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HutW from *Vibrio cholerae* Is an Anaerobic Heme-Degrading Enzyme with Unique Functional Properties

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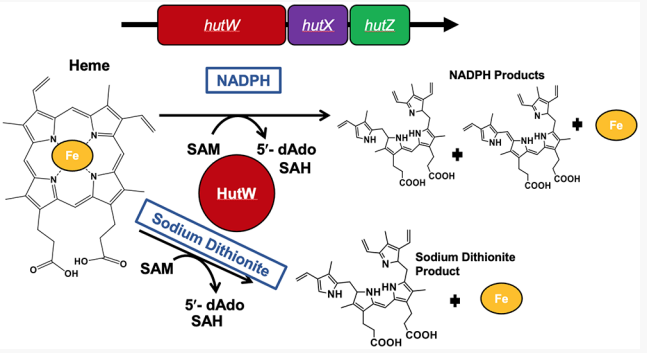
ABSTRACT: Increasing antibiotic resistance, and a growing recognition of the importance of the human microbiome, demand that new therapeutic targets be identified. Characterization of metabolic pathways that are unique to enteric pathogens represents a promising approach. Iron is often the rate-limiting factor for growth, and *Vibrio cholerae*, the causative agent of cholera, has been shown to contain numerous genes that function in the acquisition of iron from the environment. Included in this arsenal of genes are operons dedicated to obtaining iron from heme and heme-containing proteins. Given the persistence of cholera, an important outstanding question is whether *V. cholerae* is capable of anaerobic heme degradation as was recently reported for enterohemorrhagic *Escherichia coli* O157:H7. In this work, we demonstrate that HutW from *V. cholerae* is a radical *S*-adenosylmethionine methyl transferase involved in the anaerobic opening of the porphyrin ring of heme. However, in contrast to the enzyme ChuW, found in enterohemorrhagic *E. coli* O157:H7, there are notable differences in the mechanism and products of the HutW reaction. Of particular interest are data that demonstrate HutW will catalyze ring opening as well as tetrapyrrole reduction and can utilize reduced nicotinamide adenine dinucleotide phosphate as an electron source. The biochemical and biophysical properties of HutW are presented, and the evolutionary implications are discussed.



Iron is a necessary micronutrient for all cellular organisms. The importance of iron for bacterial survival and pathogenesis is evident by the variety of highly conserved and broadly employed iron acquisition strategies among bacterial pathogens.[1,2] In addition to iron-siderophore scavenging mechanisms, many bacterial pathogens also contain systems for the utilization of heme as an iron source, either as free heme or from various heme-containing proteins.[3,4] Some pathogenic bacteria have been shown to encode a heme oxygenase-type enzyme that catalyzes the regiospecific conversion of heme into biliverdin IXα, CO, and free iron.[5−10] These HO-like enzymes were thought to be the only heme-degrading enzymes; however, characterization of noncanonical enzymes from *Staphylococcus aureus* (IsdG and IsdI) and *Mycobacterium tuberculosis* (MhuD) has begun to shed light on the diversity of aerobic bacterial heme-degrading systems.[11,12]

The enterohemorrhagic pathogens *Escherichia coli* O157:H7 and *Vibrio cholerae* inhabit anaerobic environments. Both have stimulated growth rates in the presence of heme or hemoglobin and can use heme as the sole iron source.[13,14] A radical *S*-adenosylmethionine (SAM) enzyme that liberates iron from heme under strictly anaerobic conditions in *E. coli* O157:H7 was recently identified and characterized.[15] The enzyme, ChuW, is expressed in a Fur-dependent manner under iron-limiting conditions along with two other proteins, ChuX and ChuY, that are downstream in the same operon. *V. cholerae*, the causative agent of the severe diarrheal disease cholera, also expresses proteins for the uptake and utilization of heme as an iron source.[16] Three genes that are linked to the *V. cholerae* operon for heme transport and in a region containing several potential binding sites for the iron regulator protein Fur, *hutWXZ*, were discovered to be required for growth on heme as the sole iron source.[13] Specifically, similar to the *chuWXY* genes of the *E. coli* O157:H7 serotype, the *hutWXZ* genes of *V. cholerae* constitute an operon divergently transcribed from the *tonB1* operon initially identified in the uptake of extracellular heme.[16]

Upon comparison of the proteins of the *E. coli* O157:H7 (*Chu*) and *V. cholerae* (*Hut*) operons, several things are noteworthy. The level of sequence identity between ChuW and HutW is 39%, and they contain the catalytic [4Fe-4S] cluster binding motif common to all RS enzymes. Additional shared motifs include those for ligating two molecules of SAM, as is

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seen in class C radical SAM methyltransferases, and binding heme. Likewise, the sequence identity of ChuX from *E. coli* O157:H7 is 59% when compared to HutX. Alignment of the functional dimer from each of the crystal structures that are available for ChuX and HutX results in a root-mean-square deviation (RMSD) of 1.2 Å.[17,18] Both of these proteins have been shown to bind heme. In contrast to ChuW, HutW has not been isolated and has not been characterized biochemically. At the present time, no structure is available for ChuW or HutW.

A distinct difference is found in the third gene of these operons (*chuWXY* vs *hutWXZ*). Specifically, previous work has shown that ChuY (from enterohemorrhagic *E. coli* O157:H7) and HutZ (from *V. cholerae*) have different three-dimensional structures and different functions.[19−21] However, ChuY and HutZ are found in the same relative location within their respective operons. Moreover, in both cases, gene expression is Fur-regulated and similar phenotypes are observed for the knockout strains.[13,22] Finally, it has been shown that HutZ is necessary for heme utilization as an iron source,[13] while deletion of ChuY weakens the ability of the pathogen to infect human cells.[22] The two available crystal structures for ChuY [Protein Data Bank (PDB) entries 5FFQ and 5GUY] reveal a nucleotide binding motif consistent with a NADPH-dependent reductase. In keeping with this observation, it has been shown that ChuY functions to reduce the tetrapyrrole product of ChuW.[19,22] Coupled with the recent observation that both ChuX and ChuY stimulate the activity of ChuW,[23] a reasonable conclusion is that all three proteins function in a catabolic pathway responsible for the anaerobic degradation of heme. However, our understanding of heme degradation in *V. cholerae* is not clear as the function of HutW has not been addressed. In addition, we have not assessed whether similar functional synergy exists among HutW, HutX, and HutZ as has been reported for ChuW, ChuX, and ChuY.[23] Given that ChuY and HutZ have reportedly different functions and distinctly different protein structures, our hypothesis is that important functional differences exist.

