Radical new paradigm for heme degradation in *Escherichia coli* O157:H7

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All of the heme-degrading enzymes that have been characterized to date require molecular oxygen as a cosubstrate. *Escherichia coli* O157:H7 has been shown to express heme uptake and transport proteins, as well as use heme as an iron source. This enteric pathogen colonizes the anaerobic space of the lower intestine in mammals, yet no mechanism for anaerobic heme degradation has been reported. Herein we provide evidence for an oxygen-independent heme-degradation pathway. Specifically, we demonstrate that ChuW is a radical *S*-adenosylmethionine methyltransferase that catalyzes a radical-mediated mechanism facilitating iron liberation and the production of the tetrapyrrole product we termed "anaerobilin." We further demonstrate that anaerobilin can be used as a substrate by ChuY, an enzyme that is coexpressed with ChuW in vivo along with the heme uptake machinery. Our findings are discussed in terms of the competitive advantage this system provides for enteric bacteria, particularly those that inhabit an anaerobic niche in the intestines.

heme | pathogen | anaerobic | intestinal microbiome | radical SAM

Iron acquisition is essential to the survival of all cellular organisms and is a major barrier for a microorganism during pathogenesis of a mammalian host (1). For example, an enteric pathogen entering through the digestive tract must compete with the host, as well as other intestinal microflora for iron. Interestingly, the most abundant source of dietary iron is found in heme (2), a molecule that is also responsible for essential cellular processes by serving as a cofactor in a variety of enzymes (3). The degradation of heme also plays an important role in iron homeostasis and cell signaling in cyanobacteria, plants, and mammals (1, 4). Considering the bioavailability of dietary heme and the importance of iron as a nutrient, it is not surprising that pathways have evolved in pathogenic bacteria to transport and degrade heme for the sole purpose of iron acquisition (5). Furthermore, the ability to accomplish the liberation of iron from heme under strictly anaerobic conditions would be advantageous to the enteric bacteria that can inhabit certain niches of the intestines.

Enzymes that catalyze the opening of the porphyrin ring have been well characterized and are collectively referred to as heme oxygenases (6). This classification is based on the common mechanistic property of activating molecular oxygen by a "P450-like" mechanism to catalyze the oxidative degradation of the heme cofactor (7–9). Recently, two heme oxygenases have been characterized in the organisms *Staphylococcus aureus* and *Mycobacterium tuberculosis* that degrade heme to the unique chromophores staphylobilin (10) and mycobilin (11), respectively. Although molecular oxygen is still required, neither of these degradation mechanisms result in carbon monoxide production, presumably due to the pathophysiology of these organisms (12). These reports significantly advance the hypothesis that organisms evolved a variety of heme degradation mechanisms to satisfy their own physiological needs (13), thus raising the following question: how do hemolytic organisms use heme as an iron source during infection or colonization in an anaerobic environment?

Vibrio cholerae and *Escherichia coli* O157:H7 are hemolytic enteric pathogens that colonize the nonsterile region of the lower intestines. Despite their ability to use heme as an iron source, a bona fide heme oxygenase has not been characterized for either organism. However, both organisms contain a heme uptake operon that is up-regulated during iron duress (14, 15). The operon encodes for the heme uptake and transport machinery, as well as three additional genes, *chuW*, *chuX*, and *chuY*. (Fig. 1*A*) (16). ChuW is annotated as a member of the radical *S*-adenosylmethionine (SAM) superfamily, a large class of enzymes that perform a wide array of difficult chemical reactions (17, 18). Enzymes belonging to the radical SAM superfamily share common cofactor requirements and mechanistic features, such as the coordination of a redox active [4Fe-4S] cluster ligated by three protein-derived cysteine residues, often located within a CX_3CX_2C motif. This coordination environment facilitates the interaction of SAM at the unique iron site of the cluster. In the reduced state (formally 1+), the [4Fe-4S] cluster reductively cleaves SAM, forming a highly oxidative 5′-deoxyadenosyl-5′-radical (5′-dA•) that is subsequently used for catalysis. ChuW was originally annotated as the radical SAM enzyme HemN, an anaerobic coproporphyrinogen oxidase. HemN is involved in the anaerobic biosynthesis of heme, performing two subsequent decarboxylation reactions to produce protoporphyrinogen IX (19). However, previous work has shown that ChuW homologs do not retain the essential functional motifs, nor do they rescue a HemN KO in *Salmonella enterica*, and thus likely catalyze a different reaction (14, 20). A role in heme biosynthesis is also inconsistent with the location of *chuW* in an iron-regulated operon adjacent to genes that are required for heme uptake (Fig. 1).

