HasA hemophore to the outer membrane receptor HasR has

Box Figure 1

Box 1. Heme uptake: protein-mediated steps	
Gram-negatives	Gram-positives
<ul style="list-style-type: none"> ▪ Secretion of hemophores (e.g., HasA, His/Tyr) ▪ Interaction of hemophore with receptor (HasR) ▪ Interaction of receptor with TonB protein to drive heme import to periplasm ▪ Transfer to a periplasm binding protein, ABC transporter ▪ Transfer to a cytoplasmic chaperone (e.g., PhuS) 	<ul style="list-style-type: none"> ▪ Generally does not involve secreted proteins: ▪ Surface-bound proteins (e.g., iron regulated surface determinants, Isd) ▪ IsdH: <ul style="list-style-type: none"> ○ Binds heme or hemoglobin via NEAT domain ○ Multiple hemes per IsdH ○ IsdH proteins colocalize on surface ▪ Heme is relayed to other surface-bound Isds (e.g., IsdA, C), then to the heme binding element of an ABC transporter (IsdE, HtsA) ▪ Transfer from lower to higher affinity heme binding sites; driven also by energetically favorable protein-protein interactions ▪ Transfer to a cytoplasmic chaperone

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Heme uptake: protein-mediated steps.

been structurally characterized by NMR and crystallography [18^{••},19]. Exchange again occurs from a lower to a higher affinity site, with protein complex formation helping to drive the transfer.

The next step is transport of heme into the cytoplasm. Gram-positives like *S. aureus* accomplish this via the ATP-binding cassette transporter IsdDEF. IsdE is the lipoprotein component of this complex, which binds heme via a Met/His pair; binding may be loosened through interactions with the transporter's transmembrane component [20]. In Gram-negatives, heme is recognized at the outer membrane by receptor proteins. The HasR outer membrane receptor/transporter from *Serratia marcescens* interacts specifically with the TonB-like protein HasB, driving import of heme across the outer membrane. The binding of extracellular substrates like heme and hemophores (HasA) to HasR alters the interaction between the transporter (HasR) and the globular C-terminal domain of HasB, suggesting that protein-protein interactions on the outer membrane relay signals to the periplasm, permitting heme transport [21]. Another recently characterized outer membrane receptor, ShuA (*Shigella dysenteriae*), has two histidines mediating heme binding/release. The protein interacts with the TonB transporter with 1:1 stoichiometry [22[•]].

Several periplasmic heme binding/transporter proteins have recently been identified and characterized [23–26]. In an important and technically impressive step forward, the Wilks lab recently cloned and characterized the heme ABC transporter ShuUV, which translocates heme from *Shigella's* periplasm to cytoplasm [27[•]].

Inside the cytoplasm, heme must be bound, for example, by a chaperone, to keep it from damaging the cell. The

heme-binding PhuS (*Pseudomonas* sp.) binds two heme molecules and has multiple heme-bound states that are probably important for binding and release of the cofactor [28]. PhuS may serve a secondary role in sensing heme and regulating iron homeostasis [29]. Other members of the PhuS family may couple heme binding with other roles. For example, AphC is an alkyl hydroperoxide reductase that also binds heme. It has been proposed that heme protection is AphC's primary role, and its peroxidase and reductase chemistry are secondary [30].

