Time-resolved Studies of IsdG Protein Identify Molecular Signposts along the Non-canonical Heme Oxygenase Pathway

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IsdGs are heme monoxygenases that break open the tetrapyrrole, releasing the iron, and thereby allowing bacteria expressing this protein to use heme as a nutritional iron source. Little is currently known about the mechanism by which IsdGs degrade heme, although the products differ from those generated by canonical heme oxygenases. A synthesis of time-resolved techniques, including in proteo mass spectrometry and conventional and stopped-flow UV/visible spectroscopy, was used in conjunction with analytical methods to define the reaction steps mediated by IsdG from Staphylococcus aureus and their time scales. An apparent meso-hydroxyheme (forming with $k = 0.6 \text{ min}^{-1}$, pH 7.4, 10 mM ascorbate, 10 $\mu$M IsdG-heme, 22 °C) was identified as a likely common intermediate with the canonical heme oxygenases. Unlike heme oxygenases, this intermediate does not form with added H$_2$O$_2$ nor does it convert to verdoheme, although the products differ from those generated by canonical heme oxygenases. A synthesis of time-resolved techniques, including in proteo mass spectrometry and conventional and stopped-flow UV/visible spectroscopy, was used in conjunction with analytical methods to define the reaction steps mediated by IsdG from Staphylococcus aureus and their time scales. An apparent meso-hydroxyheme (forming with $k = 0.6 \text{ min}^{-1}$, pH 7.4, 10 mM ascorbate, 10 $\mu$M IsdG-heme, 22 °C) was identified as a likely common intermediate with the canonical heme oxygenases. Unlike heme oxygenases, this intermediate does not form with added H$_2$O$_2$ nor does it convert to verdoheme and CO. Rather, the next observable intermediates ($k = 0.16 \text{ min}^{-1}$) were a set of formyloxobilin isomers, similar to the mycobilin products of the IsdG homolog from Mycobacterium tuberculosis (MhuD). These converted in separate fast and slow phases to $\beta$-/d-staphylobilin isomers and formaldehyde (CH$_3$O). Control of released of this unusual C1 product may support IsdG's dual role as both an oxygenase and a sensor of heme availability in S. aureus.

Heme oxygenases (HOs) are enzymes that oxidatively liberate iron from the heme tetrapyrrole (1–3). In the well characterized HOs from animals and many bacteria, the same heme molecule acts as both the O$_2$-activating cofactor and substrate. Three successive monooxygenation steps yield Fe(II), CO, and CH$_2$O instead of CO implies that verdoheme, the green intermediate of iron. Because of the intriguing nature of the reaction, which uses heme as both cofactor and substrate (8–10), as well as the acute biological importance of HO-mediated processes, HOs are used in conjunction with analytical methods to define the reaction steps mediated by IsdG from Staphylococcus aureus and their time scales. An apparent meso-hydroxyheme (forming with $k = 0.6 \text{ min}^{-1}$, pH 7.4, 10 mM ascorbate, 10 $\mu$M IsdG-heme, 22 °C) was identified as a likely common intermediate with the canonical heme oxygenases. Unlike heme oxygenases, this intermediate does not form with added H$_2$O$_2$ nor does it convert to verdoheme and CO. Rather, the next observable intermediates ($k = 0.16 \text{ min}^{-1}$) were a set of formyloxobilin isomers, similar to the mycobilin products of the IsdG homolog from Mycobacterium tuberculosis (MhuD). These converted in separate fast and slow phases to $\beta$-/d-staphylobilin isomers and formaldehyde (CH$_3$O). Control of released of this unusual C1 product may support IsdG's dual role as both an oxygenase and a sensor of heme availability in S. aureus.

By the early 2000s, however, it was apparent that many important Gram-positive pathogens that degrade host heme did not possess an HO-encoding gene in their genomes. A new family of heme-degrading proteins known as IsdGs was subsequently discovered, with representatives found in bacteria from both Gram-positive and Gram-negative phyla (11). IsdG family proteins are evolutionarily and structurally distinct from the well studied HOs (12, 13), and they yield different end products. Instead of biliverdin IX$\alpha$ and CO, the IsdG protein from Mycobacterium tuberculosis (known as MhuD) generates triply-oxygenated linear tetrapyrroles called mycobilins (Fig. 1) (14, 15). A formyl group remains appended to pyrrole ring A or B at the site of macrocycle cleavage, and an oxo group is generated on the pyrrole ring on the opposite side. Notably, no C1 product is released (16).