Sequence alignments indicate that ChuW has homology to the radical SAM methyltransferases (RSMTs). RSMTs are a subfamily of radical SAM enzymes that function in the transfer of

Significance

The ability of pathogenic microorganisms to acquire iron from heme has been shown to be a valid antimicrobial target. However, all of the known mechanisms for heme catabolism that lead to iron release in bacteria and higher eukaryotes are dependent on molecular oxygen as a cosubstrate. The human gut is dominated by strictly anaerobic bacteria, and in this work, we provide evidence for anaerobic heme degradation in enteric pathogens. The anaerobic mechanism is significantly different from what has been observed in other bacteria and may provide a unique opportunity for a new class of antimicrobial compounds.

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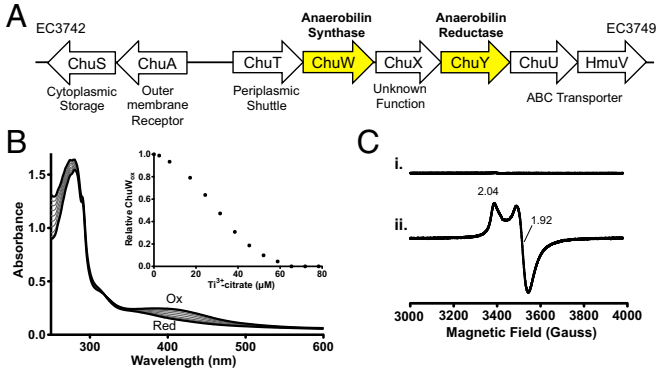

Fig. 1. Genetic organization of the heme utilization operon in *E. coli* O157:H7 and characterization of the Fe-S cluster in purified ChuW. (*A*) ChuW is genetically adjacent to the heme uptake machinery that is expressed during iron starvation. (*B*) UV-visible spectra of isolated ChuW and reduction of the Fe-S cluster. Scans were recorded after injecting with equivalents of Ti^{3+} citrate. (*Inset*) Percentage of oxidized ChuW based on the relative change in the adsorption at 400 nm plotted with respect to the concentration of Ti^{3+} citrate added. (*C*) EPR spectra of ChuW in the absence and presence of 1 mM sodium dithionite (top and bottom trace, respectively). Spectra are a result of three scans recorded at 10 K with 1-MW microwave power, a modulation frequency of 100 kHz, and modulation amplitude of 6.477 gauss (G).

methyl groups to unreactive carbon atoms and, in some cases, promote significant chemical rearrangements (21, 22). However, they are the least understood of the four distinguished classes (23), making it difficult to determine function or mechanism using only a bioinformatics approach. To address the precise function of ChuW from the enterohemorrhagic serotype of *E. coli* O157:H7, we isolated and characterized the recombinant enzyme. Our data provide in vitro evidence that ChuW functions as an anaerobic heme-degrading enzyme that uses a mechanism similar to what has been reported for other RSMTs.

Results

Isolation of ChuW with an Intact [4Fe-4S] Cluster. Similar to what has been reported for other radical SAM enzymes, the most stable form of ChuW was obtained when we coexpressed the *Azotobacter vinelandii* iron sulfur cluster (*isc*) biosynthesis genes (pDB1282 vector, a generous gift from Dennis Dean, Department of Biochemistry, Virginia Tech University, Blacksburg, VA) and *chuW* in *E. coli* using minimal media that was supplemented with exogenous iron and cysteine during expression. Isolation of ChuW using this approach yielded a protein with 3.8 ± 0.2 irons per monomer of purified enzyme, following the reconstitution and purification procedure described in *SI Materials and Methods*. We investigated the spectroscopic characteristics of the reconstituted enzyme using UV-visible spectroscopy. The purified enzyme (20 μM) exhibited a broad absorption feature with a maximum near 400 nm, indicative of the coordination of a [4Fe-4S] cluster (Fig. 1*B*). Injecting incremental aliquots of Ti^{3+}-citrate into the sample resulted in a bleaching of color and decreased the absorption feature around 400 nm. The relative percentage of oxidized ChuW was plotted with respect to the concentration of Ti^{3+}-citrate injected (Fig. 1*B*, *Inset*) and suggests a single electron reduction of the cluster. The oxidation state of the [4Fe-4S] cluster was further investigated by electron paramagnetic resonance (EPR) (Fig. 1*C*). In the presence of sodium dithionite, we observed an axial EPR spectrum ($g_{||} = 2.04$, $g_{\perp} = 1.92$; Fig. 1*C*, spectra ii) that is typical for radical SAM enzymes that contain only the catalytic [4Fe-4S] cluster (24, 25). As expected, the [4Fe-4S] cluster of ChuW was extremely oxygen sensitive, and enzyme activity was lost if exposed to any level of molecular oxygen.

