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Reactions of ferrous coproheme decarboxylase (HemQ) with O₂ and H₂O₂ yield ferric heme *b*

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Abstract

A recently discovered pathway for the biosynthesis of heme *b* ends in an unusual reaction catalyzed by coproheme decarboxylase (HemQ), where the Fe(II)-containing coproheme acts as both substrate and cofactor. Because both O₂ and H₂O₂ are available as cellular oxidants, pathways for the reaction involving either can be proposed. Analysis of reaction kinetics and products showed that, under aerobic conditions, the ferrous coproheme-decarboxylase complex is rapidly and selectively oxidized by O₂ to the ferric state. The subsequent second-order reaction between the ferric complex and H₂O₂ is slow, pH dependent, and further decelerated by D₂O₂ (average KIE = 2.2). The observation of rapid reactivity with peracetic acid suggested the possible involvement of Compound I (ferryl porphyrin cation radical), consistent with coproheme and harderoheme reduction potentials in the range of heme-proteins that heterolytically cleave H₂O₂. Resonance Raman spectroscopy nonetheless indicated a remarkably weak Fe-His interaction; how the active site structure may support heterolytic H₂O₂ cleavage is therefore unclear. From a cellular perspective, the use of H₂O₂ as an oxidant in a catalase-positive organism is intriguing, as is the unusual generation of heme *b* in the Fe(III) rather than Fe(II) state as the end product of heme synthesis.

Graphical Abstract

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Author Contributions

All authors have given approval to the final version of the manuscript.

Supporting Information. Additional supporting data include (S1) UV/vis spectra for all complexes in the manuscript; (S2–S9) further UV/vis and EPR spectroscopic characterization of reactions with O₂ and H₂O₂; (S10) redox titrations of the coproheme decarboxylase in complex with harderoheme and heme *b*. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

heme; iron; propionate; decarboxylase; gram-positive

Tetrapyrroles - hemes, corrins, and chlorophylls - give rise to many of the colors of life and mediate processes from respiration to photosynthesis. The biosynthetic routes to tetrapyrroles have evolved and diversified alongside the transitions from anaerobic to aerobic Earth and uni- to multi-cellular life. In addition to the well-known, textbook pathway for heme biosynthesis, at least two more ancient routes have been described.^{1, 2}

In the final steps of the well-described pathway, which is shared by eukaryotes and gram-negative bacteria, coproporphyrinogen undergoes decarboxylation, macrocycle oxidation, and metalation to give heme *b*.^{3, 4} By contrast, in gram-positive bacteria (Actinobacteria, Firmicutes), the same intermediate is initially oxidized and then metalated to give coproheme (isomer III). In the final step, two propionate substituents on the coproheme III (referred to as *coproheme* herein) are oxidatively decarboxylated to vinyls to produce heme *b*. The oxidation and metalation steps are catalyzed by homologs to their canonical counterparts, which act on different substrates in the non-canonical pathway. The final step, however, is catalyzed by a novel decarboxylase encoded by the *hemQ* gene.¹ Functionally, the coproheme decarboxylase (HemQ) is part of a collection of enzymes that includes heme oxygenases where the same molecule acts as both substrate and cofactor.

It was previously observed that the decarboxylation (formally involving a loss of $H^+ + 2e^- + CO_2$ per reactive propionate) can proceed with the coproheme in the ferric (Fe(III)) oxidation state and with hydrogen peroxide as the cosubstrate.^{1, 5, 6} However, whether this reaction is biologically relevant is not clear because, under biological conditions, iron is inserted into porphyrins as Fe(II).⁷ Because ferrous hemes can react with O₂, the cellular process could use O₂ and ferrous coproheme as cosubstrates, yielding H₂O₂ and a ferrous decarboxylation product (Scheme 1A). An O₂-dependent reaction of this kind is consistent with known biological oxidative decarboxylases, including those used in the canonical pathway to decarboxylate heme intermediates.^{8–11} Alternatively, a reaction between O₂ and Fe(II)-coproheme could occur by a monooxygenase pathway via a hydroxylated intermediate (Scheme 1B). This mechanism is analogous to the heme oxygenase reaction, which begins with an O₂-mediated hydroxylation of the porphyrin ring.^{12, 13} Finally, the enzyme might catalyze a reaction involving Fe(II) and H₂O₂ (Scheme 1C). While not completely unprecedented,^{14, 15} such a reaction would be highly unusual since hemes generally react with H₂O₂ when the iron is in the ferric oxidation state.¹⁶ Potential

competition from the Fenton-reaction, in which ferrous ion rapidly donates an electron to H_2O_2 to yield OH^- and $\text{OH}^\bullet$, would have to somehow be excluded.

We sought to determine which if any of these pathways is followed by the coproheme decarboxylase in the presence of both O_2 and H_2O_2 . Using the *hemQ*-encoded enzyme from *Staphylococcus aureus*, we examined complexes of the protein with its substrate coproheme and intermediate harderoheme III (here referred to as *harderoheme*), and their reactions with both oxidants from both the ferrous and ferric states. The results presented here support the conclusion that the enzyme follows none of the standard Fe(II)-dependent pathways outlined in Scheme 1; instead, it facilitates the O_2 -mediated oxidation of the ferrous iron under aerobic conditions and then acts as an Fe(III)/peroxide-dependent decarboxylase. Distinct from the canonical pathways for heme biosynthesis, the coproheme decarboxylase yields ferric heme *b*. These findings have important implications for how *S. aureus* and potentially other gram-positive bacteria have evolved to survive in and exploit an aerobic environment.

EXPERIMENTAL METHODS

Preparation of stocks and pH buffers

Ferric coproporphyrin III chloride (coproheme, Frontier Scientific) and hemin chloride (Sigma) were dissolved in dimethyl sulfoxide (DMSO) to generate 5–10 mM stocks. Harderoheme III was synthesized and purified as described previously.⁵ Hydrogen peroxide (H_2O_2) and peracetic acid working solutions (0.5–5 mM) were prepared from 50 mM stocks in 50 mM potassium phosphate (KPi) buffer (pH 7.4). Working solutions were kept on ice and replaced with fresh stocks every 2 h. Oxidant stocks were titrated with acidified KMnO_4 , which had itself been titrated against ultrapure oxalic acid, to verify their concentrations. Biochemicals (lysozyme, catalase, DNase, etc.) were from New England Biolabs. Reactions were carried out over a range of pH values in either 50 mM KPi (pH 5.8, 6.6, and 7.4) or 50 mM Tris-Cl (pH 8.2 or 8.8). For the solvent isotope studies, the 50 mM Tris-Cl buffers were made in D_2O (Cambridge isotopes, 99.8 % D) with addition of DCl (Acros Organics 99% + D) to give final pD values 6.1, 6.5, 6.8, 7.3, 8.1, 8.7, and 9.2 (pD = $\text{pH}^* + 0.41$, where pH^* is the apparent pH measuring using a standard glass electrode).¹⁷ Protein samples were prepared through multiple rounds of concentration and resuspension in deuterated buffer, resulting in only 0.1 % or less of the original buffer solution present in the final solution. D_2O_2 solutions were made from a 10.6 M H_2O_2 stock diluted 1:100 in D_2O , producing a 108 mM stock with 98 % deuterium enrichment.