In the preliminary characterization of HutZ, Wyckoff et al. stopped short of describing HutZ as a heme oxygenase due to the lack of catalytic activity under physiologically relevant conditions.[13] In contrast, Uchida et al. later showed that ascorbic acid could be used to obtain spectroscopic intermediates that were similar to what had been reported for human heme oxygenase 1 (hHO-1).[21] Of significant interest was the proposal that peroxide may be a transient intermediate. Specifically, it has long been known that hydrogen peroxide (H_2O_2) can substitute for molecular oxygen (O_2) and two reducing equivalents in the HO-1 reaction[24] resulting in the formation of the ferric HO-1/verdoheme complex when a stoichiometric amount of peroxide and heme are reacted. HutZ appears to be capable of similar chemistry, based on the observed spectroscopic signatures. Furthermore, entirely on the basis of changes in the ultraviolet−visible spectrum, the addition of a chemical reductant, like ascorbic acid, to the HutZ/verdoheme complex leads to what appears to be a HutZ/biliverdin complex, although the tetrapyrrole product of this reaction has yet to be isolated and characterized.[21]

In light of these observations, and the similar environment colonized by both pathogens, the enigma appears to be how these proteins (ChuWXY vs HutWXZ) may have functionally diverged to accomplish a similar task in the same ecological niche. Our current hypothesis posits that HutW is a radical SAM enzyme capable of opening the porphyrin ring of heme to release iron in an oxygen-independent reaction, although this hypothesis has not been tested. ChuX and HutX are structurally identical, and the RMSD for all backbone carbon atoms is <0.5 Å. Therefore, if HutW does catalyze a reaction similar to that of ChuW, then it stands to reason that HutX will function similarly to ChuX and bind free iron and/or the tetrapyrrole product of HutW. Given that ChuY and HutZ both contain nucleotide binding domains but have notably different overall folds, an exciting possibility is that the tetrapyrrole product of HutW is different from ChuW. In this case, HutZ may serve dual functions. Specifically, HutZ may be capable of catalyzing heme degradation in the presence of molecular oxygen (explaining the lack of a ChuS homologue in *V. cholerae*) but then also functioning as a reductase under anaerobic conditions to clear the product of HutW (explaining the lack of a ChuY homologue in *V. cholerae*), produced during anaerobic heme degradation. Alternatively, HutW may utilize a different mechanism, when compared to ChuW, to open the porphyrin ring of heme.

To address whether HutW is a radical SAM enzyme involved in anaerobic heme degradation, and generation of potentially new tetrapyrrole catabolites, and to address new functions for HutX and HutZ, we have isolated and characterized the recombinant proteins. Our results show that HutW is indeed a radical SAM enzyme capable of catalyzing anaerobic heme degradation, albeit with significantly different mechanistic features. Moreover, we show that while inclusion of HutX in the assay does increase the rate of HutW turnover, inclusion of HutZ does not. We also demonstrate that HutW can utilize NADPH as a substrate, a significant first for any RS enzyme. Both of these observations are in marked contrast to what was reported for ChuW from the O157:H7 serotype of *E. coli*. The physiological implications for utilization of heme as an iron source by *V. cholerae* are discussed.

MATERIALS AND METHODS

Overproduction, Purification, and Reconstitution of HutW. The gene encoding HutW was codon-optimized for expression in *E. coli* and synthesized de novo by DNA 2.0 (now ATUM, Newark, CA). This plasmid (kanamycin resistant) utilized a standard T7, isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible, promoter and was co-transformed along with the pDB1282 plasmid[25] (ampicillin resistant), containing the *isc* operon, into *E. coli* BL21(DE3). A 20 mL starter culture of LB medium was inoculated with a single colony and incubated overnight at 37 °C while being shaken at 250 rpm. Flasks (4 L) containing M9 medium (1 L) were inoculated with the overnight starter culture to initiate growth (180 rpm at 37 °C). Expression of the genes encoded on plasmid pDB1282 was induced at an OD_{600} of 0.3 with 0.2% arabinose. Expression of the *hutW* gene was subsequently induced with 0.2 mM IPTG at an OD_{600} of 0.6, and the mixture incubated overnight for 18 h at 17 °C while being shaken at 180 rpm. The cells were harvested, flash-frozen, and stored at −80 °C.

For purification, 30 g of frozen cell paste was resuspended in 150 mL of anaerobic lysis buffer containing 50 mM Tris (pH 7.5), 250 mM KCl, 10% (v/v) glycerol, PMSF (1 mM), DNase1 (100 μg/mL), and lysozyme (1 mg/mL). Resuspended cells were degassed with argon while being stirred. Solubilized cells were then lysed anaerobically by equilibrating a closed-system French press with anaerobic buffer while

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maintaining a stream of argon in the drawing and collection flasks. The lysate was centrifuged at 60000*g* for 1.5 h. The supernatant was collected and purified anaerobically by a cobalt affinity column pre-equilibrated with buffer. After application of the supernatant, the column was washed with a buffer containing 0 and 10 mM imidazole before elution with 250 mM imidazole. Protein was diluted to 1 mg/mL, and the [4Fe-4S] cluster was reconstituted following previously established methods.[25] Briefly, the protein was diluted in 100 mM HEPES (pH 8.0), 300 mM KCl, 10% glycerol, and 10 mM DTT. Then, 100 mM ferric chloride was added to 4 times the protein concentration. After the solution had been allowed to incubate for 30 min, 15 μL of 100 mM sodium sulfide was added every 10 min until reaching 1 equiv of the iron concentration. After incubation in the glovebox for 12 h, excess iron−sulfur clusters were removed by centrifugation and the supernatant was run over a DEAE-Sepharose anion exchange column in the glovebox. The reconstituted enzyme was eluted using a stepwise (0.1 M steps) gradient (0-1 M KCl) in buffer. Protein fractions were concentrated anaerobically and applied on a G25 size exclusion column pre-equilibrated with a buffer containing 100 mM HEPES (pH 8.0), 300 mM KCl, and 10% glycerol. Protein fractions were concentrated anaerobically before being stored in liquid nitrogen.

HutX and HutZ Expression and Purification. Unlike HutW, HutX and HutZ do not contain any cofactors that would require a unique expression protocol. The gene for HutZ was optimized for expression and cloned into the pD431-SR vector by ATUM. The gene for HutX was amplified from genomic *V. cholerae* DNA (a generous gift from S. Payne) and cloned into the commercially available pTrcHisA vector. Expression of both proteins was carried out in *E. coli* BL21(DE3) cells and the aforementioned IPTG-inducible expression plasmids (the HutZ expression vector is kanamycin resistant, while the HutX expression vector is carbenicillin resistant). Expression of HutZ and HutX was performed in LB medium (1 L) inoculated with a 10 mL starter culture and grown at 37 °C while being shaken at 200 rpm. Once an OD_{600} of 0.6 was achieved, IPTG was added to a final concentration of 1 mM. Cultures were grown for an additional 6 h before being harvested by centrifugation, frozen, and stored at −80 °C. Frozen cell pellets (50 g) were solubilized in 200 mL of a buffer containing 50 mM Tris (pH 7.5) and 250 mM KCl supplemented with PMSF (1 mM), lysozyme (1 mg/mL), and DNase (1 mg/mL). Cells were lysed aerobically with a French pressure cell, and the lysate was centrifuged at 100000*g* for 1.15 h. The supernatant was applied to a TALON column that was pre-equilibrated with a buffer containing 50 mM Tris (pH 7.5), 250 mM KCl, and 10% glycerol. The column was washed with the same buffer also containing 0 and 10 mM imidazole buffer before elution with a buffer containing 250 mM imidazole. Protein fractions were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) for homogeneity. Protein-containing fractions were pooled and concentrated prior to being frozen at −80 °C.