Flavodoxin Is the Electron Source for the Radical SAM Enzyme ChuW. In addition to being a radical SAM enzyme, sequence analysis indicates that ChuW is distinct from HemN and more similar to the class C RSMTs (Fig. S1). Therefore, we looked for both 5′-deoxyadenosine (5′-dA) and *S*-adenosylhomocysteine (SAH) production during ChuW turnover by HPLC. In our assays, we used the heme analog deuteroheme as the substrate due to the insolubility of hemin. Consistent with what others have reported, we observed "abortive cleavage" of SAM to 5′-dA in the absence of deuteroheme when using the chemical reductant sodium dithionite (Fig. S2*A*). Minimal SAH production was observed when deuteroheme was absent and the chemical reductant was used (Fig. S2*B*). However, when using the *E. coli* flavodoxin (EcFldA)/ferredoxin (flavodoxin):NADP+ oxidoreductase (EcFpr)/NADPH system (26), abortive cleavage was not observed, and both 5′-dA and SAH were produced at 1:1 stoichiometries only when substrate was present (Fig. S2*C*, trace *ii*). These observations further underscore the importance of using a physiological electron source for the in vitro characterization of radical SAM enzymes (26–28). Therefore, we used the physiological electron donor system in place of a chemical reductant. Quantitation of the total iron that was subject to chelation (labile iron) was also performed. Measurements of the labile iron were performed at the start of the reaction and after 30 min. It should be recognized that the catalytic cluster is fragile and also subject to chelation in contrast to iron bound to the porphyrin. Notably, we observe an increase in the labile iron during ChuW turnover; specifically, we measured 227 ± 20 μM labile iron at the start of the assay and 302 ± 27 μM after a 30-min reaction (Fig. S2*D*).

Heme, Mesoheme, and Deuteroheme Are Substrates for ChuW. No notable changes in the UV-visible spectrum of heme could be directly associated with heme-iron coordination by ChuW. However, when all of the components required for RSMT activity were present, distinct absorption changes for heme or deuteroheme (between 350 and 900 nm) were observed during a 30-min reaction (Fig. 2 *A* and *B*, respectively). Turnover of mesoheme was also monitored by UV-visible spectroscopy, and the products were analyzed by MS (Fig. S3*A*). Under our assay conditions, deuteroheme was soluble and preferentially used as a substrate, although similar results were obtained with mesoheme and hemin. For deuteroheme, turnover resulted in a decrease in its major Soret band at 407 nm, with a corresponding increase in absorbance at 439 and 780 nm (Fig. 2*A*). Based on the isosbestic point observed at 418 nm and the initial concentration of deuteroheme, we calculated an extinction coefficient for the product(s) absorbing at 780 nm to be 31.5 $mM^{-1}{\cdot}cm^{-1}$. Similar results were obtained when the physiological substrate, heme, is used in the assay, albeit with red-shifted spectral features (Fig. 2*B*). The calculated extinction coefficients for the product were used to determine the specific activities by plotting the initial rate of product formation with respect to the concentration of ChuW (Fig. 2, *A* and *B*, *Inset*). From this analysis the specific activities were calculated to be 5.5 and 8.4 $nmol{\cdot}min^{-1}{\cdot}mg^{-1}$ for deuteroheme and heme, respectively. We have not established an accurate V_{max} for any of the ChuW catalyzed reactions and therefore cannot report a turnover number. In addition, we are not using a coupled assay and cannot account for potential feedback inhibition of product, as has been observed for the canonical heme oxygenase. Finally, we cannot account for any photodegradation that may be occurring. Given this precedent, it is possible that we are underestimating the true rate and the development of a coupled assay is underway. However, to make a catalytic comparison, it is useful to look at the rate relative to the molar amount of ChuW in the assay. In this case, the enzyme is catalyzing the conversion of 0.289 and 0.442 $nmol{\cdot}min^{-1}{\cdot}(nmol\ ChuW)^{-1}$ for deuteroheme and heme, respectively. By comparison, using a coupled assay, hHO-1 has been shown to consume heme at a rate of ~3.3 $nmol{\cdot}min^{-1}{\cdot}(nmol\ of\ hHO\text{-}1)^{-1}$ (29). Consistent