Protein production and complex formation

Expression, purification, and ligand complexation with coproheme decarboxylase were carried out as previously reported.⁵ Porphyrin concentrations were determined by the pyridine hemochrome methods. Briefly: 50 μL of protein solution (at 50–300 μM) was mixed with 200 μL of 50 mM NaOH containing 20% pyridine by volume. 3 μL of 0.1 M $\text{K}_3[\text{Fe}(\text{CN})_6]$ was added and the oxidized spectrum was measured; 3–5 mg of solid sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) was then added to yield spectra for the reduced pyridine-bound hemes. Difference spectra (reduced minus oxidized, r-o) were used to determine the concentration

of metalloporphyrin released from the protein. For coproheme: $\epsilon_{\text{r-o}} 546 \text{ nm} = 23.2 \text{ mM}^{-1} \text{ cm}^{-1}$; for heme *b*, $\epsilon_{\text{r-o}} 556 \text{ nm} = 28.4 \text{ mM}^{-1} \text{ cm}^{-1}$.

Heme reduction

Solutions were made anaerobic using a double manifold Schlenk line with alternating cycles of argon gas purging and evacuation. Protein-coproheme complexes were reduced in an anaerobic chamber (Coy) using solutions of sodium dithionite prepared by dissolving the solid in deoxygenated buffer. The dithionite concentration was determined via anaerobic titration with potassium hexacyanoferrate(III) in the presence of methylene blue as indicator.¹⁸ A 1.5 molar excess of dithionite relative to coproheme was added and reduction of the heme species was monitored by UV/visible spectroscopy (UV/vis, Agilent 8453 spectrometer). Excess unreacted dithionite and dithionite oxidation products were removed using a PD-10 desalting column (GE Healthcare).

Titration with oxidants

Complexes of coproheme decarboxylase with either coproheme or harderoheme (ferrous and ferric, pH 7.4, 20 °C) were mixed anaerobically with H₂O₂ or peracetic acid (0, 0.5, 1, 1.5 or 2 eq) inside an anaerobic chamber. Products were analyzed by UV/vis, high performance liquid chromatography (HPLC) (samples 10 μM in the heme species), and electron paramagnetic resonance spectroscopy (EPR) (described below). Reactions with O₂ were carried out by exposing ferrous complexes to atmosphere (240 μM O₂, pH 7.4, 20 °C) or diluting anaerobic reaction mixtures with aliquots of buffer containing defined oxygen concentrations inside septum-sealed vials. Desired concentrations were obtained by mixing O₂-saturated and anaerobic buffers in varying proportions, then verifying the resulting concentration of dissolved O₂ via polarography using a Clark O₂ electrode. In reactions involving either O₂ or peracid, catalase was included at 1000 U/mL to remove any H₂O₂.

HPLC

Reactions were analyzed for coproheme, harderoheme, and heme *b* by HPLC on an Agilent 1100 series instrument with a Phenomenex Luna C18 3 μm column (150 mm x 4.6 mm). The solvents were H₂O with 0.1% TFA (trifluoroacetic acid) (Solvent A) and acetonitrile (ACN) with 0.1% TFA (Solvent B). All samples were run at a flow rate of 1.5 mL/min starting with isocratic 80% A for 3 min, followed by a linear gradient transitioning to 5% A over 12 min. This was followed by 5% A for 5 min and a final isocratic run of 20 % A for 3 min. UV/vis detection was at 411 nm. Standard curves (0 – 500 pmol) were generated for quantifying heme species via integration of the peak areas. Samples were quantified in triplicate and error bars are ± 1 standard deviation.

EPR

250 μL aliquots of coproheme decarboxylase in complex with its ferrous/reduced substrate (pH 7.4, 90 μM) were mixed with 0–2 eq of H₂O₂ from a 5 mM stock, loaded into EPR tubes, sealed, and removed from the anaerobic chamber in a sealed glove bag. The samples were immediately frozen and stored in liquid N₂. Spectra were measured on a Bruker EMX EPR spectrometer (X-band, 9.37 MHz) at 12 K using a Bruker Cold Edge (Sumitomo

Cryogenics) cryogen-free system with a Mercury iTC controller unit. Instrument parameters were: 3.18 MW microwave power, 100 kHz modulation frequency, and 5 G modulation amplitude. Averages of 4 scans are reported. For spin quantitation, horse heart myoglobin (Sigma) was used as a standard. Spectra were double integrated over 500–4000 gauss (OriginLab) to include the range of Fe(III) species produced during the reaction with H₂O₂.

Transient kinetics

Data were measured using KinetAssyst stopped-flow spectrometer (Hi-Tech Scientific) in single mixing mode with diode array detection. For the ferric substrates, the decarboxylase-coproheme complex (5 μ M) was rapidly mixed (<5 ms) with variable concentrations of H₂O₂ or peracetic acid prior to measurement of spectra. For ferrous substrates, the spectrometer was made anaerobic by overnight incubation with protocatechuate dioxygenase (PCD) and its substrate, protocatechuic acid (PCA).¹⁹ Dithionite-reduced anaerobic samples were prepared in an anaerobic chamber as described above and sealed in an airtight tonometer that interfaced with the stopped flow sample handling unit. Deoxygenated buffer, H₂O₂, and peracetic acid solutions were prepared in the glove box, sealed in gastight syringes, and then introduced to the sample handling unit for reaction. Data were measured at varying time points and fit using the Kinetic Studio (Hi-Tech Scientific) software to exponential decay functions to determine rate constants (k_{obs}). For each experimental condition, all data were measured in at least triplicate and averaged. Plots of k_{obs} versus oxidant concentration were fit with linear least squares regression analysis to determine second order rate constants (Kaleidagraph).

Testing for oxygenase activity

To test for oxygenase activity in the ferric state, 5 μ M ferric coproheme-decarboxylase complex was treated with 5 mM (1000 eq) ascorbate in the presence of 100 U catalase (50 mM KPi, pH 7.4) and spectral changes were monitored every min for 30 min. The same assay conditions led to complete oxidation of the heme *b* substrate in studies of bacterial IsdG-type heme oxygenases.²⁰ To screen for oxygenase activity from the ferrous state, 10 μ M coproheme-bound decarboxylase was reduced with dithionite under anaerobic conditions (excess reductant removed as described above). The complex was mixed 1:1 v/v in the stopped flow with freshly prepared aerobic solutions of reductant (2 mM dithionite or 20 mM ascorbate, 50 mM KPi, pH 7.4) and spectral changes monitored over time via diode array detection. The products in each case were quantified by HPLC.