Ultraviolet−Visible (UV−vis) Activity Assays. All UV−vis spectra were recorded on an HP 8453 diode array spectrophotometer running on OlisWorks using a Peltier temperature controller set to 25 °C and a stir speed of 1200 rpm. Unless indicated in the figure legend, assays were conducted anerobically with degassed buffer containing 5% DMSO (from a porphyrin stock), 10 μM HutW, 1 mM SAM, and 20 μM heme, protoporphyrin IX, deuteroheme, or deuteroporphyrin IX. Initially, sodium dithionite (2 mM) was used as the reducing agent until it was discovered that NADPH could drive the reaction. Turnover was initiated by the addition of SAM unless indicated otherwise. Spectra were recorded from 350 to 900 nm every minute. Assays were run for as long as 30 min, but for the majority of porphyrins investigated, all spectral changes were completed within 4−5 min. Extinction coefficients were also determined by the isosbestic point at 418 nm using the relationship $A = l\varepsilon(C_{substrate} + C_{product})$.

Quantification of Fe−S Cluster Content and Labile Iron after Turnover. Quantification of the iron content in active preparations of HutW (presumably containing a [4Fe-4S] cluster) was performed as previously described.[26] To quantify the labile iron present in assays pre- and post-turnover, the assay was modified to include HutW (10 μM), heme (50 μM), SAM (1 mM), and either NADPH (10 mM) or sodium dithionite (2 mM) as the reductant. Aliquots for quantification were taken at the indicated time points. Iron standards were prepared in acid-washed glassware at a concentration of 0.5 mM ferrous ammonium sulfate heptahydrate and diluted to various concentrations between 0.012 and 0.2 mM in 50 mM Tris (pH 8.0), 250 mM KCl, and 10% glycerol. After acid precipitation and heat incubation at 80 °C, 750 μL of dH_2O was added, and samples were centrifuged. Supernatants were transferred to new microcentrifuge tubes followed by addition of 50 μL of 10% hydroxylamine and 250 μL of 0.1% bathophenanthroline. Samples were incubated at room temperature for 1 h, and the absorbance at 535 nm was taken. All iron standards were performed in triplicate as well as the samples for HutW. The same protocol was used to detect the total amount of iron available to the chelator in the HutW assay before and after turnover. Any protein-bound iron is released during acid hydrolysis, whereas heme-bound iron is not.

Electron Paramagnetic Resonance (EPR) Spectroscopy. Samples were prepared, sealed, and flash-frozen in an anaerobic chamber. For all samples, the protein concentration was 400 μM in the presence of 3 mM sodium dithionite. When SAM was added to the sample, it was at a concentration of 1 mM. All EPR spectra were recorded at 12 K with a microwave power of 0.1 mW, a microwave frequency of 9.352 GHz, a modulation amplitude of 4.0 G, and a modulation frequency of 100 kHz.

High-Performance Liquid Chromatography (HPLC) Analysis. Turnover assays of HutW were performed anaerobically in a 2 mL reaction volume with final concentrations of 1 mM SAM, 100 μM heme, 100 μM HutW, in 50 mM Tris (pH 8.0), 250 mM KCl, and 10% glycerol buffer. The reductant was either sodium dithionite (2 mM) or NADPH (10 mM). Due to the detection limits of our HPLC instrument, aliquots were taken at various time points during an assay. These are noted in the respective figure legends and include 0 min, 15 min, 30 min, 60 min, 90 min, and overnight (approximately 12 h). Sample aliquots (150 μL) were taken and quenched with 1% TFA (v/v, final) and centrifuged to remove the precipitant, and the supernatant was applied to a C18 HPLC column (15 cm × 4.6 mm). SAM cleavage products were separated by a gradient of acetonitrile in 0.1% TFA at a flow rate of 1 mL/min and monitored at 260 nm. The column was washed following each injection for 5 min with 100% acetonitrile. A standard curve was constructed by spiking the assay solution with

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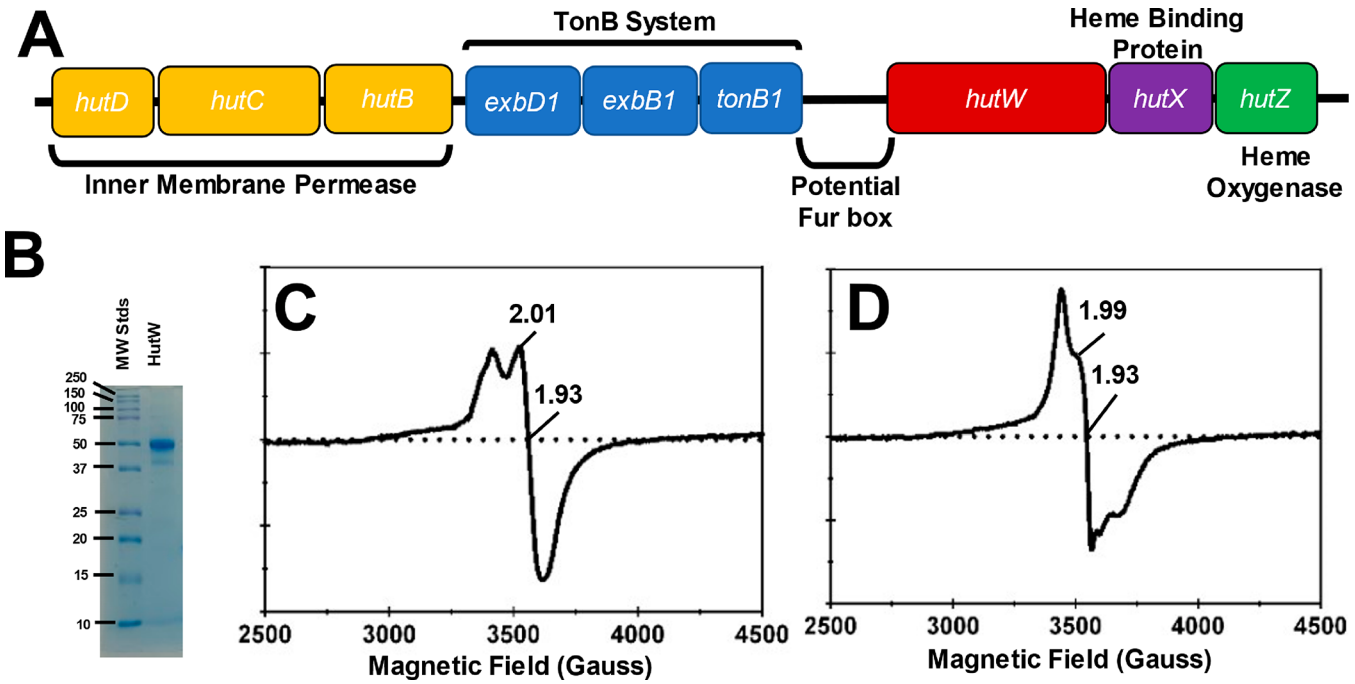

Figure 1. Genetic organization of the heme utilization operon in *V. cholerae*, SDS−PAGE of purified HutW, and EPR spectra. (A) HutW is genetically adjacent to the heme uptake machinery that is expressed during iron starvation. (B) SDS−PAGE showing molecular weight standards and the isolated HutW used in this study. EPR spectra of (C) purified HutW with 3 mM sodium dithionite before and (D) after the addition of 1 mM SAM. All EPR spectra were recorded at 12 K with a microwave power of 0.1 mW, a microwave frequency of 9.352 GHz, a modulation amplitude of 4.0 G, and a modulation frequency of 100 kHz.

various concentrations of SAM, SAH, or 5′-dA and integrating the respective peak areas.