Although homologous to MhuD, the IsdG from Staphylococcus aureus degrades heme to yet a third set of products. The macrocycle is not cleaved at the $\alpha$-meso- but rather at either the $\beta$- or $\delta$-meso-carbon. Oxo groups are generated on both the carbon backbone and the pyrrole rings at the cleavage site, generating tetrapyrrole products known as staphylobilins (Fig. 1) (17). It was recently shown that a C1 product is indeed released by the S. aureus IsdG; however, quite unexpectedly, the major C1 product was determined to be formaldehyde (CH$_2$O) instead of CO (18). Unlike CO, formaldehyde may be undetectable by animal immune systems, offering a potential selective advantage for heme-feeding pathogens that use IsdG-type enzymes (5, 19, 20). Mechanistically, the observation that CH$_2$O instead of CO implies that verdoheme, the green inter-
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Experimental Procedures

IsdG Purification, Expression, and Reconstitution with Heme—Recombinant IsdG with an N-terminal His$_6$ tag (pET-15b) was expressed in *Escherichia coli* Tuner (DE3) cells (Merck/Novagen) grown in Terrific Broth supplemented with ampicillin (100 µg/ml). Protein was expressed and purified, as described previously (12), with some modifications. Briefly, cultures were grown at 37 °C in a shaker incubator (250 rpm) to mid-logarithmic phase (absorbance at 600 nm = 0.4). 1 mM isopropyl β-D-thiogalactopyranoside was added to induce protein expression, and the temperature was lowered to 20 °C. After 16 h, cell pellets were collected by centrifugation and stored at −80 °C.

Pellets were thawed/resuspended in Buffer A (50 mM potassium phosphate (KPi) buffer, pH 7.4, 600 µg/ml lysozyme, and 1 mg of DNase) and lysed by pulsed sonication on ice. The lysates were clarified by centrifugation, and supernatants were loaded onto a 20-ml PerfectPro nickel-nitrilotriacetic acid-agarose (5 PRIME) column. Pure protein was eluted in 3-ml fractions using an AKTA protein purification system (GE Healthcare) (20–500 mM linear gradient of imidazole in Buffer A, flow rate 2 ml/min). Eluted proteins were screened by SDS-PAGE, and pure fractions pooled/buffer-exchanged into Buffer A using SnakeSkin dialysis tubing (10,000 molecular weight cutoff (MWCO), ThermoScientific). For His tag cleavage, the dialyzed protein was incubated with His$_6$-tagged tobacco etch virus protease (50:1 w/w) for 16 h at 4 °C with stirring. Tag cleavage was monitored by SDS-PAGE and MS (see below). Cleaved protein was loaded onto a 10-ml PerfectPro nickel-nitrilotriacetic acid-agarose column to remove the tobacco etch virus protease. The flow-through and a column wash (20 mM imidazole in Buffer A) were pooled and dialyzed into Buffer B (0.1 M KPi, pH 6.8).

The dialyzed IsdG was incubated with 1 eq of hemin chloride (Calbiochem) from a dimethyl sulfoxide (DMSO) stock and incubated overnight at 4 °C with stirring. Excess hemin was removed by centrifugation followed by further purification of the protein by size exclusion chromatography (Sephacryl S200 HR, GE Healthcare). Pure protein was dialyzed into Buffer A, concentrated to 10 mg/ml using an Amicon stirred cell concentrator (10,000 MWCO), frozen in liquid N$_2$, and stored at −80 °C. All IsdG-heme concentrations are given as heme-bound monomer, where [heme] and [protein] were determined by the pyridine hemochrome and Bradford assays, respectively.

IsdG-Heme Reaction with O$_2$ and Reducing Equivalents Monitored by UV/Vis Spectroscopy—To initiate the heme decomposition reaction, 10 µM IsdG-heme was treated in air with 10 mM sodium ascorbate (i.e. 1000 eq) in the presence of 100 units of catalase (50 mM KPi, pH 7.4, 22 °C). These conditions were known from prior work to lead to readily observable loss of the IsdG-heme chromophore. Ascorbate stocks were prepared immediately prior to use and maintained under N$_2$. Spectral changes were monitored over time with a Cary600i spectrophotometer (Agilent Technologies). The absorbance at the heme Soret band maximum (411 nm) plotted versus time was fit by linear least squares regression analysis to exponential decay curves to obtain first-order rate constants ($k_{obs}$).
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All kinetic data plotting and fitting were carried out using KaleidaGraph 4.0 except where noted. All reported $k_{\text{obs}}$ values are averages of $\pm 3$ measurements (error $= \pm 1$ S.D.).