Porphyrin synthesis and breakdown

Heme breakdown by canonical heme oxygenases (HOs) is the presumed fate for imported heme in many bacteria. However, some bacteria, including important pathogens, either do not have a HO homolog or have otherwise been shown to possess a non-canonical enzyme for heme degradation (Box 2). For example, the paralogous heme oxygenases IsdG and IsdI from *S. aureus* have been crystallographically characterized and shown to be structurally and mechanistically distinct from known heme oxygenases [31^{••}]. The product of these enzymes is moreover not biliverdin as with HO, but rather the oxo-bilirubin chromophore staphylobilin [32]. Finally, the structures of IsdG/I have highly ruffled and therefore strained porphyrins, facilitating cleavage of the macrocycle at the β -meso or δ -meso (rather than α -meso) carbons via a heme-bound hydroperoxide [31^{••},32]. Recently, an unusual IsdG/I homolog was discovered in *Mycobacterium tuberculosis*. The protein, MhuD (Mycobacterial heme utilization, Degradar), is able to bind and degrade heme. Its contains an unusual diheme structure, where the individual porphyrin rings are not as ruffled as in IsdG/I [33]. In several Gram-negative bacteria, including *Shigella dysenteriae*, *Salmonella typhi*, and *E. coli* pathogenic strains, homology searches have failed to identify

Box Figure 2

Box 2. Heme degradation: proteins involved, sources and defining characteristics		
Heme oxygenase (HO)	Found in eukaryotes, many bacteria	Canonical means of degrading heme to biliverdin and CO
IsdG/I family proteins	<i>Staphylococci</i> , other Gram-positives	Strongly ruffled porphyrin oxidized at δ - or b-meso position to staphylobilin
MhuD	<i>Mycobacterium tuberculosis</i>	Diheme active site
EfeB	Pathogenic <i>Escherichia coli</i>	Removes iron from protoporphyrin IX, leaving ring intact

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Heme degradation: proteins involved, sources and defining characteristics.

orthologs to any known heme degrading enzymes, though they can use heme as an iron source. The EfeUOBM proteins from *E. coli* have been proposed to have roles in iron import [24,34], with EfeB serving specifically as the enzyme responsible for removing iron from heme via an unusual reaction that leaves the porphyrin intact [35^{••}]. Complete biochemical characterization of EfeB is still pending.

At the other end of heme homeostasis, heme biosynthesis is a multi-step, multi-enzyme process that, as with degradation, is complicated in bacteria by the absence of some expected enzymes and variability in others [36]. Recent work has shed light on the terminal steps. Protoporphyrinogen oxidase (PPO) catalyses the oxidation of protoporphyrinogen IX to protoporphyrin IX. The ring is then metallated by ferrochelatase (HemH) or Mg-chelatase to heme or chlorophyll, respectively. Eukaryotes and many aerobic/facultative Gram-negative bacteria contain an O₂/FAD-dependent PPO, HemY. This protein and ferrochelatase are membrane bound in Gram-negatives, and soluble in Gram-positives. Many γ -Proteobacteria, including *Escherichia coli*, contain a soluble PPO (HemG) that is quinone-dependent and O₂-independent [37]. In studies using *hemY* and *hemH* genes from Gram-positive bacteria, an additional gene (designated *hemQ*) was shown to be necessary for complementing *E. coli* Δ *hemG*/ Δ *hemH* double mutants. The suggested function of HemQ was as a locally acting catalase [38^{••}]. A recent analysis of structure, sequence, and structure for the superfamily proteins to which HemQ belongs also suggests possible non-redox roles, for example in stabilizing a HemY/HemH complex or chaperoning the protoporphyrin IX substrate (DuBois *et al.*, submitted; see also **Respiration** below). A third type of PPO, a putative membrane-spanning protein that is distantly related to the M subunit of NADH dehydrogenase complex I, was discovered in the cyanobacterium *Synechocystis sp.* PCC6803 [39]. Interestingly, a survey for homologs of

all 3 PPOs in the Gen-Bank database by the authors did not identify hits in the majority of the Archaea and several bacterial phyla, suggesting that they may contain still unidentified types of PPO.