Mass Spectrometry. Samples were diluted to approximately 10 μM in methanol containing 0.1% formic acid. The solutions were infused directly into the electrospray source of the ion mobility mass spectrometer (Waters Synapt XS) at a flow rate of 660 μL/h using an external syringe pump following mass calibration to 1 ppm over the range of m/z 50−1200. Data were acquired in positive ionization mode (capillary, +3 kV; source temperature, 120 °C; cone gas, 10 L/h; desolvation gas, 500 L/h at 350 °C) over a period of 5 min with a scan time of 1 s. The ion mobility separation was achieved using nitrogen drift gas and traveling wave settings of 550 m/s and 40 V. The tandem MS experiment was performed using a 25 eV collision energy that was applied to the transfer cell of the instrument. The exact calculated masses of the products were extracted from the ion mobility arrival time distribution within a window of 0.01 Da.

RESULTS

Isolation and Purification of HutW. HutW is the first of three genes expressed in an iron-dependent (Fur promoter) manner as part of a larger heme utilization (Hut) operon (Figure 1A). In addition to the similar organization of the operon, HutW shares 39% sequence identity with ChuW, a radical SAM methyl transferase (RSMT) involved in anaerobic heme degradation.[15] Therefore, similar to the expression of other radical SAM enzymes, expression of HutW was performed using an additional plasmid to increase the level of iron−sulfur cluster biosynthesis during expression.[23,25] Anaerobic isolation (Figure 1B) and reconstitution of HutW yielded a purified enzyme with 4.4 ± 0.7 iron atoms per protein. The UV−vis spectrum had a broad adsorption feature with a maximum near 400 nm, indicative of the coordination of a $[4Fe\text{-}4S]^{+}$ cluster (Figure S1). To further investigate the cluster content, the EPR spectra of HutW in the presence of sodium dithionite were recorded and revealed a broad signal centered on a g of 1.93 (Figure 1C). These observations are similar to what has been observed for other radical SAM enzymes that contain only the catalytic [4Fe-4S] cluster in the reduced state (formally 1+).[27] In addition, when SAM is added to the mixture, a significant change in the line shape of the EPR signal ($g_{\|}$ = 1.99, and $g_{\perp}$ = 1.93) occurs that is consistent with SAM binding to the unique iron site (Figure 1D) of the $[4Fe\text{-}4S]^{+}$ cluster.

Finally, when purified HutW was passed through a calibrated size exclusion column, the elution volume was consistent with a monomer (Figure S2).

Heme Binding. To investigate heme binding by HutW, we monitored the absorption spectrum of a buffered solution of heme following the addition of purified HutW. A red shift in the major Soret band (from 385 to 422 nm) is observed and can be utilized to follow heme binding (Figure 2). Under reducing conditions, the observation of a major Soret band at ∼420 nm, and an α band at 556 nm, for heme-bound HutW, are spectral properties consistent with those of a reduced ferrous six-coordinate low-spin heme complex.[24,28,29] Addition of an increasing concentration of HutW to a hemin solution (20 μM) produces a binding isotherm (Figure 2) that can be used to calculate binding affinities.

Specifically, HutW−heme titration was performed under strictly anaerobic conditions using either ferrous or ferric heme. The dissociation constant (K_d) for the HutW−hemin or −heme complexes was determined by fitting the data to a rectangular hyperbole giving K_d values of 1.4 ± 0.5 and 3.6 ± 0.9 μM for the binding of ferric and ferrous heme, respectively.

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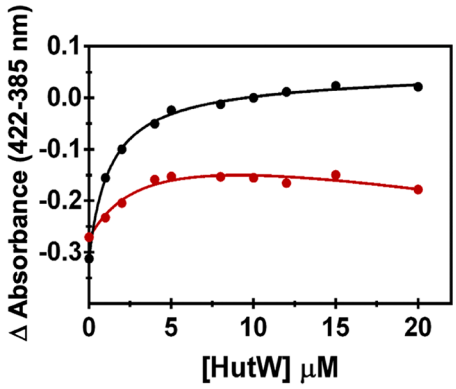

Figure 2. Ferric (red circles) and ferrous (black circles) heme binding to ferric HutW. Titration of increasing amounts of HutW (1−20 μM) into a solution of hemin (10 μM). The shift in the heme Soret peak was monitored and plotted as a function of protein concentration. Saturation was reached at a 1:1 protein:heme ratio (10 μM). The data were fit to a standard binding isotherm represented by a rectangular hyperbola using PRISM as described in the text. Dissociation constants were 1.27 and 3.64 μM for the ferrous and ferric heme, respectively.

These K_d values are in a range that is similar to what has been reported for other bacterial heme-degrading enzymes.[12,30,31] The binding also appears to saturate at 10 μM, which is a 1:1 HutW:heme ratio.

Turnover of HutW Using Metalated and Nonmetalated Porphyrins. Despite the 39% sequence identity shared with ChuW, HutW is phylogenetically distinct[32] with significant sequence divergence occurring in an extended N-terminal domain. Therefore, although we hypothesized that HutW is a RSMT involved in anaerobic degradation, it is possible that significant differences in the catalytic mechanism exist. To assess our hypothesis and the porphyrin-degrading activity of HutW, we performed assays monitoring the UV−vis spectrum during incubation of the protein with one of several porphyrins or metalated porphyrins. This was performed in the presence of the chemical components typically required of a radical SAM enzyme, specifically, SAM as well as a source of low-potential electrons. No changes were seen if SAM was omitted from the assay, regardless of the source of electrons. We observed distinct changes in the absorption spectra for all of the porphyrins investigated (Figure 3). Progress curves for heme turnover are shown in Figure S3. The data in Figure 3 show the changes observed for heme (Figure 3A), deuteroheme (Figure 3C), protoporphrin IX (Figure 3B), or deuteroprotoporphyrin IX (Figure 3D). Rates were calculated from the progress curves, as described in Materials and Methods (Table 1), and reveal that the physiological substrate, heme, has the fastest turnover rate (Figure 1A), resulting in a decrease in the intensity of the major Soret band at 407 nm and Q-bands concurrent with an increase in absorbance at 451 and 795 nm.

Table 1. Specific Activities for HutW-Catalyzed Porphyrin Degradation Reactions

protein component in assay	substrate	specific activity [nmol min^{-1} (mg of HutW)$^{-1}$]
HutW	heme	300.6 ± 15.6[a]
HutW (NADPH)[b]	heme	148.3 ± 14.0
HutW	deuteroheme	132.3 ± 46.7
HutW	protoporphyrin IX	191.1 ± 4.3
HutW	deuteroporphyrin IX	174.8 ± 14.5
HutW and 1 equiv of HutX	heme	359.8 ± 4.0
HutW and 2 equiv of HutX	heme	412.6 ± 1.9

[a]The error represents the average deviation of the mean for three measurements. [b]NAPDH was the reductant in these assays. Unless stated otherwise, sodium dithionite (2 mM) is the electron source.