IsdG-Heme Reaction with $O_2$/Ascorbate Monitored by Time Resolved in Proteo Mass Spectrometry—Reactions were prepared exactly as above but at volumes allowing for multiple successive injections. Alternatively, reactions were prepared at elevated concentrations (50 $\mu$M IsdG-heme) to increase the concentrations and thereby the intensity of the signals emitted by intermediates. Reactions and automated analysis used a 1290 ultrahigh pressure (UPLC) series chromatography stack (Agilent Technologies) coupled directly to an electrospray-time of flight mass spectrometer (Bruker Micro-TOF). Rapid reverse-phase chromatography (5 min) was carried out with a PLRP-S 100 Å column (50 mm, 3 $\mu$m, flow rate 600 $\mu$L/min, autosampler temperature 20 °C). The gradient was as follows: 0.8 min, 20% B; 0.8–4.3 min, 20–95% B; 4.8 min, 20% B at 37 °C column compartment temperature; solvent A = 0.1% formic acid (FA, Sigma) in water (Burdick & Jackson) and solvent B = 0.1% FA in acetonitrile (ACN, Burdick & Jackson). No carry-over of heme-associated products or protein was observed in blank runs carried out as controls. Electrospray conditions were as follows: drying gas 8.0 liters/min, dry temperature 180 °C, and capillary exit 150 V. Scan range was 200–3000 m/z. Data processing and analysis were performed using Bruker Data Analysis package 4.0. Mass spectra for the protein and heme products were concurrently obtained and used to verify identity/mass. Measured m/z values for all reported compounds and isomers were <5 ppm from calculated monoisotopic values.

IsdG-Heme Reaction with $O_2$/Ascorbate Monitored by UV/Vis Stopped-flow Spectroscopy—Reactions were carried out at 20 °C on a Hi-Tech stopped-flow instrument (1.5-ms mixing time) with diode array detection (250–700 nm). Aerobic IsdG-heme (20 $\mu$M) was mixed 1:1 volume:volume with ascorbate in 50 mM KP$_2$, pH 7.4, prepared immediately prior to use at varying concentrations under N$_2$ (gas) from a 2 M stock. Additionally, the concentration of O$_2$ was varied by equilibrating KP$_2$ buffer with O$_2$/N$_2$ gas mixtures and measuring the resulting [O$_2$] with a Clark-type O$_2$ electrode. The variable [O$_2$] buffer was mixed with IsdG-heme equilibrated to air to reach the final [O$_2$]. Spectra were measured over time at 1–100 ms intervals. Absorbance at selected wavelengths near the Soret and visible maxima was fit to one or more exponential curves using both the Hi-Tech data analysis software and KaleidaGraph 4.0, yielding values of $k_{\text{obs}}$. Full spectra (300–700 nm) were fit in parallel to a two-step model via singular value decomposition using the SpecFit software. Reported rate constants are averages of three measurements (error $= \pm 1$ S.D.).

Shunt Reactions with H$_2$O$_2$ and Peracetic Acid—5 $\mu$M IsdG-heme in 50 mM KP$_2$, pH 7.4 (2 ml), was rendered anaerobic by multiple cycles of headspace gas exchanges with argon carried out on a vacuum manifold and then brought into an anaerobic glove box (Coy). Reactions were initiated by addition of 1–5 eq of H$_2$O$_2$ or peracetic acid. Spectra were recorded every 2 min at 22 °C until they ceased changing. The anaerobic reactions were exposed to air, and changes to UV/vis spectra were subsequently measured. Samples for MS analyses were frozen in liquid N$_2$.

Extraction of Tetrapyrrrole Products—Three methods were applied to remove bound products from IsdG at the end of the heme-decomposing reaction. First, concentrated HCl was used to adjust the pH to 2 followed by addition of 2 volumes of 2-butanol. The mixture was vortexed and centrifuged, and the organic layer containing the extracted products was removed and washed with 150 mM NaCl. Second, 1 volume of a 1:1 H$_2$O/ACN mixture with 0.1% trifluoroacetic acid (TFA) was added to the IsdG/product mixtures, followed by centrifugation to pellet the precipitated proteins. The supernatants were subsequently filtered through Centricon Plus spin filters (5 kDa, Millipore) at 4000 rpm. Finally, reactions were applied to a superclean C-18 solid phase extraction column (Supelco), and tetrapyrrole products were eluted twice with 200 $\mu$L of 50 mM KP$_2$, pH 7.4 (flow rate 1 mL/min). Extracted products were lyophilized in a mini-SpeedVac.