Spectroelectrochemical titrations

Fe(III)/Fe(II) reduction potentials (E) were determined via titration of the decarboxylase bound to coproheme, harderoheme, or heme *b* (6 μ M) with dithionite in the presence of the redox mediator methyl viologen (2 μ M) and a reducible dye (10–15 μ M) in an anaerobic glove box (50 mM KPi, pH 7.0, 20 °C). The dyes used were indigo disulphonate (–125 mV, coproheme and harderoheme complexes) and indigo trisulphonate (–85 mV, heme *b*) (all potentials are reported versus the standard hydrogen electrode, SHE, at pH 7).²¹ Changes in the UV/vis spectrum were recorded following each dithionite addition. Absorbances for the oxidized and reduced dyes (dye_{ox}, dye_{red}) and the ratio of the oxidized/reduced protein-heme absorbances (Enz_{ox}/Enz_{red}) were recorded at their maxima: 612 nm, oxidized indigo

disulphonate; 593 nm, indigo trisulphonate; 424 nm, coproheme; 418 nm, harderoheme; 428 nm heme *b*. Plots of $\ln(P_{ox}/P_{red})$ versus $\ln(dye_{ox}/dye_{red})$ were fit to a modified Nernst equation:

$$\ln(Enz_{ox}/Enz_{red}) = (nF/RT)(E_{dye} - E_P) + \ln(dye_{ox}/dye_{red}) \quad (1)$$

R is the universal gas constant, F is Faraday's constant, and n is the number of reducing equivalents involved. Titrations were carried out in triplicate, averaged, and ± 1 standard deviation reported as the error.

UV/visible and resonance Raman characterization

UV/vis spectra were measured on either a Cary 50 spectrometer under ambient atmosphere, or an Agilent 8453 spectrometer housed inside an anaerobic chamber. Resonance Raman (rR) spectra were obtained from enzyme samples ranging from 20 to 100 μ M in coproheme at 20 °C. Spectra were recorded using the 135° backscattering geometry and f1 collection. Excitation of Raman scattering was achieved with either 441.6-nm emission from a HeCd laser, or the 413.1-nm line from a Kr⁺ laser. The laser beam was focused to a line on a spinning 5 mm NMR tube. Scattered light was passed through a holographic notch filter and a polarization scrambler then f-matched to a 0.67-m, f/4.7 Czerny-Turner spectrograph fitted with a 1200 groove/mm grating. The Raman spectrum was detected using a liquid N₂ cooled CCD camera (1340×400 array of 20×20 μ m pixels, 26.8×8.0 mm² image area). Dimethylformamide, toluene, acetone, and methylene bromide were used as external standards for spectral calibration. Laser power at samples ranged from 2 to 8 mW; no spectral artifacts due to photoinduced chemistry were observed with these irradiation powers.

RESULTS

The purified decarboxylase forms stable complexes with hardero- and coproheme

The decarboxylase purified in yields of 15–20 mg/L culture. Following incubation with either coproheme or harderoheme and further purification by gel filtration chromatography, nearly 1:1 ratios of protein monomer:heme species were measured.⁵

UV/vis features of the oxidized and reduced enzyme-heme complexes are distinct

Spectra for the oxidized and reduced complexes of the decarboxylase with its substrate (coproheme), intermediate (harderoheme), and product (heme *b*) were measured and their features summarized in Table 1 (Supplementary Information Figure S1). As previously noted,⁵ the ferric complexes had Soret and visible bands typical of five coordinate high spin ($S = 5/2$) Fe porphyrins with the Soret bands shifting to the red as the number of propionates (and therefore the charge) of the tetrapyrrole diminished (coproheme to harderoheme to heme *b*). Reduction of the iron led to a significant red shift of the Soret band (>20 nm) for each ferrous complex relative to the ferric value and the appearance of a prominent visible band near 550 nm with a higher energy shoulder. These spectral changes are consistent with

those observed for other histidine-ligated heme proteins, and are suggestive of the formation of five coordinate high spin (5cHS) ferrous complexes.^{22, 23}

Reactions between ferrous heme species and O₂ do not lead to decarboxylation

Complexes of the decarboxylase with coproheme or harderoheme were reduced to their ferrous forms under anaerobic conditions. Following exposure to air in the presence of catalase, both ferrous complexes converted to ferric within the manual mixing time ($\sim$ 5 s, pH 7.4, 20 °C, Figure S2). HPLC analysis of the tetrapyrrole products indicated no change in the coproheme amount or retention time, indicating that the propionate side chains were not converted to vinyls (Figure S2). It was concluded that propionates 2 and 4 are not converted to vinyls via an Fe(II)/O₂ mediated oxidation (Scheme 1A).

The ferrous enzyme-coproheme complex and O₂ rapidly form an intermediate that converts to ferric coproheme

Reactions between the ferrous enzyme-coproheme complex and O₂ were monitored via UV/vis stopped flow (pH 7.4, 20 °C). An intermediate with a Soret band at 408 nm (350 nm shoulder) and visible bands at 539 and 573 nm (Table 1) formed rapidly (100 ms, Figure 1A). The rate constant for Fe(II)/O₂ intermediate formation depended linearly on O₂ concentration under pseudo first order conditions, yielding second order rate constant $k = 1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 1B, Table 2). The intermediate converted in a slower, O₂-independent, single-exponential phase to a final product ($k = 0.20 \pm 0.02 \text{ s}^{-1}$, Table 2) with UV/vis features closely resembling the ferric enzyme-coproheme complex. A two-intermediate model (sum of 3 exponentials) was tested but did not substantially improve the quality of the data fit. The identity of the product as coproheme was confirmed via comparison of its HPLC retention time with those of standards (Figure S2).⁵

The oxidative decarboxylations do not occur via a monooxygenase intermediate

A monooxygenase reaction starting from complexes of the decarboxylase with ferric states of coproheme or harderoheme and leading to hydroxylation of the propionate side chain may require both O₂ and additional electrons. In studies of both canonical and IsdG-type heme oxygenases, the electrons have been supplied by excess ascorbate, dithionite, or other chemical donors that can act as surrogates for enzymatic reductases proposed to act in concert with the oxygenases in the cell.^{13, 20, 24, 53, 54} Under conditions that led to reactivity in IsdG-type heme oxygenases (excess ascorbate, O₂ and catalase to consume H₂O₂),^{13, 20} no reaction was observed for the decarboxylase-coproheme complex (i.e., no changes in the UV/vis spectrum and no loss/conversion of coproheme was observed by HPLC, Figure S2). Reactions were subsequently carried out starting from the ferrous coproheme-decarboxylase complex. Upon addition of O₂ and in the presence of either 10 mM ascorbate or 1 mM dithionite the same intermediate formed that was observed in the Fe(II)/O₂ reaction in the absence of reductants (Figure 1A, Table 1). No changes in the rate of intermediate formation or decay were detected, and the only observable product (via UV/vis and HPLC) was ferric coproheme (Figure S2). This suggests that the presence of excess reducing equivalents along with O₂ is not sufficient to promote oxidative decarboxylation. This further indicates that the coproheme decarboxylase either does not react as a monooxygenase (Scheme 1), or that,

unlike known heme oxygenases, it has an obligate requirement for a specific physiological reductant.