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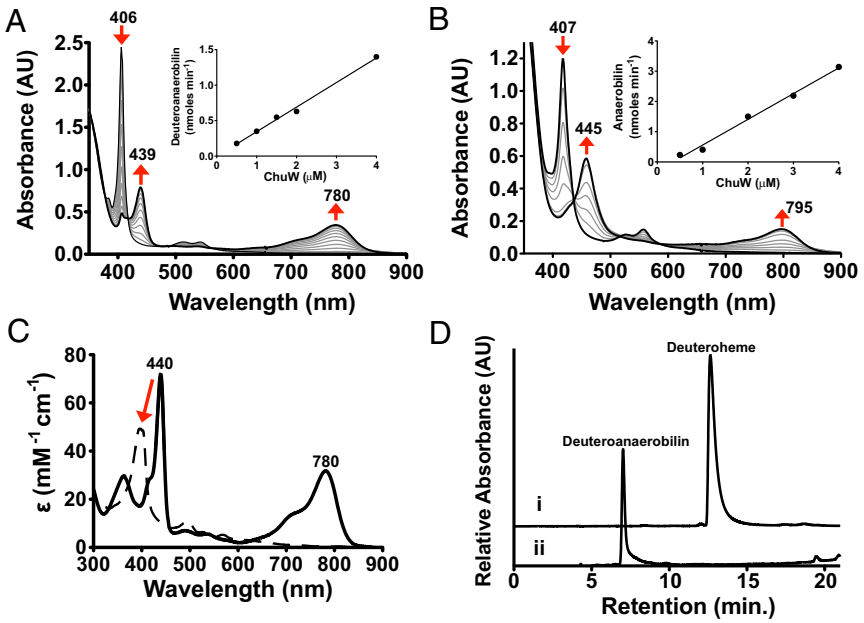

Fig. 2. Characterization of anaerobic ChuW-dependent heme degradation by UV-visible spectroscopy. UV-visible spectroscopic assays were performed by adding ChuW (2 μM) to a solution containing 10 μM deuteroheme (*A*) or heme (*B*), respectively, as well as the EcFld/EcFpr electron donor system (5 and 2 μM, respectively) with 200 μM NADPH. Assays were initiated by the addition of SAM (250 μM), and spectra were recorded every 2 min over the course of 30 min. The red arrows indicate the direction of spectral changes during the course of the experiment. (*Insets*) Plot of the product formation rate at various ChuW concentrations. (*C*) The UV-visible spectrum of isolated DAB in methanol resulted in similar spectroscopic features observed in the enzyme assay (solid spectra). Exposure to sunlight resulted in significant changes to the spectra (dashed line). (*D*) HPLC chromatogram (detection at 397 nm) following the isolation of the deuteroheme substrate and the product of ChuW turnover, herein termed DAB.

with the heme oxygenase literature, we have named products of ChuW-catalyzed heme, deuteroheme, and mesoheme degradation "anaerobilin," "deuteroanaerobilin," and "mesoanaerobilin," respectively.

To assess the properties of the tetrapyrrole that was produced, we isolated the deuteroheme-derived product, deuteroanaerobilin (DAB). Exposure of DAB to sunlight for 5 min resulted in a significant change in the UV-visible spectrum (Fig. 2*C*, dashed spectrum). In contrast, if the isolation procedure was performed while avoiding light exposure of the sample, the isolated tetrapyrrole had a spectra that was identical to the absorption spectrum observed at the end of the assay (Fig. 2*C*, solid spectrum). These data demonstrate that, similar to biliverdin, DAB is photolabile and must be isolated in the absence of light. HPLC analysis of DAB was performed to ensure ChuW catalyzes the formation of a single product. Although deuteroheme elutes at 12.6 min, DAB eluted at 7 min and appeared as a single peak (Fig. 2 *D*, traces *i* and *ii*, respectively).

ChuW Is a Radical SAM Methyltransferase That Catalyzes the Anaerobic Liberation of Iron and Formation of a Tetrapyrrole. The light sensitivity of anaerobilin and DAB has prohibited the accumulation of product that is sufficient for NMR studies at this time. Therefore, we used nanospray ionization MS (NSI-MS) to address