Tetrapyrrrole Product Analysis by Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS)—Analyses were performed on an Agilent 1290 UPLC series chromatography instrument coupled to a 6538 UHD Q-TOF mass spectrometer (Agilent Technologies) operated in both positive and negative ion modes. Samples (15 $\mu$L) were separated on a Zorbax RRHD Eclipse Plus C-18 column (150 $\times$ 2.1 mm; 1.8 $\mu$m, Agilent Technologies) at 700 $\mu$L/min using the following conditions: 2 min, 5% B; 2–15 min, 5–90% B; 15–17 min, 90% B (50 °C), where solvent A = 0.1% FA in water and solvent B = 0.1% FA in ACN. Electrospray conditions in both targeted and auto-MS/MS modes were as follows: drying gas flow 12.0 liters/min at 350 °C, nebulizer 55 pounds per square inch, and capillary 3500 V, fragmentor 120 V, and skimmer 45 V. Scan range was 50–1300 m/z (auto-MS/MS) and 50–800 m/z (targeted MS/MS) with isolation width 4 m/z and an acquisition rate of 1 spectrum/s. The collision energy was fixed at 35 V in targeted MS/MS mode, whereas a linear voltage gradient was applied for molecules fragmented in auto-MS/MS experiments. Data acquisition and spectral analysis were performed using MassHunter (Qualitative Analysis version B.04.00, Agilent Technologies).

Quantification of C1 Products—IsdG-heme (200 $\mu$L) was incubated with ascorbate/air under the same conditions used for time-resolved UV/visible spectroscopy and in proteo MS (see above) and then analyzed for formaldehyde (the major C1 product previously detected) using Nash’s reagent (see below and see Ref. 18). Reactions were filtered in a 3-kDa centrifuge concentrator to remove the IsdG protein and any tightly bound products. The filtrate was reacted 1:1 (v/v) with Nash’s reagent, in addition to no-ascorbate controls. The retentate, extracted retentate, and a whole reaction sample in which the protein had not been removed by filtration were all independently reacted with Nash’s reagent, in addition to no-enzyme and no-ascorbate controls.

Samples were incubated at 37 °C for 30 min to allow acetyl acetone to couple to any formaldehyde that might be present. A set of formaldehyde standards (0–30 $\mu$L) was generated using incubation conditions identical to those above. The samples
and standards were analyzed via HPLC on an Agilent 1100 series instrument with UV/vis detection (412 nm). For HPLC, a 150 × 5 mm Phenomenex Luna C-18 column was used at a flow rate of 1.5 ml/min. Elution conditions were as follows: 3 min, 5% B; 3–12 min, 5–95% B; 12–15 min, 95% B at 37 °C; solvent A = 0.1% TFA; solvent B = ACN + 0.1% TFA. A standard curve of the integrated peak area for the formaldehyde/acetyl acetone product (sharp peak, 7.1 min) was constructed from the formaldehyde standard samples, and the curve was used to calculate [formaldehyde] in each sample.

Results and Discussion

Little is known about the steps by which proteins from the IsdG family degrade heme. The reaction can be readily monitored via the disappearance of the intense UV/vis Soret band associated with the aromatic heme macrocycle (λ_max (Soret) = 411 nm; ε = 91 mM⁻¹ cm⁻¹) (16–18). Heme degradation is typically initiated by adding excess ascorbate in the presence of air and H_2O_2-scavenging catalase to the IsdG-heme complex. Fig. 2A illustrates the subsequent changes in the heme UV/vis spectrum monitored over time for 30 min, when changes to the UV/vis spectra appear to cease (10 μM IsdG-heme, ascorbate, 50 mM KP_i, pH 7.4, 22 °C). Despite the likelihood that the reaction occurs via multiple microscopic steps, the Soret band diminished over time in an apparent single exponential phase. This was fit to give a first-order rate constant (k_Soret = 0.13 ± 0.03 min⁻¹; all reported first-order rate constants are averages of ≥3 independent measurements; error = ±1 S.D.). No intermediates were detectable, and the final spectrum resembled those previously published for similar experiments (λ_max = 412 nm and 460 nm shoulder) (17). The value for k_Soret was insensitive to pH over pH 5–10 (data not shown), a pH range over which the reductant (pKa values of 4.2 and 11.6) is expected to exist predominantly in its singly deprotonated, anionic form. This insensitivity is despite the observation of a transition to an alkaline species with a distinct UV/vis spectrum in this pH range, which was ascribed to formation of the ferric-OH complex at high pH in the related IsdI enzyme (23). However, k_Soret depended linearly on the initial ascorbate concentration (k = 10 ± 0.1 M⁻¹ min⁻¹, see Fig. 2B). This suggested that the reaction step that controls k_Soret is likewise dependent on reducing equivalents and independent of whether the enzyme is in its acidic or alkaline form.