Decarboxylation of the ferrous coproheme or harderoheme complexes does not occur following reaction with H₂O₂

We next examined the possibility of a ferrous decarboxylase reaction using H₂O₂. If the enzyme acts efficiently, then 0–1 eq of H₂O₂ should result in stoichiometrically equivalent amounts of vinyl groups forming with no oxidation of the iron. To test this hypothesis, ferrous complexes of the coproheme decarboxylase with both the substrate and intermediate harderoheme were prepared, mixed anaerobically with 0–2 eq of H₂O₂, and the tetrapyrrole and Fe(III) products quantified by HPLC, EPR, and UV/vis spectroscopy (pH 7.4, 20 °C, Table 2).

UV/vis spectra did not exhibit changes consistent with the oxidation of the propionates; rather, both UV/vis and EPR spectra indicated that the substrate Fe(II) was oxidized to Fe(III) (Figure S3–S4). Iron oxidation did not occur stoichiometrically with added H₂O₂. Instead, full oxidation of the iron for either the coproheme or harderoheme complex required 1.5 eq of H₂O₂, at which point 0.58 or 0.14 vinyl groups (per coproheme or harderoheme molecule, respectively, Table 2) had also formed, with both harderoheme and heme *b* as products. We note that, though the mechanism of propionate oxidation is not possible to discern from these experiments, 0.45 vinyl groups formed following addition of 1.5 eq of H₂O₂ to the ferric coproheme-decarboxylase complex, suggesting that the observed propionate oxidation could have occurred following oxidation of the coproheme iron. Together, these results demonstrated that H₂O₂ preferentially oxidized Fe(II), accompanied by a small amount of oxidation of the propionates of either coproheme or harderoheme.

The ferrous coproheme-decarboxylase complex reacts more rapidly with air than with H₂O₂

Even though it does not lead to stoichiometric decarboxylations, we asked whether H₂O₂ might nonetheless kinetically outcompete O₂ for reaction with the ferrous iron of coproheme or harderoheme bound to the decarboxylase. The reactions between these complexes and H₂O₂ were therefore examined in the absence of O₂ by stopped flow.

After mixing the enzyme-coproheme complex with 1 eq H₂O₂, an intermediate spectrum with UV/vis features similar to the Fe(II)/O₂ intermediate formed (maximizing at ~100 ms, pH 7.4, Table 3, Figure 2A). This intermediate slowly converted to a ferric product (within 20 s at pH 7.4, $k_{\text{obs}} = 0.48 \text{ s}^{-1}$, Table 3). EPR confirmed that the majority of this end product was in the ferric state (Table 2, Figure S4). Its UV/visible features resembled those measured for ferric decarboxylase-coproheme or –harderoheme complexes, or a mixture of the two (397 nm [Soret]; 508, 632 nm [visible bands]) (Table 1). This result is consistent with HPLC analysis of the products showing predominantly Fe(III) production and incomplete oxidation of the propionates (Table 2).

The reaction was subsequently monitored as a function of [H₂O₂]. The first order rate constants for intermediate formation (average $k_{\text{obs}} = 32 \text{ s}^{-1}$) or decay were relatively insensitive to [H₂O₂], remaining roughly constant for the two respective phases (Figure S5).

This suggested that the initial step leading to intermediate formation is either independent of H_2O_2 or that a saturating value for the rate had been reached at $5 \mu\text{M}$ H_2O_2 (Table 3). If we take the latter interpretation as more likely, then we may compare this saturating rate constant to the second order rate constant for the ferrous decarboxylase-coproheme/ O_2 reaction. At $27 \mu\text{M}$ of either O_2 or H_2O_2 , the two oxidants yield equivalent values for k_{obs} . Above this concentration, the reaction with O_2 becomes faster than the reaction with H_2O_2 . Notably, the value for k_{obs} measured at air-saturating O_2 (300 s^{-1} , Figure 2B) is 10-fold higher than the saturating rate constant for the reaction with H_2O_2 . These results suggest that, under the aerobic conditions where *hemQ* is presumed to be expressed,¹ dioxygen is the kinetically preferred (faster) reaction partner for the ferrous decarboxylase-coproheme complex, with $\text{O}_2^{\bullet-}$ as the presumed co-product.

A similar intermediate forms whether O_2 , H_2O_2 , or peracetic acid is the oxidant or harderoheme is the tetrapyrrole substrate

To gain further insight into the likely identity of the intermediate(s) formed in reactions between O_2 or H_2O_2 and ferrous coproheme-decarboxylase, reactions with alternate substrates were examined via stopped flow spectroscopy (Table 3). Peracetic acid ($\text{CH}_3(\text{CO})\text{OOH}$, PAA) typically reacts as an oxygen atom donor, oxidizing heme species by two electrons and forming a ferryl [from Fe(II)] or ferryl porphyrin π -cation radical [from Fe(III)]. When peracetic acid was used as the oxidant (1 eq, pH 7.4, 20°C), an intermediate formed with the same UV/vis bands as the others and on a similar time scale, maximizing at approximately 100 ms (Figure 3A). The intermediate converted to ferric coproheme within 10 s ($k_{\text{obs}} = 0.47 \pm 0.04 \text{ s}^{-1}$).

The reaction between the ferrous decarboxylase-harderoheme complex and 1 eq H_2O_2 led to a similar intermediate (408 nm [Soret], 350 nm [shoulder]; 537, 572 nm [visible bands]) (Figure 3B, Table 1) that formed on a comparable time scale as the coproheme complex and then converted to the ferric product (first order rate constant $k_{\text{obs}} = 0.72 \text{ s}^{-1}$, Table 3). The observation of an intermediate with similar UV/vis features with either coproheme or harderoheme as the substrate or in the presence of O_2 , H_2O_2 , or peracetic acid as oxidants suggests that a similar species that is potentially an Fe/oxidant adduct formed in each case.

Reactions of ferric coproheme- or harderoheme-decarboxylase complexes with H_2O_2 are slow and pH dependent

Since the decarboxylase appears to kinetically favor O_2 -mediated oxidation of the ferrous coproheme iron in air (Figure 1), both of the decarboxylation reactions are expected to take place in an $\text{Fe(III)}/\text{H}_2\text{O}_2$ dependent manner under aerobic conditions. The ferric coproheme-decarboxylase reaction with H_2O_2 was monitored as a function of H_2O_2 concentration and at a series of pH values. An intermediate was not clearly detectable under any condition. Instead, the initial reaction phase led to a heme *b* complex that subsequently decomposed (Figure 4A). The two observed phases could be fit to single exponential curves to obtain k_{obs} values. The first phase exhibited a strong, linear dependence on H_2O_2 concentration (Figure 4B) and a sigmoidal dependence on pH, yielding second order rate constants ranging from 110 to $330 \text{ M}^{-1} \text{ s}^{-1}$ (pH 5.8–8.8). Fitting the data to eq (2) yielded $\text{p}K_a = 7.4 \pm 0.3$ (Figure 5):

$$\log k = \frac{k_2 \times 10^{(pH-pK_a)} + k_1}{(1 + 10^{pH-pK_a})} \quad (2)$$

The constants k_1 and k_2 describe the low and high pH forms of the enzyme-substrate system.