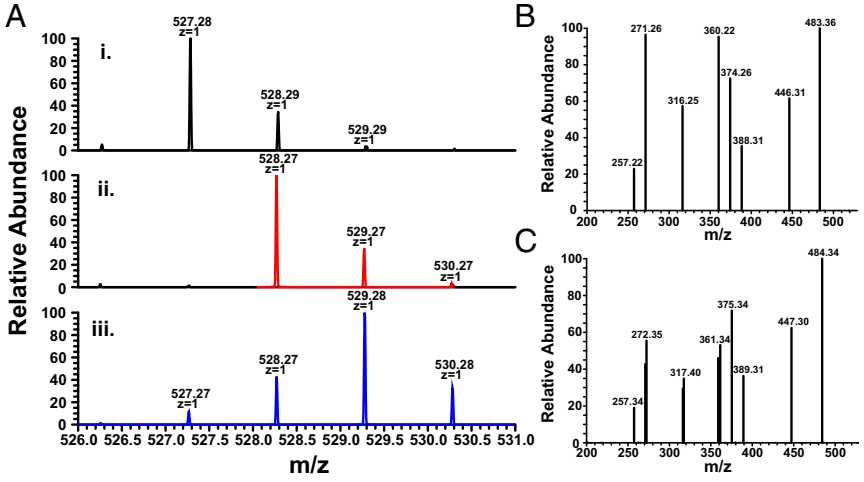

Fig. 3. MS of isolated DAB. (*A*) NSI-MS of DAB was performed as described in *SI Materials and Methods* using deuteroheme and (trace *i*) (^{12}C-methyl) SAM, (trace *ii*) (^{13}C-methyl) SAM, or (trace *iii*) (d_3-methyl) SAM as substrates. (*B*) The MS/MS of DAB or (*C*) ^{13}C-methylated DAB revealed multiple fragments when using CID at 40%. Predicted fragment structures are shown in Fig. S4.

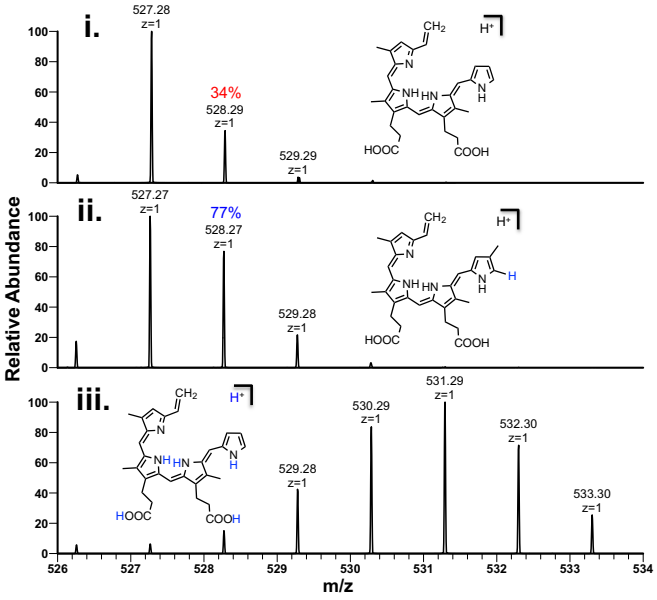

Fig. 4. MS following solvent-derived deuterium incorporation into DAB. ChuW was assayed in buffered H_2O (trace *i*) and 70% buffered D_2O (trace *ii*). DAB was isolated as described in *SI Materials and Methods* and analyzed by NSI-MS. When assayed in buffered D_2O, we observed a 43% enrichment of the A+1 peak at 528.27 *m/z* for the product (trace *ii*). NSI-MS of isolated DAB in d_4-methanol (trace *iii*).

the structure of DAB by direct infusion of the isolated product. When unlabeled SAM was used in the ChuW assay, we observed a dominant peak of 527.28 *m/z* $[M+H]^+$ (Fig. 3, spectra i). The natural abundance of heavy isotopes is represented by the [A+1] peak in the mass spectrum. In this case, the [A+1] is predominately determined by the number of carbon atoms in the molecule. For DAB, the 528 *m/z* [A+1] peak is 34% of the parent ion, suggesting a molecule containing 31 carbon atoms. This observation was further substantiated by analysis of the MS for the product of mesoheme turnover, mesoanaerobilin. Mesoanaerobilin has a $[M+H]^+$ of 583 *m/z* (Fig. S3*B*), which corresponds to the difference in ethyl groups on the 3 and 8 positions of the porphyrin ring compared with deuteroheme. The [A+1] peak for mesoanaerobilin was ∼38% of the parent ion and is in agreement with a 35-carbon molecule.

To further address the methyl transfer reaction, [^{13}C-methyl]-SAM was used in the assay, and the isolated product exhibited an enrichment of ^{13}C with a dominant $[M+H]^+$ of 528 *m/z* (Fig. 3, spectra ii). This observation provides direct evidence that ChuW catalyzes the transfer of a methyl group from the SAM moiety to the product. To assess the mechanism of methyl transfer, we used [d_3-methyl]-SAM in the ChuW assay. The use of the deuterium-labeled SAM molecule resulted in a DAB mass shifted by two units yielding a primary peak of 529.28 *m/z* (Fig. 3, spectra iii), thereby confirming the transfer of only two deuterium atoms from the SAM-derived methyl group. The use of [d_3-methyl]-SAM in the assay resulted in a kinetic isotope effect (KIE) of 2.3 based on specific activities.