Deuteroheme (Figure 3C), a more soluble heme analogue, exhibited similar spectroscopic changes, including a decrease in the intensity of the major Soret band as well as increases in the absorption at longer wavelengths (448 and 775 nm). For ChuW, the mechanism of porphyrin degradation has also been shown to be independent of the metal ion,[23] and therefore, we wanted to address if the metal ion was required in the HutW

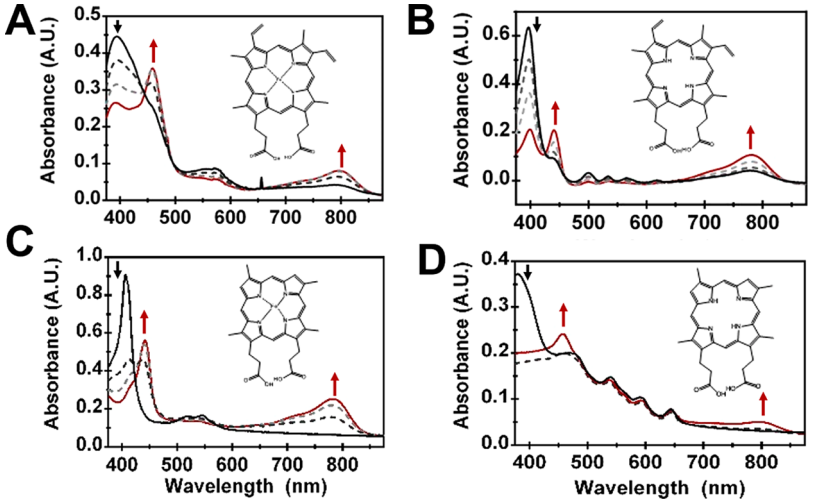

Figure 3. UV−vis absorbance spectra monitoring the anaerobic degradation of (A) heme, (B) protoporphyrin IX, (C) deuteroheme, and (D) deuteroporphyrin IX catalyzed by HutW. HutW assays were performed as described in Materials and Methods, and spectral changes were recorded every 2 min for 6 min. The structure of each substrate is shown as an inset in the respective panels, and the arrows indicate the direction of spectral changes during the assay.

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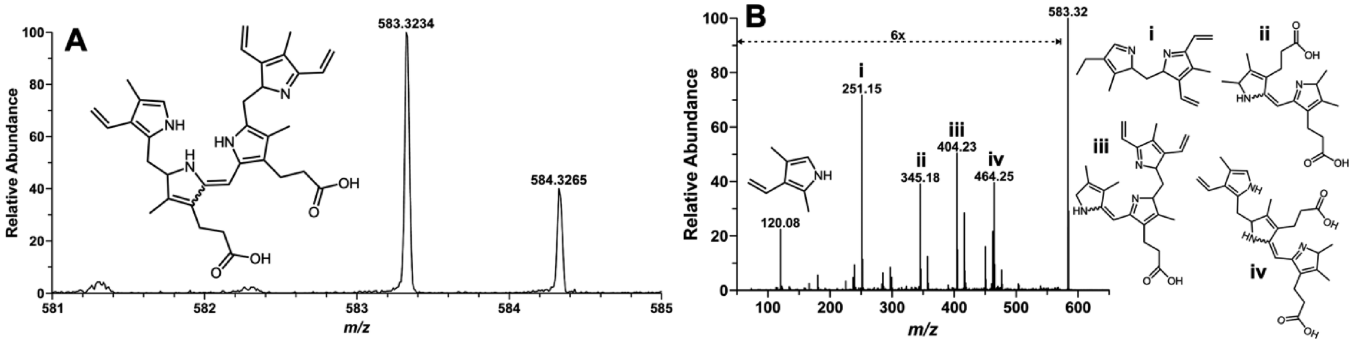

Figure 4. (A) Ion mobility mass spectrometry (IM-MS) of the tetrapyrrole product produced in the HutW reaction and (B) MS/MS fragment peaks and some representative fragment structures. Pre- and post-turnover samples were prepared and analyzed by IM-MS as described in Materials and Methods. Comparison of pre- and post-turnover data showed the appearance of a peak at m/z 583.3 (A) that was subjected to fragmentation (B). The five most abundant fragment peaks and structures are labeled (B). One hundred six of the 107 fragments predicted by MetFrag were observed with intensities of >200 counts.

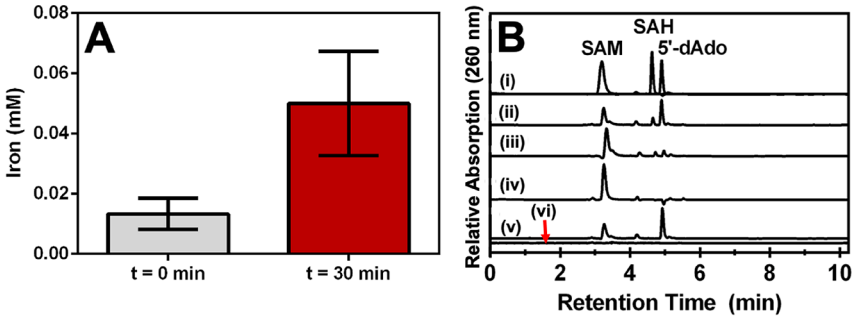

Figure 5. (A) Detection of labile iron and (B) SAM turnover products for the dithionite-driven HutW reaction. The total labile iron was measured, and HPLC analysis was performed as described in Materials and Methods. (A) The labile iron was measured prior to the initiation of the reaction (t = 0) and after 30 min (t = 30). (B) HPLC traces showing the chromatogram for some standards (i) and the results of a 90 min assay containing the complete reaction mix (ii) or in the absence of dithionite (iii), the absence of enzyme (iv), the absence of heme (v), and the absence of externally added SAM (vi).

mechanism. Similar to heme and deuteroheme, the equivalent nonmetalated porphyrin molecules, protoporphyrin IX (Figure 3B) and deuteroporphyrin IX (Figure 3D), were also viable substrates. The absorption changes observed for all of the porphyrin substrates investigated are consistent with turnover regardless of the metalation state of the substrate. In addition, mesoheme and coproporphyrin III exhibited similar spectral changes (Figure S4). No spectroscopic changes were observed when *N*-methylprotoporphyrin was investigated, suggesting that "bent" porphyrins are not viable substrates.

Analysis of the Tetrapyrrole Product by Ion Mobility Mass Spectrometry. The tetrapyrrole product of ChuW has been shown to be a hydrophobic, light/redox sensitive compound that is difficult to isolate and/or solubilize,[15] thus making analysis difficult. However, the field of lipidomics has experienced similar challenges that have resulted in new mass spectrometry methods to characterize hydrophobic compounds.[33,34] To this end, we analyzed HutW assay solutions before and after turnover using hydrophobic liquid chromatography (HILIC) paired with ion mobility mass spectrometry (IM-MS). When sodium dithionite was used as the electron source, a major peak was observed at m/z 583.3 (38.5% m + 1 for ^{13}C), consistent with a more reduced form of anaerobilin (Figure 4). Initially, this is not that surprising, given that the midpoint reduction potential of dithionite approaches −0.8 V at pH 8.0.[35] Specifically, compared to the anaerobilin molecule produced by ChuW, at least two more electrons and protons appear to have been added giving rise to the tetrapyrrole shown in Figure 4A. To further assess the structure of the tetrapyrrole product, fragmentation of the peak at m/z 583.3 was performed (Figure 4). Interestingly, of the 107 likely fragments predicted by the MetFrag[36] software (https://ipb-halle.github.io/MetFrag/), we observe 106 of the fragments with intensities of >200 counts (Figure S5). These results confirm that a more reduced form of anaerobilin is the product of HutW heme degradation (Figure 4A), again with the caveat that sodium dithionite is the reductant in these experiments. However, given that previous experiments using titanium(III) citrate as the reductant for ChuW did not result in production of a more reduced form of anaerobilin,[23] we favor a model whereby HutW is indeed functionally different from ChuW. Sodium dithionite and titanium(III) citrate are strong reductants, so reduction should be thermodynamically favorable. However, unknown kinetic factors related to the pathway for electron transfer cannot be ruled out at this time.