Although a convenient probe of the reaction, conventional UV/visible spectroscopy has several drawbacks as a tool for discerning the mechanism. First, spectra for the starting material, several potential intermediates, and products likely overlap. Second, the acute intensity of the Soret absorbance is attributed to aromaticity in the heme macrocycle; k_Soret is therefore expected to reflect predominantly the kinetic step involving loss of aromaticity (for example, scission of the macrocycle). The existence of other steps and intermediates may consequently be masked by monitoring the reaction at the Soret maximum. Finally, conventional UV/visible spectroscopy with manual mixing has relatively poor time resolution. Rapid or early events in the overall reaction, or sequential events with similar time constants, are not expected to be observed by this method.

We therefore sought means for monitoring the IsdG-mediated reaction that might be more sensitive to the presence of tetrapyrrole intermediates and therefore more powerful mechanistic probes. Time-resolved in proteo MS was of particular interest because the anticipated changes in mass associated with the reaction, including addition of three oxygen atoms (16 Da each) and loss of CH_3OH (30 Da) from the tetrapyrrole, are large. Moreover, the reaction is relatively slow (t_1/2 (Soret) ~5 min; 50 mM KP_i, pH 7.4, 22 °C). Time-resolved MS therefore seemed ideally suited for monitoring IsdG intermediates and products, particularly ones that have less intense or overlapping absorbance in their UV/visible spectra relative to heme or which are too unstable to be extracted from the protein in unaltered form.
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FIGURE 3. IsdG reaction was monitored using time resolved in proteo MS. A, mass spectrum was measured at t = 0, showing the heme (616.17 Da) and protein envelope. B, integrated intensities of the extracted ion chromatograms (m/z ± 0.01) measured over time for the species with exact masses 616.17 (red), 611.24 (green), and 599.24 (orange) Da. Intensities for the 616.17 Da species (heme) are given on the left y axis; intensities for the other two species are given on the right y axis. The data were fit to single exponential curves as described in the text. Data from a representative experiment are shown; reported rate constants are the average of three values. Conditions were the same as reported in Fig. 2A.

In proteo MS conditions were adjusted to allow quantitative tracking of tetrapyrroles bound to IsdG (Fig. 3A). The disappearance of a species with an exact mass/charge ratio (m/z) of 616.17 (heme b, Fig. 1) occurred in a single exponential kinetic phase with a rate constant $k_{616.17} = 0.52 \pm 0.09 \text{ min}^{-1}$ (Figs. 3B and 4A), four times the value of $k_{\text{Soret}}$.

In further contrast with the UV/vis data, at least three heme-derived species could be detected by time-resolved MS, starting with a species appearing transiently in positive ion mode at 0.5 min after initiation of the reaction. Reactions measured at 1.5 and 2 min did not contain this species, suggesting that it is relatively short lived. Its exact mass (632.15 m/z), is consistent with the addition of an oxygen atom (16.01 m/z) to the heme, possibly at one of the meso-carbons of the tetrapyrrole (Fig. 1). Even at concentrations nearing the saturation limit of the detector (50 µM IsdG-heme), too little of this species accumulated to allow for further characterization of its structure by collision-induced dissociation (CID) MS/MS fragmentation. However, the exact mass and isotope pattern measured at t = 0.5 min (Fig. 4B) are consistent with the expected formula for a meso-hydroxymyoglobin ($\text{C}_{34}\text{H}_{42}\text{FeN}_{6}\text{O}_{5}$, [M$^+_{\text{H}}$]). The [M$^+_{\text{H}}$] species was previously observed in prior studies of meso-hydroxymyoglobin generated inside H$_2$O$_2$-treated metmyoglobin (24). Furthermore, the presence of iron in this molecule, as in the isotopically resolved mass spectrum for heme (Fig. 4A), is definitively reflected by the observation of two low intensity, low molecular weight isotopes (m/z = 630.15 and 631.15). Hence, this intermediate appears to correspond to heme with one oxygen atom incorporated. Although further in-depth characterization of the intermediate is needed, the available data are consistent with its proposed description as β- or δ-meso-hydroxymyoglobin.

A second, iron-free species with an m/z of 611.24 (Fig. 4C) accumulated to a more significant level, forming with a first-order rate constant that was the same within error as $k_{\text{Soret}}$: $k_{611.24} = 0.16 \pm 0.03 \text{ min}^{-1}$ (Fig. 3B). This species grew to substantial intensity before decaying very slowly over a period of >12 h. The final species detected by in proteo MS had the exact mass of staphylobilin (599.24 m/z, Fig. 4D), forming with a fitted first-order rate constant $k_{630.14} = 0.22 \pm 0.05 \text{ min}^{-1}$ (Fig. 3B).