To investigate the state of the pyrrolic ring system post turnover, we acquired the fragmentation pattern of DAB (Fig. 3*B*). The MS/MS revealed multiple fragments consistent with the cleavage of the macrocycle. Upon fragmentation of ^{13}C-methylated DAB, some of these fragments also shifted by one mass unit, indicating the retention of the isotopic label (Fig. 3*C*). A similar fragmentation profile was observed when MS analysis was performed on the product mesoanaerobilin (Fig. S3*C*). These data are in stark contrast to the fragmentation typically observed for closed porphyrin ring systems, which only results in the cleavage of substituents surrounding the ring. This observation suggests that ChuW ultimately catalyzes the opening of the porphyrin ring. This conclusion is also supported by the increase in labile iron (Fig. S2). A proposed fragmentation pathway was constructed based on the predicted structure of DAB (Fig. S4).

We further probed the methyl transfer mechanism through NSI-MS analysis of HPLC-isolated 5′-dA when [d_3-methyl]-SAM was used in the assay (Fig. S5). The [A+1] peak for 5′-dA increased from 11% to 60% (Fig. S6, compare traces *i* and *ii*). These data are consistent with what has been reported for other RSMTs and indicate that the 5′-dA•, generated on one SAM molecule, abstracts a hydrogen atom from the methyl group of another SAM molecule. In addition to sequence similarities between ChuW, HemN, and several other class C RSMTs, these data would further support the presence of two distinct SAM binding sites. In fact, two SAM binding sites would be consistent with the proposed HemN mechanism and what has already been confirmed for some RSMTs.

Because lyase reactions may require proton transfer or incorporation, we performed the assay in buffered ^2H_2O (70%) using deuteroheme as the substrate to determine if a solvent-derived proton is incorporated into DAB. Upon purification in methanol, NSI-MS of DAB resulted in a 43% increase in the [A+1] peak to the parent ion (Fig. 4, compare traces *i* and *ii*). This signal was retained after incubation with methanol for 24 h, suggesting that a deuteron is incorporated during the reaction and originates from solvent. Similarly, the number of exchangeable hydrogens that are present on the product was determined by analyzing isolated DAB in d_4-methanol. NSI-MS analysis resulted in a Gaussian distributed signal centered at 531 *m/z* (Fig. 4, trace *iii*).

DAB Is a Substrate for ChuY. To further confirm the role of ChuW in an anaerobic pathway, we isolated and characterized ChuY, the protein that is expressed downstream from ChuW in the heme uptake operon. The current function for ChuY is annotated as an NAD(P)H oxidoreductase, so we performed activity assays using ChuY in the presence of NADPH and isolated DAB. On addition of enzyme into the assay mixture, we observed a decrease in the UV-visible features of DAB with respect to time (Fig. S6). Increasing the amount of ChuY present in the assay resulted in a linear increase in that activity, indicative of an enzyme-catalyzed reaction. No biliverdin reductase activity was observed for ChuY, although the function appears to be similar, specifically to reduce a potentially toxic product of heme degradation.

Discussion

In this investigation, we present evidence for enzyme-catalyzed oxygen-independent heme degradation using isotope labeling, activity assays, and MS to gain insight into the reaction catalyzed by ChuW from enterohemorrhagic *E. coli* (Fig. 5). Our data suggest that ChuW is a radical SAM enzyme and specifically a member of the class C RSMT subgroup based on the domain structure, sequence analysis, and similar mechanisms for methylation that have been proposed for these enzymes (23). The current mechanistic paradigm for all heme oxygenases reported to date involves activation of molecular oxygen. In stark contrast, the data presented herein suggests that ChuW uses the oxidizing power of a primary-carbon radical to catalyze methyl transfer and rearrangement of the porphyrin ring, resulting in the liberation of iron. Similar to other radical SAM enzymes, any exposure to molecular oxygen inactivates ChuW, likely due to the degradation of the catalytic [4Fe-4S] cluster. Without question, the ability to acquire iron from a porphyrin macrocycle under low oxygen tension would provide a microorganism with a distinct competitive advantage. Such a competitive advantage is especially important for an enteric organism that is competing for survival in an anaerobic environment, such as the human intestine, where heme is readily available from dietary sources.