Iron Release and Other Turnover Products. HutW is part of an operon that is required for *V. cholerae* to use heme as an iron source; therefore, we determined the concentration labile iron before and after turnover (Figure 5A). Similar to what has been reported for ChuW, we observe an increase in the labile iron that is proportional to the amount of heme degraded during the assay. As mentioned previously, sequence homology also indicates that HutW is a class C radical SAM methyltransferase (RSMT). Class C RSMTs require two molecules of SAM per turnover. They have been shown to use a mechanism whereby one SAM molecule is converted to 5′-

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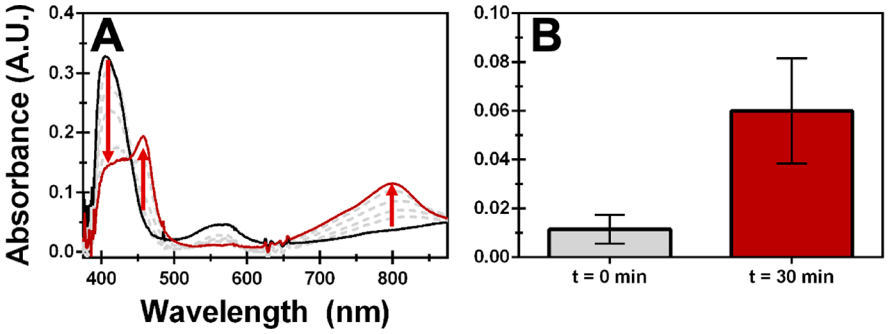

Figure 6. NADPH-dependent turnover of HutW monitored by following (A) the UV−vis spectra and (B) changes in the concentration of the labile iron in the assay. Assay conditions were identical to those shown in Figure 3, except that NADPH (10 mM) was used as the reductant instead of sodium dithionite. In addition, the assay was conducted for 30 min, with a scan recorded every 6 min. The assay was performed as described in Materials and Methods to include HutW (10 μM) and heme (20 μM) and initiated by the addition of SAM (1 mM).

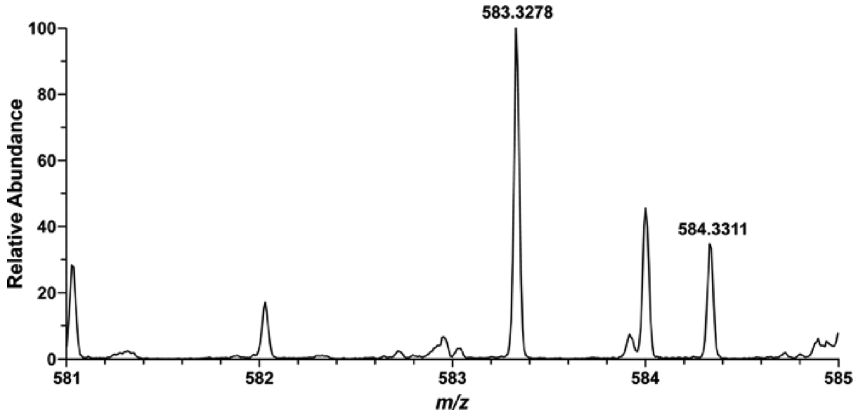

Figure 7. Ion mobility mass spectrometry (IM-MS) of tetrapyrrole product produced in the NADPH-dependent HutW reaction. The NADPH-dependent assay was performed and subjected to IM-MS as described in Materials and Methods. Similar to the dithionite-driven assay (Figure 4), a strong peak at m/z 583.3 appeared in the post-turnover sample.

dA for radical generation while the second molecule of SAM is converted to *S*-adenosyl-L-homocysteine (SAH).[37] Therefore, we employed HPLC to analyze the products of the dithionite-driven HutW reaction. It is important to note that, due to the detection limits of our HPLC system and the low solubility of the tetrapyrrole products, these assays are run for an extended period and do not represent a steady state. However, this does provide insight into the relative ratio of products. On the basis of the data shown in Figure 5B (trace ii), and integration of the respective peaks, 340 μmol of SAH and 745 μmol of 5′-dA have been produced when dithionite is used as the reductant. This is indicative of the "abortive cleavage" of SAM, an observation that has been reported for other RS enzymes.[38]

Due to the extended length of the assay required for HPLC analysis, a small amount of breakdown products is observed in the control experiment in the absence of externally added dithionite (Figure 5B, trace iii), but this is in marked contrast to the fact that no SAH is produced in the absence of heme and abortive cleavage observed only when dithionite is added (Figure 5, trace v). These observations are not unexpected and, as we will demonstrate, are most likely an artifact of the electron source, as others have also reported.[38]

HutX Increases the HutW Turnover Rate. The enzymes HutW, HutX, and HutZ are expressed from the same operon, suggesting that they may work synergistically during heme acquisition and degradation. To address this, we investigated the effect of HutX and HutZ on the turnover rate observed for HutW. The rationale for this experiment is based on the observation that product release has been shown to be the rate-limiting step for the heme oxygenase reaction. This can be altered by the inclusion of biliverdin reductase in the assay, resulting in a significantly faster turnover rate.[39,40] Similarly, when HutX was included in the HutW assay, the turnover rate appeared to increase (Table 1).

However, inclusion of purified HutZ, the third enzyme from the same operon in the *V. cholerae*, failed to increase the rate of turnover. This is distinctly different from the increase in specific activity reported for enterohemorrhagic *E. coli* O157:H7 when the third gene (*chuY*), shown to be an "anaerobilin reductase", is included.[19] In fact, we hypothesize that one reason HutZ does not accelerate the HutW reaction is due to the higher affinity for heme (K_d = 0.052 ± 0.004 μM at pH 8.0) that has been reported.[20] These observations are consistent with previous work supporting a role for HutZ in heme degradation under aerobic growth or oxidizing conditions.[21] These observations may also explain the lack of a ChuS homologue in *V. cholerae*, where ChuS, from *E. coli* O157:H7, has been shown to catalyze heme degradation under aerobic conditions in vitro.[31]

NADPH Is an Acceptable Electron Donor for the HutW Reaction. Every radical SAM enzyme requires a source of low-potential electrons. Ultimately, in vivo these electrons come from NADH or NADPH by way of ferredoxin or flavodoxin and the respective oxidoreductases. However, in the course of expression and isolation of these enzymes, it may or may not be possible to obtain the physiologically relevant

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electron transfer proteins. Therefore, chemical reductants such as sodium dithionite or titanium(III) citrate are often used in the laboratory. As others have observed, and we clearly see in Figure 5B, strong chemical reductants can lead to unproductive cleavage of SAM. Interestingly, HutW is phylogenetically distinct from ChuW and contains a glycine rich NAD(P) binding motif (^{328}GCGAGGNMGG^{337}).[32,41] Given this observation and the lack of a ChuY homologue in *V. cholerae*, we decided to test whether NADPH would support HutW turnover. Surprisingly, utilization of NADPH as the electron donor in the HutW assay resulted in changes in the absorption spectra that were similar to what was observed when sodium dithionite was used as the electron donor (Figure 6A).