Taken together, these results are consistent with the initial rapid conversion of heme to a short lived meso-hydroxymyoglobin (632.15 Da) intermediate and then to a species resembling mycobilin (exact mass 611.24 Da, see Fig. 1). Macrocycle cleavage would presumably occur not at the heme α-meso-carbon but rather at the β- or δ-meso-carbons, with formyl and oxo groups generated on pyrrole rings B/C or A/D to make a formyloxobilin mixture. The similarity in the measured values for $k_{\text{Soret}}$ and $k_{611.24}$ suggested that loss of heme aromaticity is kinetically coupled to formation of this putative formyloxobilin. In the simplest interpretation of the data, both rate constants could be ascribed to the roughly concomitant opening...
(k_{Soret}) and second oxygenation (k 611.24) of the heme macrocycle. The final observed tetrapyrole appeared to be staphylobilin (599.24 Da). The comparable values for k 611.24 and k 599.24 (the same within error) and the failure of 611.24 intensity to diminish as the 599.24 grew argues against a precursor-product relationship between these two species. Instead, although a formyloxobilin would logically form prior to staphylobilin, the kinetic data suggest that the formyloxobilin and staphylobilin appear roughly concurrently.

To test this hypothetical model, the 611.24- and 599.24-Da species, both of which accumulated at the apparent end of the reactions shown in Figs. 2A and 3B (t = 30 min), were further characterized by UPLC-MS/MS. Species with m/z = 599.24 eluted cleanly as two UPLC peaks (at 9.6 and 9.9 min). Each yielded UV/vis (Fig. 5A) spectra and MS/MS fragment patterns (obtainable in both positive and negative ion modes) identical to those previously published for staphylobilin isomers β and δ (data not shown) (17).

By contrast, the UPLC trace for the species with m/z = 611.24 was far more complicated (Fig. 5B), and it was composed of several temporally resolved peaks. This result is perhaps less surprising in light of the four formyloxobilin isomers that might, in principle, be observed: two cleaved at the δ-meso-carbon, which is converted to a formyl group appended to ring A or D; and the analogous pair of isomers cleaved at the β-meso-carbon with the formyl group on ring B or C.

The most prominent LC peak (retention time = 6.6 min) was extensively characterized by CID MS/MS (Fig. 5C). The intensity of the data in negative ion mode was too low; therefore, the positive ion mode was analyzed. The most abundant fragments were generated by formation of cations containing both pyrrole rings A and B and an appended formyl group, with molecular ion labels are color-coded to match fragments containing formyl

The prominent 6.6-min peak therefore appeared to correspond to either 10-formyl,11-oxo-bilin or 20-formyl,19-oxo-bilin, or a mixture of the two.

Systematic MS/MS analysis of m/z 611.24 peaks eluting between 4 and 8 min confirmed that the fragments diagnostic of formyloxobilin (m/z = 279.11 and 251.11) were ubiquitous. Moreover, several of the same fragments as in Fig. 5C were detected in the peak at 4.8 min, although less prominently, in addition to fragments diagnostic of formylation at the C and/or D rings (m/z = 241.11 and 267.11, [M]⁺). Together, these results suggest that the m/z 611.24 species is a formyloxobilin that is present as more than one structural isomer (see Fig. 1).

To further explore the intermediacy of a meso-hydroxyl heme, we attempted to isolate this species using either H_2O_2 or peracetic acid. Classic experiments with human HO-1 (hHO-1) showed that the ferric heme-HO complex converted to the α-meso-hydroxyl heme within 4 min of the anaerobic addition of 1 eq of H_2O_2 (23 °C) (21). Exposure of this species to air brought about its rapid (within ~5 s) and quantitative conversion to verdoheme and CO, where verdoheme (visible absorbance ~690 nm) is an intermediate on the pathway to biliverdin-IXα. Peracids yielded neither verdoheme nor biliverdin-IXa, suggesting that a ferric heme-hydroperoxy species was an obligate hHO-1 intermediate.
Using the ferric IsdG-heme complex, the same experiments were repeated here. Following anaerobic treatment with either H$_2$O$_2$ or peracetic acid, the Soret peak diminished slightly, consistent with the possible formation of meso-hydroxyheme (Fig. 6). However, upon exposure of the oxidant-treated species to air, the UV/vis spectra changed little. In particular, the prominent absorbance band centered near 690 nm and known to be responsible for the characteristic green color of verdoheme (21) was not observed. MS analyses of the air-treated products revealed neither staphylobilin nor biliverdin IX$\alpha$. Instead, the overall amount of heme appeared to diminish, and a very small amount of 611.24 species formed specifically in the H$_2$O$_2$-treated sample. These results suggest that either the meso-hydroxyheme is not an intermediate in the IsdG-catalyzed reaction or that this intermediate forms and reacts in a manner that is distinct from its hHO counterpart.