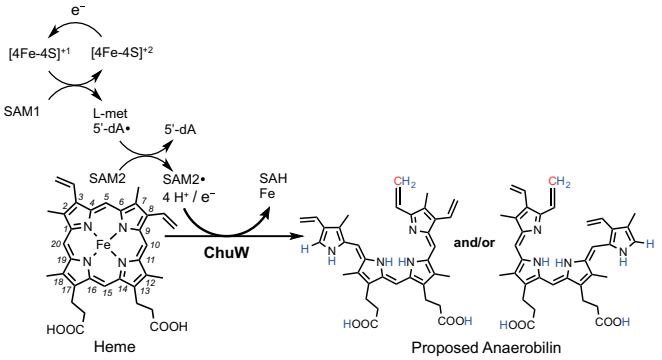

Fig. 5. Proposed reaction scheme and products for ChuW. Predicted placement of hydrogens observed during deuterium incorporation are labeled in blue, whereas the SAM-derived methyl group is shown in red.

Radical SAM enzymes are known to perform difficult chemical rearrangements and, in some cases, oxygen-using enzymes have taken over a particular metabolic step, presumably after the appearance of oxygen-evolving photosynthesis. The current broad classification of genes as a "HemN" is ambiguous due to the limited scope of the sequence motif used for the classification (20). However, distinction between genes is coming to light. Bioinformatic analyses have identified more than 300 similar sequences across 36 genera within the Structure-Function Linkage Database (SFLD) (30), all from organisms that do not encode a heme oxygenase (HO). Evolutionarily speaking, it is interesting that two distinct enzymes, HemN and ChuW (HutW in *V. cholerae*), contain a similar functional domain, yet one enzyme is important for the anaerobic formation of heme (HemN), whereas the other enzyme can catalyze the anaerobic degradation of heme (ChuW).

A radical-based mechanism, instead of the activation of molecular oxygen, makes mechanistic sense; however, it is unclear how such a mechanism proceeds. Clues to the ChuW mechanism come from what has been recently observed for other class C RSMTs. All known class C RSMTs catalyze methylations of sp^2-hybridized carbon atoms that, in some cases, lead to considerable structural rearrangement (22, 23, 31, 32). Although ChuW/HutW's appear to be evolutionarily distinct (Fig. S7) compared with other RSMTs or HemN, all of these enzymes have been shown to use the reductive cleavage of SAM as the starting point in their catalytic cycle. In addition, activation of the substrate or the methyl group of one SAM moiety by reductive cleavage of another SAM molecule has already been presented for several RSMTs (23). Building on our observations and these postulated mechanisms, Fig. 5 depicts the proposed reaction scheme for the ChuW-dependent decyclization of the porphyrin ring. Although the complete mechanism is incomplete, our data indicate that after generation of the 5′-dA•, this radical abstracts a hydrogen atom from the methyl group of a second SAM molecule. The newly formed methylene radical attacks the substrate directly before causing a rearrangement of the porphyrin ring that results in the liberation of iron. At the present time, there are other possible product structures that would satisfy this goal and our MS data (Fig. S8), and this is under further investigation. Regardless, the most likely mechanism for ring opening would involve the formation of an intermediate that spontaneously results in a distortion of the porphyrin structure.

Several intermediates can be considered based on our observations and the observations for other class C RSMTs. Of particular interest are the products of YtkT and Jaw5 that have been shown to contain other "high energy" C1-derived moieties, such as cyclopropane rings (22, 31). Such a moiety may be unstable if placed across the 4–5 or 5–6 carbon–carbon bond of deuteroheme (Fig. 5) or heme and would provide structural reasoning for any of the products presented depending on resolution of the cyclopropane ring cleavage (Fig. 5 and Fig. S8). Similar to what has been described for the class C RSMTs YtkT and Jaw5 (22), whether or not such a mechanism involves radical addition or nucleophilic attack by the methylene radical or ylide, respectively, is also not clear. Either mechanism for methyl transfer and ring opening requires an additional electron, a problem discussed in a recent review (23) but that is still unresolved for these class C RSMTs.

The fragmentation pattern that we observe for DAB is particularly useful in that cleavage at the central bridging methine carbon produces a pyrrolium ion $[M'+H]^+$ at 271 *m/z* and a pyrrolic ion $[M^+]$ at 257 *m/z* (Fig. S4), which is similar to what has been observed in the mass spectral analysis of biliverdin (33). To this end, we compared structures that coincided with ring cleavage at either the α or γ methine bridge while accounting for the addition of a methyl group at or near the cleavage site. The resulting even mass fragments suggest a loss of one nitrogen atom and are best explained by fragmentation at either the β or δ methine bridges. It is unclear if there is a preference for which methine bridging carbon–carbon bond is ultimately broken (C4-C5 or C5-C6) during catalysis, but both outcomes are possible (Fig. 5). Although our observations for MS/MS analysis of DAB are explained by the proposed structure (Figs. 4 and 5), other potential isomers are described in Fig. S8 that cannot be ruled out at this time. Due to the photolability and oxygen sensitivity of the reaction products, efforts to isolate DAB, anaerobilin, and mesoanaerobilin to a purity and quantity sufficient for NMR analysis have proven challenging.