This value is well below the pK_a of H_2O_2 (11.7), suggesting that it is due to a deprotonation event on the decarboxylase-coproheme complex rather than deprotonation of free H_2O_2 . The second phase was far slower and independent of both H_2O_2 concentration and pH (Table 3).

The reaction of the ferric harderoheme complex was likewise characterized by two phases. The first led to a complex of the decarboxylase with heme *b* (Soret 408 nm; visible bands 540, 572 nm) and the second to heme destruction (Figure S6). Second order rate constants were slightly faster with harderoheme than coproheme as substrate (190 to 350 $M^{-1} s^{-1}$, pH 5.8–8.8) and exhibited the same pK_a (7.3 ± 0.2) (Figure 5). The heme decomposition phases for coproheme or harderoheme as substrates (Figure S6) were indistinguishable kinetically or spectroscopically, suggesting that the degradation process occurred after heme *b* formed.

Reactions between ferric coproheme-decarboxylase complex and peracetic acid are fast and pH-independent

When PAA was used as the oxidant in place of H_2O_2 , analogous conversion of the substrate to a heme *b* complex followed by degradation was observed in stopped flow experiments (Figure S7). However, the phase leading to heme *b* was approximately an order of magnitude faster with the peracid than with H_2O_2 ($k = 3600\text{--}5200 M^{-1} s^{-1}$). Because PAA is expected to react as an oxygen atom donor, the fact that both it and H_2O_2 are productive oxidants suggests that the ferryl porphyrin π -cation radical ($Fe(IV)=O$ por π^+ , Compound I) is a common intermediate on both the H_2O_2 and peracid pathways. Finally, neither the second order formation nor the first order decay rate constants had a sigmoidal dependence on pH (Figure 5). This may reflect the relatively low pK_a of the peracid (8.2), which would obviate the need for basic residues to catalyze the formation of a ferric-acetylperoxy species.

Solvent isotope effect on the ferric coproheme-decarboxylase reaction indicates rate limiting proton transfer

The slow second order kinetics of the ferric coproheme reactions suggests that a bimolecular step involving H_2O_2 partly limits the rate at which heme *b* forms. The pH dependence of the rate constant (Figure 5) suggests that this step includes transfer of H^+ . To further explore these hypotheses, the decarboxylase-coproheme complex reaction with D_2O_2 was monitored in D_2O at varying pD. The second order rate constants diminished in magnitude, with an averaged solvent kinetic isotope effect (SKIE) on k (k_H/k_D) of 2.2 over the measured pH/pD range. The plot of k versus pD adhered closely not to a one but rather a two pK_a model:

$$\log k = \frac{k_1 \times 10^{(pH-pK1)} + k_2}{(1 + 10^{(pH-pK1)})} + \frac{k_2 \times 10^{(pH-pK2)} + k_3}{(1 + 10^{(pH-pK2)})} \quad (3)$$

Here, k_1 , k_2 , and k_3 describe the rate constants for each of the 3 pH-dependent forms of the enzyme-substrate system. This fit yielded pK_a s of 6.5 and 9.3.

We conclude that D₂O clearly influences both the rate of the second order reaction and the observed number and magnitude of acid dissociation equilibria for the coproheme-decarboxylase complex. Substitution of ²H for ¹H is known to perturb acid dissociation equilibrium constants by varying amounts depending on the acid's structure (the pK_a shifts 0.41 units for D₂O versus H₂O, e.g., to 7.41).²⁵ It is possible that the pK_a s 7.4 and 9.3 measured in H₂O and D₂O, respectively, correspond to the same acid dissociation event, and that the second pK_a (6.5 in D₂O) was out of the pH range used in protonated buffer. Alternatively, the introduction of deuterium could have altered the rate limiting steps of the mechanism, and therefore the chemical event contributing the most to the second order rate constant. The origins of these pK_a s will be explored further in future work.

Resonance Raman (rR) spectra for ferrous decarboxylase-coproheme complex indicate a pentacoordinate high-spin coproheme with a neutral His ligand

The five-coordinate, high-spin (5cHS) nature of the ferrous HemQ complexes was confirmed by the coordination- and spin-state marker band ν_3 and oxidation state (or π^* electron density) marker band ν_4 at 1467 and 1353-cm⁻¹, respectively, in the high frequency rR spectra (Figure 6A). The ferrous coproheme, harderoheme, and heme *b* complexes exhibit very similar ν_3 and ν_4 shifts.

Additionally, the rR spectra report on the nature and strength of the proximal iron-ligand bond. In 5cHS ferrous heme proteins having proximal imidazole ligands, 441.6-nm Raman excitation elicits well-enhanced bands attributable to their iron-histidine stretches, $\nu_{\text{Fe-His}}$. By virtue of its intensity and frequency, the 214 cm⁻¹ band is tentatively assigned to the $\nu_{\text{Fe-His}}$ mode of 5cHS ferrous decarboxylase-coproheme (Figure 6B). The $\nu_{\text{Fe-His}}$ frequency for ferrous decarboxylase complexes of harderoheme and heme *b* are within 1 cm⁻¹ of that for the coproheme complex.

The 214-cm⁻¹ $\nu_{\text{Fe-His}}$ frequency of decarboxylase-coproheme is 8, 12, and 15 cm⁻¹ lower than those of the closely related, heme *b*-dependent chlorite dismutases (Clds) from *Dechloromonas aromatica* (*DaCld*, 222 cm⁻¹), *Nitrospira defluvii* (*NdCld*, 226 cm⁻¹), and *Klebsiella pneumoniae* (229 cm⁻¹), respectively.^{26–28} Mutation of the distal pocket in *DaCld* (*DaCld*(R183Q)) to mimic the predicted pocket in the decarboxylase²⁹ does not result in a significant change of its $\nu_{\text{(Fe-His)}}$ frequency.³⁰ The proximal histidine ligands of *DaCld* and *NdCld* have some imidazolate character due to H-bonding interactions with nearby glutamate side chains. Since the H-bond accepting glutamate in the Cld proximal pockets is not conserved in the decarboxylases,³¹ Hofbauer and coauthors predicted correctly that the $\nu_{\text{Fe-His}}$ frequency for the heme complexes would be similar to that of *NdCld*(E210A) which they reported at 216 cm⁻¹.²⁷ This result suggests that any proximal hydrogen bond network in the decarboxylases does not include strong participation by the proximal ligand. It further suggests that the electronic “push” exerted by the proximal His is closer to that in the globins than in His-ligated peroxidases.