Roles for heme proteins: respiration

A major role for heme proteins in bacteria is in mediating redox processes, including respiration. Bacteria are respiratory experts, coupling the reduction of nearly any available compound or material to the generation of an energy-transducing, electrochemical gradient. Respiratory substrates include O₂ (e.g. [40[•],41–43]) and NO₃⁻ but also more exotic oxidants, such as perchlorate (ClO₄⁻). This toxic, man-made anion serves as a respiratory substrate for a variety of Proteobacteria, though when and how this metabolism evolved is the subject of debate (reviewed in [44]). Perchlorate-respiring bacteria reduce ClO₄⁻ to ClO₃⁻ and then ClO₂⁻ (chlorite). Chlorite is subsequently detoxified by the heme enzyme chlorite dismutase (Cld), yielding Cl⁻ and O₂ gas. The Cld from *Dechloromonas aromatica* was heterologously expressed [45] and structurally [46^{••}] and mechanistically characterized [47,48^{••}]. (See [49[•]] for the structure of Cld from *Azospira oryzae*.) The enzyme has a highly unusual active site structure, with a His-ligated heme and a sterically confined distal pocket containing a sole polar and possibly weakly acidic arginine residue that is important for the reaction with chlorite [48^{••}]. The *Dechloromonas* enzyme is part of a large protein family, of which most members (including HemQ, above) probably do not convert chlorite to O₂. A sequence and structure based study suggests structural variability across the protein family in the active site, particularly at the critical Arg. Striking similarities between the monomer structures of Clds, dye-decoloring peroxidases, and EfeB proteins were also identified. These proteins possess a conserved fold and common membership in a structural superfamily, though their primary sequences separate into 3 clades [46^{••}] (and DuBois *et al.*, submitted). Further work will be needed

to delineate chemical and biological roles for these proteins.

Another exciting area of recent research is the dissimilatory reduction of extracellular Fe(III)-oxides (e.g. by *Shewanella* sp.). This is a formidable process: electrons from the inner membrane (IM) quinone pool must traverse the periplasmic space and outer membrane (OM), to be received by an extracellular respiratory oxidant. This process can be broken into three parts, based on the three aforementioned locations, all of which involve multiheme cytochromes. The electrical conduit joining the IM quinone pool with the OM originates with the IM-bound tetraheme CymA. CymA also supplies electrons for various dissimilatory reductions that occur in the periplasm via soluble oxidoreductases, a role recently expanded to include arsenate reduction (*Shewanella* sp.) [50]. Although the periplasmic component responsible for shuttling the electrons from the IM to the OM remains unconfirmed for *Shewanella oneidensis* (So), one abundant candidate is the small tetraheme cytochrome c (STC). This protein was implicated in Fe(III) reduction in the related organism, *Shewanella frigidimarina* (Sf). The crystal structure of SfSTC showed a heme-binding fold similar to that in SoSTC, but very different from a well-studied *Desulfovibrio* tetraheme cytochrome c with analogous function [51]. The two STCs have similar heme geometries, though they differ significantly in primary structure and heme redox potential, resulting in different orders of oxidation for their hemes [51,52^{••}]. NMR and visible spectroscopic studies were used to distinguish redox effects (heme–heme electrostatic interactions) from redox-Bohr effects resulting from the aggregate effect of several acid–base groups of the protein [52^{••}]. Reduction and protonation were shown to be thermodynamically coupled at physiological pH. Reduction of the most redox-active heme (III) causes the protein to switch from a protonated to a deprotonated microstate [52^{••}]. The switch is independent of electron donor/acceptor, suggesting a role for this protein as a nonspecific electron harvester [53[•]]. In both SoSTC and SfSTC, the heme with the lowest potential serves as the site of electron entry and exit, and since it is the last to be oxidized, provides a driving force that allows all four hemes to accept electrons.

A biochemical basis for directional electron transfer has also been proposed for the decaheme cytochromes at the end of the process in the OM, MtrA (periplasmic side), and MtrC (extracellular side) that form a complex with the β -barrel porin MtrB (MtrCAB). Evidence of direct interaction between MtrA and MtrC supports a model of the latter two cytochromes ‘reaching out’ to each other for electron transfer while partially embedded in MtrB. An elegant experiment by Hartshorne *et al.*, using methyl viologen encased in MtrCAB-embedded liposomes, showed that MtrC has a more positive potential than

the MtrCAB complex [54[•]]. This suggests a negative shift in redox potential upon incorporation of MtrC into the complex, which could provide a driving force for directional electron transfer. MtrC serves as the electron donor toward the Fe mineral.