Heme has limited solubility in aqueous solution; therefore, the standard protocol for assaying HOs is to load the enzyme with 1:1 equivalents of heme:protein followed by initiating turnover with cytochrome P450 reductase and near stoichiometric amounts of NADPH. These experiments are essentially a single turnover reaction that is often slow and that can be the result of nonenzymatic coupled oxidation when catalase is not present in the assay (13). Therefore, the identification of genuine HOs can be difficult. ChuS from *E. coli* O157:H7 was originally identified as a HO (34), and although the authors reported activity in the presence of catalase, a later report revealed significantly decreased activity in the presence of catalase for a homologous protein (35). Similarly, the heme utilization protein HutZ from *V. cholerae* was described as a HO based on spectroscopic assays (36); however, there was no indication that catalase or superoxide dismutase was included in the assay reported for the initial characterization, and the products were never identified, nor was this activity observed by others (14). Consequently, the benefit of studying an anaerobic heme-degrading enzyme in vitro is the minimal risk of nonenzymatic coupled oxidation artifacts. However, if ChuS and HutZ were bona fide heme degraders, this would allow enterohemorrhagic *E. coli* and *V. cholerae* the ability to acquire iron from heme in varying oxygenic environments.

The existence of an anaerobic mechanism for heme degradation is consistent with (*i*) the recent revelations in the heme biosynthetic pathways (20, 37), (*ii*) the characterization of radical SAM enzymes involved in catalyzing important steps in the anaerobic biosynthesis of protoheme (19, 25), and (*iii*) recent observations that heme degradation has evolved to suit the physiological needs of the organism (12). Our in vitro data suggest that ChuW functions as a RSMT that methylates heme as part of a decyclization mechanism, resulting in an open tetrapyrrole and the release of the essential nutrient iron (Fig. 5). Many of our observations overlap with what has been published for the class C RSMTs. We further demonstrated that the product of the ChuW reaction is a photosensitive substrate that is reduced by ChuY in a NADPH-dependent reaction. We propose that ChuX may facilitate the transfer of anaerobilin or the removal of iron from a ChuW-generated intermediate. In regard to the latter, a zinc ion has been observed bound to a conserved histidine residue in the structure of a ChuX homolog (Protein Data Bank ID code 3FM2). However, despite a reported

dissociation constant (K_D) of 1.99 ± 0.02 μM, a structure for ChuX with heme bound is not available at this time. We propose that both the iron atom and anaerobilin may be toxic to the cell, so a logical role for ChuX could be to chelate the exiting iron atom and/or transport anaerobilin to ChuY.

In addition to iron acquisition, the products of anaerobic heme or porphyrin degradation may have more diverse functions. When our findings are considered in light of the data for HemN, and the fact that the products of other class C RSMTs have antitumor and antibiotic properties, further characterization of these radical mechanisms will have profound implications. In particular, the presence of an enzyme that catalyzes a radical-mediated porphyrin degradation reaction in a select group of enteric pathogens will open the door to research and discovery of a new class of antimicrobial compounds.

Materials and Methods

A typical assay for *E. coli* O157:H7 ChuW, expressed and purified as described *SI Materials and Methods*, consisted of 1 μM enzyme, 5 μM EcFld, 2 μM EcFpr 250 μM NADPH, and 10 μM of hemin, mesoheme, or deuteroheme. Assays were performed anaerobically and initiated by the injection of SAM to a final concentration of 250 μM. Deuteroanaerobilin and mesoanaerobilin were isolated anaerobically in the dark using a disposable C1 column. NSI-MS was performed using a linear ion trap mass spectrometer (LTQ-Orbitrap Discovery; Thermo Fischer Scientific). Unless stated otherwise, all experimental procedures were performed in an anaerobic chamber under positive nitrogen:hydrogen (95:5) pressure with continuous scrubbing to remove any molecular oxygen. To ensure that there was no molecular oxygen present in the glovebox during experiments, we continuously monitored the atmosphere of the chamber using an electronic detector, as well as a chemical mixture of reduced methyl viologen. All additional experimental procedures are described in *SI Materials and Methods*.

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