The reduction potential for the decarboxylase-coproheme complex is peroxidase-like and becomes more positive as the propionates convert to vinyls

Fe(III)/Fe(II) potentials for coproheme (−190 mV), harderoheme (−170 mV), and heme *b* (−160 mV) bound to the decarboxylase were measured spectroelectrochemically (versus SHE, pH 7, 20 °C, Figures 7 and S8). The value for the coproheme complex is similar to the one recently reported for the same protein (−207 mV) at neutral pH.³² These positive shifts in reduction potential are consistent with the loss of negative charge from the porphyrin periphery as each propionate is converted to a vinyl group. Other histidine-ligated heme proteins offering relevant comparisons include heme peroxidases, which react in the ferric oxidation state and have heme Fe(III)/(II) reduction potentials ranging from −150 to −300 mV vs SHE.³³ The reduction potential of *Da*Cld, which is homologous (31.4% similarity, EMBOSS Needle alignment) to and structurally superimposable with the decarboxylase/HemQ, is −21 mV.³⁴ Clds use ferric heme *b* as a catalytic cofactor for converting ClO₂[−] to Cl[−] and O₂. By contrast, positive potentials are associated with heme proteins that operate in the Fe(II) state. Ferrous myoglobin, which reversibly binds O₂, has a low, positive reduction potential of 45 mV,³⁵ while the potential for bifunctional dehaloperoxidase, which reversibly binds O₂ and reacts with H₂O₂ in its ferrous form, is 222 mV.²²

DISCUSSION

The assembly of biological cofactors requires the controlled combination of reactive components within the cellular environment. In the final step of heme biosynthesis in many bacteria, including most facultative gram positives,¹ a ferrous-porphyrin precursor undergoes the selective oxidative decarboxylation of two propionate groups under conditions where both cellular O₂ and H₂O₂ are known to be present.^{1, 6} Coproheme is the substrate and cofactor in this unusual autocatalytic reaction, carried out by a coproheme decarboxylase encoded by the *hemQ* gene. Because iron is inserted into coproheme as Fe(II),^{1, 6, 36} the reaction could, in principle, occur via mechanisms that are classically oxidase-, oxygenase-, or peroxidase-like (Scheme 1). Each invokes unique chemical steps and distinct biological strategies for directing the reactivity of the redox-active substrates and product. We sought to determine which of these is the most likely pathway for the decarboxylase under biological conditions.

In nearly all cases where decarboxylation is accompanied by C-C bond oxidation, the two electrons from the substrate are funneled into a cofactor (e.g., flavin or pyridoxal phosphate).³⁷ The cofactor electrons may be subsequently discharged onto NAD(P)⁺ or O₂. The coproheme decarboxylase lacks a cofactor; however, we reasoned that the substrate itself could act as a conduit for removing two electrons from each propionate side chain and delivering them to O₂, generating H₂O₂. This hypothetical reaction (Scheme 1A) is similar to the one catalyzed by coprophorphyrinogen oxidase: the cofactor-free enzyme from the canonical pathway that catalyzes an analogous reaction with a metal-free, reduced, and unconjugated substrate.¹⁰ When we examined the ferrous coproheme decarboxylase for oxidase activity, however, none was observed.

Structurally, the decarboxylase closely resembles bacterial heme oxygenases from the IsdG family, which are found in the same bacterial taxa.³⁸ The heme oxygenase reaction is

likewise autocatalytic, with ferrous heme *b* acting concurrently as the substrate and cofactor. In the proposed mechanism for MhuD, the IsdG-family member from *Mycobacteria*,¹³ heme *b* reacts with O₂ to form a ferrous heme-O₂ complex. Addition of H⁺ and an electron leads to a ferric-hydroperoxy intermediate, which hydroxylates one of the pyrrole-bridging *meso*-carbons. The biological source of the electron has not been identified for any heme oxygenase, though various surrogates have been used under *in vitro* conditions. Following the initial hydroxylation, a series of further reactions leads to ring opening and release of formaldehyde and the triply oxygenated linear tetrapyrrole product.^{20, 39}

An analogous reaction mechanism can be proposed for the coproheme decarboxylase, though with hydroxylation directed not toward a *meso*-carbon, but rather the β-carbon of a reactive coproheme propionic acid (Scheme 1B). Consistent with an oxygenase mechanism, UV/vis spectroscopic evidence suggests that the ferrous coproheme substrate rapidly forms intermediates with both O₂ and H₂O₂ that are spectroscopically similar (Figures 1A and 2A). However, under conditions that would have resulted in heme oxygenation by IsdG and its homologs, no reaction occurred (Figure S3). Barring the requirement for a yet-unknown physiological reductant, the evidence here suggests that coproheme decarboxylase does not work according to an oxygenase mechanism.

We next considered whether the decarboxylase might react in the ferrous state with H₂O₂. Such a “ferrous peroxidase” mechanism would be highly unusual though not completely unprecedented. The heme-dependent marine worm dehaloperoxidase (DHP) provides the sole example of H₂O₂/heme reactivity occurring under biological conditions from the ferrous state.^{14, 15, 23} A ferrous-hydroperoxy heme species forms as the initial intermediate in DHP, followed by heterolytic cleavage of the O-O bond to yield Compound II (Fe(IV)=O porphyrin). Compound II acts as a one-electron oxidant toward each of a pair of exogenous substrates, returning the heme cofactor to the ferrous oxidation state.

Consistent with this pathway, the ferrous coproheme-decarboxylase appeared to form an intermediate in the reaction with H₂O₂, which is potentially a ferrous-peroxy complex (Figures 2–3). However, the subsequent steps did not lead to decarboxylation (Table 2). Instead, the reaction with 1–2 eq of H₂O₂ brought about the complete conversion of the coproheme Fe(II) to Fe(III). Moreover, while the maximal second order reaction rate between the ferrous coproheme-decarboxylase and H₂O₂ was fast, the reaction with ambient O₂ (240 μM at pH 7, 20 °C, in Bozeman, MT) was faster still. The ferrous reaction with O₂ is expected to be kinetically favored over H₂O₂ under aerobic conditions or whenever dissolved O₂ exceeds 30 μM (Table 3).

We therefore hypothesized that the decarboxylase might react as an “oxidase-peroxidase.” According to this model, the ferrous coproheme reaction with O₂ would generate the ferric coproheme and O₂•⁻ in the first step. Once in the ferric state, the coproheme- and harderoheme-decarboxylase complexes would have to react with H₂O₂, since ferric heme species are not known to be oxidized by O₂.