In addition, a similar increase in the concentration of labile iron was also observed (Figure 6B). The specific activity using NADPH was calculated from the linear portion of the progress curves (Figure S3), as described in Materials and Methods, and resulted in a rate of 148.3 ± 14.0 nmol min^{-1} mg^{-1}. To identify the tetrapyrrole product(s), we subjected the NADPH-dependent turnover samples to IM-MS (Figure 7).

Similar to the turnover sample using sodium dithionite as the reductant, the major product was associated with a peak at m/z 583.3 (Figure 7). However, in contrast to the assay using sodium dithionite, an additional peak that was not observed in the preturnover sample but was present post-turnover, albeit at much weaker level, was a peak at m/z 581.3 (Figure 7). This peak is consistent with an intermediate state of reduction as anaerobilin is reduced in two-electron/proton increments. Finally, we subjected the NADPH-dependent turnover samples to HPLC analysis and found production of SAH and 5′-A (Figure 8). Again, the detection limits of our HPLC system require that we run the assay for an extended period. The integrated areas indicate the production of 634 μmol of SAH and 742 μmol of 5′-A. While not quite stoichiometric, this is a significant improvement over what was observed when sodium dithionite was used as the electron source.

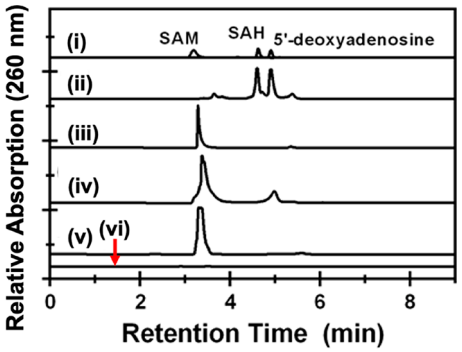

Figure 8. HPLC analysis of the NADPH-driven turnover reaction of HutW. HPLC analysis was performed as described in Materials and Methods. Traces show the chromatogram for standards (i), and the results of an overnight assay containing the complete reaction mix (ii) or in the absence of NADPH (iii), the absence of enzyme (iv), the absence of heme (v), and the absence of externally added SAM (vi).

Regardless, comparison of the data in Figure 8, with the data from the dithionite-driven turnover presented in Figure 5, indicates a significant decrease in the rate of abortive cleavage. More importantly, the observation that the physiological reductant, NADPH, can be used to drive the ring-opening reaction catalyzed by HutW, as well as reduction of the initial tetrapyrrole product, is significant in light of the lack of a ChuY homologue in *V. cholerae*.

DISCUSSION

The human gut represents a set of complex and unique microbial habitats where organisms, including the host cells, must compete for the available micronutrients.[42] A new theory has emerged that suggests commensal organisms live synergistically with one another and the host. In contrast to this collaborative relationship, enteric pathogens appear to have evolved mechanisms to rapidly acquire or steal nutrients from the commensal flora and host cells, an advantage that is amplified during systemic infection and the induction of certain host defense mechanisms. However, it is reasonable to assert that our current understanding of the human microbiome is still in its infancy. In part, understanding the human microbiome is challenging because a significant portion of the gut ecology is represented by strictly anaerobic commensal organisms that are difficult to culture. Moreover, many commensal organisms form consortiums, dependent on one another for specific, sometimes unknown, metabolites. Given the rise in antibiotic resistance, understanding how pathogenic bacteria compete in this environment is a major global interest. The utilization of heme as an iron source represents a nutrient acquisition mechanism that is found in pathogenic organisms, but not commensal bacteria, and is often essential to pathogenesis.[13] Therefore, a complete understanding of the novel mechanisms for pathogenic heme degradation, and the secondary messaging that triggers pathogenesis, represents a largely unexplored avenue of antibiotic development. In particular, while extensive work has been performed on the aerobic pathways for heme catabolism,[43] our understanding of unique anaerobic pathways remains deficient.

Anaerobic Heme Degradation by HutW in *V. cholerae*. It is clear that survival in the complex ecology of the human microbiome requires an organism to rapidly adapt to nutrient availability. In this work, we demonstrate that HutW is a radical SAM enzyme involved in anaerobic heme degradion in *V. cholerae*. The protein sequence, iron analysis, and EPR spectra of HutW presented in this work are all consistent with the presence of a single [4Fe-4S]$^{+}$ cluster in the active enzyme (Figure 1). Moreover, the EPR signal for the [4Fe-4S]$^{+}$ cluster undergoes SAM-dependent changes that are consistent with what has been reported for other radical SAM enzymes containing only the catalytic [4Fe-4S] cluster.[44] Finally, the presence of this cluster is required for heme degradation.

On the basis of changes in the major Soret band of heme, we found that HutW binds ferrous heme with a K_d of 3.6 ± 0.9 μM (Figure 2), consistent with other proteins involved in heme degradation.[17,30,45] Moreover, upon anaerobic incubation with SAM and sodium dithionite, the UV absorption spectra reveal changes consistent with opening of the porphyrin ring (Figure 3). Care must be taken when powerful chemical reductants such as sodium dithionite or titanium(III)citrate are utilized as this can lead to product artifacts. Regardless, similar to what was reported for ChuW in *E. coli* O157:H7, the iron atom does not appear to be involved in the mechanism of ring opening.[23] Analysis of the pre- and post-turnover assay by IM-MS confirmed the formation of a linear tetrapyrrole (Figure 4). We also observe a concurrent increase in the level of labile iron in the assay when metalated porphyrins were used as the substrate, consistent with the

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liberation of iron from the porphyrin macrocycle (Figure 5). Taken together, these data support a role for HutW in the anaerobic degradation of heme and iron release. Similar to what was reported for ChuW, from enterohemorrhagic *E. coli*, the mechanism of ring opening involves two molecules of SAM, one being required for radical generation and the other involved in formation of a transient methylene radical. Consistent with what has been recently reported for other class C RSMT enzymes, both 5′-dA and SAH are produced (Figure 5). However, the ability to utilize NADPH directly is an unexpected and significant difference in the mechanism of HutW. This observation must be considered in the larger context of heme acquisition/utilization by enteric pathogens and the enzymes involved. Specifically, while titanium(III) citrate was not capable of reducing the tetrapyrrole product of ChuW,[23] this organism has an additional enzyme, ChuY, that utilizes NADPH to catalyze a four-electron reduction.[19] To the best of our knowledge, there is no ChuY homologue in *V. cholerae*. In that context, the observation that HutW can catalyze the ring opening and four-electron reduction of anerobilin, regardless of electron source, certainly makes sense. However, when considering electron transfer reactions, it is important to recognize that there is a thermodynamic component and a kinetic component. The former is dependent on the midpoint reduction potential of the donor and acceptor, while the latter is often hidden within the subtle elements of protein structure and the specific electron transfer pathway. This is being investigated further, but the ability to utilize NADPH directly, instead of via a flavodoxin and ferredoxin/flavodoxin NADP oxidoreductase, would seem advantageous.