The latter explanation is plausible because the heme environment in HOs and IsdGs is highly distinct (25, 26). In particular, the IsdG-bound ferric heme, uniquely, is profoundly distorted from planarity (ruffled) (12). The vicinity of the meso-carbons, unlike in HOs, is devoid of water or conserved polar residues. Moreover, although a mechanism leading from this intermediate to either the IsdG/hHO products can be drawn, only the hHO meso-hydroxyhemeproducts are expected to convert to verdoheme plus CO. The IsdG enzyme would therefore have to have some way of blocking the analogous conversion of a $\beta$- or $\delta$-meso-hydroxyheme intermediate. Hence, inside the IsdG environment, the meso-hydroxyheme would almost certainly have to be structurally or electronically distinct from its hHO counterpart.

We next asked whether the higher resolution kinetic analysis afforded by stopped-flow UV/visible spectroscopy might provide evidence for early or other reaction intermediates. Indeed, like the time-resolved MS, stopped-flow experiments monitoring the reaction between aerobic solutions of IsdG-heme and ascorbate gave evidence for a multistep heme degradation process. Time traces for absorbances at the Soret (411 nm) (Fig. 7A)
and charge transfer band (500–600 nm) (Fig. 7B) regions were both best modeled as sums of single exponential functions, indicating at least two detectable kinetic events.

Qualitatively similar results were obtained for both regions of the UV/vis spectrum. For the Soret (Fig. 7A) maximum, a fast phase ($k_1 = 0.63 \pm 0.06 \text{ min}^{-1}$) followed by a second slower decay phase ($k_2 = 0.26 \pm 0.04 \text{ min}^{-1}$; 10 mM ascorbate, 50 mM KPi, pH 7.4, 22 °C) could be readily modeled. The majority of the fast phase ($t_{1/2} = 1 \text{ min}$) was complete before the Soret band diminished appreciably in intensity ($t_{1/2}$ (Soret) = 5 min), consistent with the possible conversion of strongly absorbing heme to a slightly less strongly absorbing meso-hydroxyheme, as expected from work with hHO (21). Instead, most of the Soret intensity was lost in the slower second phase ($k_2$), which dominates the kinetic trace at 411 nm and has a rate constant that is approximately the same as both $k_1$ and $k_2$. The full spectral data set was subsequently fit using singular value decomposition, yielding values for $k_1$ and $k_2$ and an intermediate component spectrum (Fig. 7C) that were consistent with analysis of the data at the Soret maximum.

Together, these results suggested that $k_2$, $k_{Soret}$, and $k_611.24$, measured by three different methods, are each predominantly controlled by the same reaction step. This step is most plausibly associated with the conversion of a meso-hydroxyheme intermediate to a formyloxobilin in which the macrocycle is cleaved and the iron has been released. Notably, neither $k_1$ nor $k_2$ exhibited a strong dependence on $[O_2]$, although each had a measurable linear dependence on ascorbate concentration (second-order rate constants for $k_1$ and $k_2$ are 69 and 13 m$^{-1}$ min$^{-1}$, respectively, see Fig. 7A, inset). This again indicated that the kinetic events described by these constants are rate-limited by the delivery of reducing equivalents (ascorbate).

In combination, these results suggest a model (Fig. 8) whereby the ferric IsdG-heme complex rapidly ($t_{1/2} \sim 1 \text{ min}$) converts to a ($\beta/\delta$)-meso-hydroxyheme intermediate, which then more slowly ($t_{1/2} \sim 3 \text{ min}$) goes on to form a set of formyloxobilin isomers. A subpopulation of these converts rapidly to $\beta/\delta$-staphylobilins. However, a sizable fraction stays unconverted and appears to remain associated with the protein in the formyloxobilin precursor state (~55% based on integrated extracted ion chromatogram intensities measured by UPLC for the 611.24- and 599.24-Da species and assuming identical ionization efficiencies). Measurements of the product distribution over longer time periods indicated that the formyloxobilins and staphylobilins, which are known to degrade readily under ambient light (17), both decay over a period of ~12 h.

If both formyloxobilin and staphylobilin species are present at the end of the IsdG reaction (as assessed by monitoring changes at the Soret maximum by UV/vis, Fig. 2A), why have only staphylobilins been isolated in previous analyses of IsdG (tetrapyrrrole) products? We hypothesized that the staphylobilins observed in Fig. 3A arose from one, possibly isomer-dependent, group of formyloxobilin precursors that spontaneously released formaldehyde. The other group might be more stable when associated with IsdG. Release of the protein into the buffer medium could induce their spontaneous conversion to staphylobilin isomers and formaldehyde. In reaction mixtures from which the protein was extracted, only staphylobilins would be detected.