Related work has addressed structure–function relationships in multiheme cytochromes involved in sulfate reduction in *Desulfovibrio*. The 16-heme cytochrome HmcA, part of a protein complex responsible for bridging periplasmic H₂ oxidation and cytoplasmic sulfate reduction, contains a glycosylation site that may tether it to the cytoplasmic membrane [55]. This protein takes on a ‘V’ shape (with glycosylation at the vertex). The different electrostatic environment of each arm may allow interaction with various electron donors.

Signaling

A second major role for bacterial heme proteins is in sensing. Heme sensor proteins specifically bind diatomic O₂, NO, and CO, transmitting the received signal to a protein domain capable of executing a response. Several relevant papers have emerged recently. Wan *et al.* describe a globin-based O₂ sensor from *Bordetella pertussis*, BpeGReg [56[•]], which couples O₂ binding at heme to stimulation of diguanylate cyclase activity in a GGDEF domain. O₂ is therefore the first ligand shown to induce production of the bis-(3′–5′)-cyclic diguanosine monophosphate second messenger. While the full effect of this second messenger is unknown, it has been shown to regulate production of biofilms, which are important for the establishment and maintenance of many bacteria in their most virulent forms.

On the NO front, the first structure of a putative NO-sensing domain belonging to a bacterial transcription factor (dissimilative nitrate respiration regulator, DNR) was reported [57]. It was shown to contain a pentacoordinate heme-NO species reminiscent of the liganded form of guanylate cyclase. DNR regulates the denitrification pathway of *Pseudomonas aeruginosa*, in which NO is an intermediate. At the same time, NO is a product of the human immune system and a eukaryotic/prokaryotic signaling agent. Nanomolar concentrations of NO, for example, have been shown to disperse biofilms of *P. aeruginosa*. How the NO signal is concurrently processed for its many roles, by DNR and possibly other signaling proteins, remains an open question.

Production of the NO signal occurs at heme-dependent nitric oxide synthases (NOSs). Work with prokaryotic NOSs has been pursued as a means of obtaining more experimentally manageable proteins than those from eukaryotes, and in order to understand the roles, origins, and diversity of NO-related biochemical pathways in prokaryotes. An NOS from *Sorangium cellulosum* was isolated that, like its eukaryotic counterparts, has both

reductase and oxidase domains in the same polypeptide [58]. An NOS-like protein from *Deinococcus radiodurans* was identified even though the organism lacks genes for biosynthesis of tetrahydrobiopterin, a necessary electron-shuttling cofactor [59]. It was shown to depend instead on tetrahydrofolate [60].

In some cases, heme itself is the sensed entity. In some species of α -Proteobacteria, the concentration of cellular heme, sensed by the iron response regulator (Irr), indirectly regulates cellular iron concentration. In *B Bradyrhizobium japonicum* and under conditions of iron sufficiency, Irr binds heme from the terminal heme biosynthesis enzyme (ferrochelatase) and degrades [61•]. By contrast, the Irr from *Rhizobium leguminosarum* does not degrade. Rather, DNA binding affinity is greatly reduced upon heme binding [62]. The *B. japonicum* protein was also shown to have a broader cellular role, responding not only to cellular Fe, but manganese [63]. Manganese binds directly to Irr, altering its secondary structure and inhibiting its heme binding. Irr is thereby stabilized under high Mn and low Fe concentrations, where it positively regulates transcription of genes involved in Fe uptake.

Conclusions

Genetic work in bacteria has led to the discovery of interesting, novel metabolic pathways that involve heme proteins in unexpected ways. These discoveries can be expected to carry on as the wealth of prokaryotic genomic data continues to be explored.

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