HutW Is a Class C RSMT. The results reported herein are consistent with HutW functioning as a class C radical SAM methyltransferase (RSMT) that shares some sequence and mechanistic homology with ChuW but is phylogenetically distinct.[32] Currently, the proposed mechanism for ChuW and other class C RSMT involves two molecules of SAM, as first observed in the HemN crystal structure.[49] The first molecule of SAM is required for generation of the 5′-deoxyadenosyl radical (5′-dA•). This radical, proposed to be common to all radical SAM enzymes, abstracts a hydrogen atom from the second molecule of SAM (SAM2) yielding a methylene radical. A unique aspect of the class C RSMT mechanism is that the methylene radical on SAM2 is added to a double bond, methylating an otherwise unreactive sp^2-hybridized carbon center.[46] This is in contrast to an enzyme that uses the methylene radical to abstract a hydrogen atom from the substrate molecule. The latter has now been shown to be the case for HemN, as a SAM2−prophyrin adduct was recently captured,[47] confirming an evolutionary/mechanistic connection between HemN and the class C RSMTs. Specifically, in the HemN mechanism SAM2 functions as part of a "hydrogen atom relay" network, while the methylene radical in class C RSMTs serves to methylate what would be otherwise unreactive sp^2-hybridized carbon centers.[27,37,48,49] Therefore, an equal ratio of 5′-deoxyadenosine (5′-dA) to the *S*-adenosylhomocysteine (SAH) product is expected for any class C RSMT. We looked for both 5′-dA and SAH production during HutW turnover by HPLC and observed a much larger peak for 5′-dA when using the chemical reductant sodium dithionite (Figure 5), compared to when NADPH is used (Figure 8). This is consistent with the "abortive cleavage" of SAM that others have reported for radical SAM enzymes when chemical reductants are used.[38]

Mechanism and Function of HutW versus ChuW. A significant, and potentially problematic, step in the catabolism of heme is the production of reactive tetrapyrrole intermediates. Specifically, production of the biliverdin or anaerobilin intermediate occurs via the aerobic or anaerobic pathway, respectively. Accumulation of these intermediates is often toxic, and therefore, organisms contain specific reductases to prevent accumulation.[19,50,51] The source of the toxicity appears to be ring conjugation and the general aromaticity of the compounds, making the tetrapyrrole highly reactive and insoluble. Therefore, it is not surprising that these reductases eliminate some of the toxicity by reducing the double bonds between the pyrrole ring and a bridging carbon atom.[19,52] While we have shown that HutW is an anaerobic heme-degrading enzyme, like ChuW, functional differences between the enzymes must be considered in the larger context of what is known about utilization of heme as an iron source as well as the organization of these genes in the two organisms.

Pathogenic strains of *E. coli*, such as serotype O157:H7, and *V. cholerae* contain similar operons for the utilization of heme as an iron source.[1,53] Likewise, expression of genes within both operons has been shown to be regulated in a Fur-dependent manner and they have been shown to encode a number of proteins with homologous functions. For example, homologous proteins are involved in heme uptake, transport/storage within the periplasm, and the ATP-dependent transport from the periplasm into the cytoplasm. However, following transport into the cytoplasm, the fate of heme seems to differ in the two organisms. For pathogenic *E. coli* O157:H7, evidence has been presented that identifies ChuS as both a heme oxygenase[54] and a heme storage/transfer protein.[23] The latter role is consistent with the structural homology and significant sequence identity (66%) to the heme storage protein PhuS in *Pseudomonas aeruginosa*.[55,56] Moreover, little is known about how the externally obtained heme is incorporated into the pathogen's own heme proteins or how the degradation products of heme are sequestered. Specifically, how do the pathogens detoxify the tetrapyrrole products or transport or store the iron that is released following catalysis? These are important questions because the only single-gene deletions that have been shown to result in a fitness phenotype during growth on heme as the sole iron source in pathogenic *E. coli* O157:H7 or *V. cholerae* are deletions of ChuY or HutZ, respectively.[13,22] Similar to the role of biliverdin reductase, ChuY has been shown to utilize NADPH to reduce anaerobilin.[19] Even though the *hutZ* gene is found in an orientation similar to that of the *chuY* gene, HutZ has been shown to be a peroxide-dependent heme oxygenase, potentially explaining the phenotype of the deletion strain. However, there is no ChuY homologue in *V. cholerae*. Therefore, the discovery that HutW can utilize NADPH to catalyze not only the opening of the porphyrin ring but also the reduction of anaerobilin suggests that HutW performs the function of both ChuW and ChuY. However, deletion of HutZ would still be problematic as it is required for aerobic heme degradation, consistent with the observed phenotype for the deletion strain.[13] This conclusion is also consistent with sequence motifs unique to HutW and the observation that HutW is phylogenetically distinct, as discussed above.

HutX Stimulates HutW Catalysis. HutX is transcribed from the gene immediately following HutW in the same operon. Therefore, it is plausible that HutX is also involved in the anaerobic breakdown of heme, by binding the freed iron atom, the linear tetrapyrrole, delivering heme to HutW, or

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through some other mechanism, such as allosteric modulation of HutW activity. From the current literature, HutX is annotated as a heme binding protein. Therefore, we hypothesize that HutX could function to deliver heme to HutW under anaerobic conditions, or HutZ aerobically for degradation, fulfilling its role as a general heme binding protein. Additionally, recently a new role for ChuX (the homologous protein from *E. coli* O157:H7) was proposed where the ChuX protein works synergistically with the ChuW protein as a chaperone to transiently sequester the iron atom and/or the tetrapyrrole product.[23] The evidence presented here demonstrates that HutX stimulates the heme degradation activity of HutW (Table 1). Whether this is through binding and facilitation of removal of iron or the tetrapyrrole product is currently under investigation. Regardless, HutX binding one of the products of HutW catalysis is in agreement with both the organization of the genes within the heme utilization operon and our evidence that HutX stimulates HutW activity.

Conclusions. We have provided experimental evidence that HutW is a class C RSMT involved in the liberation of iron from heme. HutW is expressed along with several other proteins involved in heme acquisition under iron-depleted conditions.[13] The observation that NADPH will catalyze the ring opening and subsequent reduction of the tetrapyrrole product is a significant new observation that is consistent with the lack of a ChuY homologue in *V. cholerae*. In evolutionary terms, the conservation of an enzyme that releases iron from heme under anaerobic conditions across enteric pathogens makes sense, as any nutritional advantage would be significant in the crowded and competitive environment of the human gut. Future work will interrogate the precise mechanism of NADPH-dependent turnover. Specifically, to provide insight into the reaction mechanism and how interactions with HutX modulate the mechanism, these studies will be coupled to biophysical investigations, to shed light on potentially unexplored avenues for antimicrobial development. Finally, this work, and future investigations, will expand our understanding of the poorly understood class C RS enzymes involved in the synthesis of natural compounds with therapeutic properties.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00950.

UV−vis spectra of as-isolated HutW, analysis of HutW on a calibrated size exclusion column, progress curves for HutW turnover (with a heme substrate) using Dt and NADPH, and complete breakdown of MS fragmentation (PDF)

Accession Codes

Information about the enzymes being studied in this work can be found as UniProt entries A0A3G4VEL8 and Q9KL40 for HutW and HutX, respectively.

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Notes

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