This is an attractive proposition since heme enzymes generally react with H₂O₂ in the ferric state, and since we know the decarboxylase reaction can occur in an Fe(III)/H₂O₂-dependent

manner.⁵ We observed here (Figures 6 and 7) that the Fe(III)/(II) reduction potential and coordination state for the decarboxylase-coproheme or -harderoheme complexes are reminiscent of typical heme peroxidases, suggesting that these complexes have a sufficient driving force for reacting in the ferric state. We further observed that the reaction between the ferric coproheme-decarboxylase complex and H₂O₂ was second order and pH dependent, growing faster with a pK_a near 7.4 (Figure 5). The observation of a solvent kinetic isotope effect further indicated that a proton transfer – for example, from H₂O₂ or to a ferric-OOH complex to generate water and Compound I – partly limited the rate of this second order step. Acid-base catalysis of Compound I formation, usually involving a His-Arg pair, is a cornerstone of proposed mechanisms for H₂O₂ activation by heme peroxidases, and could be in effect here.⁴⁰

However, the second order reaction between the decarboxylase-coproheme complex and H₂O₂ was exceedingly slow, with rate constants (110–330 M⁻¹s⁻¹) that are 10²–10⁴ times lower than expected for typical peroxidases.¹⁶ Further, instead of forming Compound I as an initially observable intermediate, the first species observed in reactions with either the ferric coproheme- or harderoheme-decarboxylase complex and H₂O₂ is the complex of the protein with ferric heme *b* (Figure 4). Compound I may nonetheless be on the reaction pathway. Peracetic acid, an oxygen-atom donor which typically forms Compound I from ferric heme, very efficiently generates the expected harderoheme intermediate and heme *b* product from the ferric decarboxylase-coproheme complex. We would not expect Compound I to be directly observed if it reacts quickly once it forms. The lifetime of Compound I in peroxidases is highly variable, and appears to depend strongly on the number and proximity of redox-active amino acids that can react with it.^{41, 42} The structure of the decarboxylase with coproheme bound is not yet available; however, consistent with a short lived intermediate, it has multiple Trp and Tyr residues (5 and 12, respectively) in its 250 amino acid sequence, and its two oxidizable substrates are covalently attached to the heme. Thus, if the oxidative decarboxylation proceeds via Compound I, its lifetime may be fleeting.

Compound I-catalyzed decarboxylations could occur by one of at least two possible mechanisms (Scheme 2). The first is analogous to the classic, two-step peroxidase catalytic cycle (Scheme 2 path **A**).¹⁶ In the first step, a net hydrogen atom (H⁺ plus e⁻) is transferred from the β-carbon of the propionate to the ferryl porphyrin cation radical, filling the hole on the porphyrin to generate Compound II and protonating the ferryl oxygen atom. Second, migration of the remaining unpaired electron from the β-carbon of the propionate to the Fe(IV) might occur with concomitant loss of the carboxylate group as CO₂. A second proton would need to be brought in to make H₂O. Alternatively, one could imagine the same protonated Compound II [Fe(IV)-OH] “rebounding” with the propionyl radical to yield a hydroxylated intermediate (Scheme 2 path **B**). The rebound reaction would likely only take place if the ferryl were adjacent to the reactive propionate. As this is difficult to envision, the rebound mechanism is less probable. The peroxidase-like mechanism, by contrast, could involve amino acid side chains as conduits for the transferred electrons and protons. In either case, the oxidation state of the metal remains unchanged at the beginning/end of the reaction, and the ferric coproheme is first converted to ferric harderoheme. A second catalytic cycle between Fe(III) harderoheme and H₂O₂ would be needed to generate heme *b*.

Do the two, sequential oxidative decarboxylation reactions occur in the same manner? The fact that the second order rates for the ferric coproheme/H₂O₂ and harderoheme/H₂O₂ reactions leading to heme *b* are roughly the same indicates that the second propionate conversion (from harderoheme to heme *b*) limits the overall reaction rate for coproheme, though whether the H₂O₂-activating steps are the same is not clear.

A decarboxylation mechanism involving Compound I is plausible, but the electronic structure of the coproheme bound to the decarboxylase presents a conundrum. A “push-pull”/distal acid-base model is used to explain heterolytic peroxide cleavage in the peroxidase mechanism.⁴⁰ The “push” effect correlates with the basicity of the proximal ligand which, in the case of heme peroxidases, is typically a His side chain whose basicity is modulated by H-bond acceptors. The strongest, anionic H-bond acceptors lead to the most basic His ligands, which in turn stabilize the high-valent iron centers of Compounds I and II. The extent of the “push” effect is reflected in the iron histidine stretching frequency, which ranges from 233–246 cm⁻¹ in heme peroxidases.^{43, 44} By contrast, the low $\nu_{\text{Fe-His}}$ frequency of 214 cm⁻¹ shows that the imidazole ligand in coproheme decarboxylase is not associated with an anionic H-bond acceptor, predicting a relatively weak proximal “push”. Clds, which are closely related to these HemQ-decarboxylases, also exhibit lower $\nu_{\text{Fe-His}}$ frequencies (222 – 229 cm⁻¹) than heme peroxidases, suggesting a weaker proximal “push”, but perhaps greater than in coproheme decarboxylase.^{5, 45, 46} However, their modest push, in conjunction with a strong “pull” from their distal Arg drives heterolytic cleavage of an O–Cl bond in the ClO₂⁻ substrate. As in the heme peroxidases, this is thought to direct both oxidizing equivalents of the substrate to the heme cofactor thereby yielding compound I. The distal environment of the coproheme decarboxylase is not known, though analyses of available substrate-free apo-protein structures and structure-based sequence alignments predict a distal Gln.^{31,32} This might partly compensate for the weak Fe-His interaction via the “pull” of positive charges. By the same token, it is possible that the negatively charged propionate groups 2 and 4 could act together with the protein environment to stabilize the ferric oxidation state, potentially supporting heterolytic cleavage of bound H₂O₂.³³ Alternatively, heme oxygenases have neutral His heme ligands ($\nu_{\text{Fe-His}}$ = 216–218 cm⁻¹)^{47, 48} and do not react via Compound I. Rather, an Fe(III)-OOH species directly hydroxylates one of the pyrrole-bridging carbons, without prior cleavage of the O–O bond,^{49, 50} and an analogous mechanism using ferric coproheme can be proposed here (Scheme 2, path C).

A pathway invoking O₂-mediated oxidation of Fe(II) followed by Fe(III)/H₂O₂ dependent decarboxylations has at least three important implications for how heme synthesis is organized in the cells that use this noncanonical mechanism. First, the well-known biosynthetic pathway from gram-negative bacteria and eukaryotes generates ferrous heme *b*. The production of the ferric heme in gram-positives suggests distinct ways of subsequently chaperoning the cofactor and incorporating it into proteins in the cell. Second, a reaction cycle that begins from the ferrous coproheme complex produces a stoichiometric quantity of superoxide per coproheme/heme *b* conversion as the metal oxidizes from Fe(II) to Fe(III) (Scheme 2). Although the fate of the O₂^{•-} is not clear, catalytic disproportionation of superoxide in the presence of a proton source rapidly yields H₂O₂.⁵¹ The susceptibility of

free ferric coproheme to bleaching in the presence of even small amounts of H₂O₂ suggests that oxidation of the iron must occur inside the decarboxylase rather than freely in the cell.⁵ The upstream catalyst, ferrochelatase (HemH), may therefore directly relay the substrate to the decarboxylase, as some existing data already support.⁵² Finally, though *S. aureus* like many gram-positives is a catalase-rich organism, this unique oxidative decarboxylation reaction depends on H₂O₂, suggesting that the delivery of both H₂O₂ and the ferrous coproheme to the enzyme are under some type of cellular control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACN	Acetonitrile
Cld	Chlorite Dismutase
DHP	Dehaloperoxidase
DMSO	Dimethyl Sulfoxide
EPR	Electron Paramagnetic Resonance Spectroscopy
5cHS	Five Coordinate High Spin
HPLC	High Performance Liquid Chromatography
PAA	Peracetic Acid
PCD	Protocatechuate Dioxygenase
PCA	Protocatechuic Acid
SKIE	Solvent Kinetic Isotope Effect
TFA	Trifluoroacetic Acid