To address this hypothesis, IsdG reaction mixtures were fractionated and analyzed quantitatively for their formaldehyde and bilin content (Table 1). When the protein was filtered from

![Summary of the kinetic scheme determined using in proteo MS and conventional time-resolved and stopped-flow UV/vis approaches.](image)

**TABLE 1**

Stoichiometries of formaldehyde produced following the IsdG-mediated breakdown of heme

| Sample/fraction | eq of CH$_2$O |
|-----------------|---------------|
| IsdG-heme (control) | 0 |
| Whole reaction mixture (untreated) | 0.4 ± 0.1 |
| Whole reaction mixture (solid phase extracted) | 0.9 ± 0.1 |
| Filtrate (3000 MWCO) | 0.5 ± 0.1 |
| Retentate (solid phase extracted) | 0.5 ± 0.1 |
the reaction mixture following 40 min of reaction time, much of the characteristic orange color of the tetrapyrrole product remained associated with the protein-containing retentate. MS analysis of this fraction showed that it contained both staphylobilin and formyloxobilin, with a roughly even ratio in their integrated extracted ion chromatogram peaks. By contrast, the filtrate contained no observable bilins.

Parallel analyses for formaldehyde showed that 0.5 (±0.1) eq could be detected in the filtrate and no CH₂O in the washed, protein-containing retentate fraction. Following solid phase (C-18 column) extraction to remove protein-bound tetrapyrroles from the retentate, the protein component became colorless. An additional 0.5 (±0.1) eq of CH₂O emerged in the extract. Moreover, the bilin component of the extract shifted strongly in favor of staphylobilins (93:7), according to their integrated extracted ion chromatograms. HPLC analysis further confirmed that, in reaction mixtures in which ACN was used to precipitate the protein or either 2-butanol or a C-18 column to extract the tetrapyrroles, only staphylobilins were detected.

Consistent with these and prior observations (18), a total of 0.9 (± 0.1) eq of CH₂O were detected in whole reaction mixtures that had been passed over the solid phase extraction column. Collectively, these results suggest that some formaldehyde remained protein-associated, likely appended to the 611.24-Da species, until the tetrapyrrole was released from the protein. Notably, the addition of further equivalents of ascorbate and/or O₂ did not accelerate conversion of this protein-associated species to staphylobilin and CH₂O.

Conclusions

The results presented here are consistent with a model for the S. aureus IsdG reaction that has elements in common with both HO and MhuD. An initial intermediate, tentatively assigned as a mixture of β/δ-meso-hydroxyhemes, is analogous to the α-meso-hydroxyheme of HOs, although the two enzyme families carry the intermediate forward toward different fates.

The observation of a formyloxobilin as a second likely staphylobilin intermediate is chemically intuitive and satisfying from a biological perspective. As described above, the formyloxobilin known as mycobilin is the final reaction product of the IsdG homolog, MhuD (Fig. 1). Formyloxobilins are also observed as intermediates in plant chlorophyll degradation, as products of O₂-mediated chlorin ring cleavage by the enzyme pheophorbide a oxygenase (27). Interestingly, a cytochrome P₄₅₀ was recently discovered that oxidatively frees formaldehyde from the chlorophyll derivative (28), converting the formyloxobilin into a staphylobilin-like dioxygen product (28). There is no obvious candidate P₄₅₀ to carry out the analogous reaction in S. aureus. However, enzymes in the IsdG family belong to a class of cofactor-independent monooxygenases, where reactions very similar to the proposed oxidative deformylation are known to occur autocatalytically (29). Auto-catalytic O₂-dependent release of the formyl group would therefore appear to be plausible for members of the IsdG family. However, uncatalyzed conversion of the formyloxobilins to staphylobilins plus CH₂O appears to occur when the former are released from the protein and into aerobic aqueous solution.

Why the MhuD product does not spontaneously release formaldehyde, and why the product of the S. aureus IsdG appears to generate formaldehyde from only some of its formyloxobilin population are not clear. Given the biological roles of the latter enzyme as both a catalyst and a sensor of S. aureus cellular heme status (30,31), it may be that the tendency of IsdG to retain at least some of the formyloxobilin, which is slowly released to yield formaldehyde, has some yet-known biological role. It is likewise possible that the affinity of the formyloxobilin for IsdG may be isomer-dependent or that another protein is involved in facilitating formyloxobilin release. These are questions that will be addressed in future work.

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