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Figure 1. The reaction between the ferrous coproheme-decarboxylase complex and O₂ leads to an intermediate, followed by oxidation of the coproheme iron

A. The ferrous coproheme-decarboxylase complex (5 μM, blue line) was rapidly mixed with one eq of O₂ and the reaction monitored by stopped flow UV/vis spectroscopy (50 mM KPi, pH 7.4, 20 °C). Conversion to an initial Fe/O₂ intermediate after 100 ms (green line) was observed, followed by formation of the ferric coproheme product (15 s, red line).

Intervening spectra are shown at 0.015, 0.045, 2, and 5 s (lighter lines). The inset shows the visible bands on an expanded scale. B. The progress of the reaction was monitored over time at 408 nm at varying final concentrations of O₂ (6 [red], 60 [orange], 120 [blue], and 240 μM [purple]). Each curve was fit to the sum of two exponentials. Inset: values of k_{obs} for the initial phase leading to the green intermediate in (A) were plotted as a function of [O₂], yielding $k = 1.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$.

Figure 2. The ferrous coproheme-decarboxylase complex reacts faster with O_2 /air than with H_2O_2

A. The ferrous coproheme-decarboxylase complex (5 μ M, blue line) was rapidly mixed with one eq of H_2O_2 and the reaction monitored by stopped flow UV/vis spectroscopy (50 mM KPi, pH 7.4, 20 $^\circ\text{C}$). Conversion to an initial Fe/ H_2O_2 intermediate after ~ 70 ms (green line) was observed, followed by formation of the ferric product (15 s, red line). Intervening spectra are shown at 0.015, 0.045, 2, and 5 s (lighter lines). The inset shows the visible bands on an expanded scale. B. The progress of the reaction monitored over time at 408 nm is shown for the reactions of ferrous coproheme-enzyme complexes with 1 eq H_2O_2 (blue), 1 eq peracetic acid (PAA) (green), or O_2 (half and full air saturation, 120 and 240 μM O_2 , red and black points respectively). The data points are shown in fits to sums of two exponential curves. (Fits to the initial phase for the stoichiometric reactions are included as a visual aid.)

Figure 3. The ferrous coproheme decarboxylase forms intermediates with similar UV/vis spectra whether coproheme/harderoheme is the oxidizable substrate or O₂/H₂O₂/PAA is the oxidant
 A. The ferrous coproheme-decarboxylase complex (5 μ M, blue line) was rapidly mixed with one eq of PAA and the reaction monitored by stopped flow UV/vis spectroscopy (50 mM KPi, pH 7.4, 20 $^{\circ}$ C). Conversion to an initial Fe/PAA intermediate after ~150 ms (green line) was observed, followed by formation of the ferric coproheme product (10 s, red line). Intervening spectra are shown at 0.015, 0.045, 1, and 3.5 s (lighter lines). The inset shows the visible bands on an expanded scale. B. The ferrous harderoheme-decarboxylase complex (5 μ M, blue line) was rapidly mixed with one eq of H₂O₂ and the reaction monitored by stopped flow UV/vis spectroscopy (50 mM KPi, pH 7.4, 20 $^{\circ}$ C). Conversion to an initial Fe/H₂O₂ intermediate after ~225 ms (green line) was observed, followed by formation of the ferric harderoheme product (15 s, red line). Intervening spectra are shown at 0.015, 0.045, 1, 3, and 10 s (lighter lines). The inset shows the visible bands on an expanded scale. The UV/

visible features of the intermediates and ferric products resemble those measured for the reactions using either O₂ or H₂O₂ as oxidants (Figures 1–2).

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Figure 4. Oxidative decarboxylation of ferric coproheme occurs slowly and without observable intermediates when H_2O_2 is the oxidant

A. The ferric coproheme-decarboxylase complex (7 μM , red line) was rapidly mixed with 10 eq of H_2O_2 and the reaction monitored by stopped flow UV/vis (50 mM KPi, pH 7.4, 20 $^\circ\text{C}$). Conversion to a complex with heme *b* was observed after ~35 s (purple line), followed by heme decomposition over several minutes (illustrated by the 500 s curve, gray). Intervening spectra are shown at 5, 10, and 20 s (lighter lines). The inset shows the visible bands on an expanded scale. B. The progress of the reaction monitored over time at 411 nm is shown for the reaction of ferric coproheme-decarboxylase with increasing concentrations of H_2O_2 (in direction of arrow: 75, 125, 250, 500, 1250, 2500, and 500 μM ; 50 mM KPi, pH 7.4, 20 $^\circ\text{C}$). Each curve was fit to the sum of two exponentials. The first kinetic phase corresponded to the generation of a heme *b* complex and the second to heme *b* decomposition (k_{decay}). Inset: Values of k_{obs} for the first phase (red squares) were plotted versus $[\text{H}_2\text{O}_2]$ and used to generate second order rate constant $210 \text{ M}^{-1} \text{ s}^{-1}$.

Figure 5. The rates of reactions between the ferric coproheme-decarboxylase and H₂O₂ are pH and solvent-isotope dependent

Reactions between the ferric coproheme- and harderoheme-decarboxylase complexes and H₂O₂, D₂O₂ (in D₂O), or PAA were monitored as a function of pH (pD). An initial phase corresponding to heme *b* formation (k_{heme}) followed by heme decomposition (k_{decay}) was observed in every case. Second order rate constants measured as shown in Figure 4B were plotted as a function of pH: red circles, coproheme/H₂O₂; blue squares, harderoheme/H₂O₂. The initial phase grew faster with increasing pH and was fit to a single pK_a equation, yielding $pK_a = 7.3$ (harderoheme) or 7.5 (coproheme). The same rate constants measured for the coproheme reaction with D₂O₂ (in D₂O) (blue diamonds) were fit to a two- pK_a model yielding 6.5 and 9.3; average SKIE = 2.2. Finally, rate constants for the coproheme reaction with PAA (orange triangles) were much larger and did not exhibit a sigmoidal trend. Rate constants describing the decay phase did not display sigmoidal dependence on pH (see Figure S8).

Figure 6. Vibrational characterization of the ferrous iron tetrapyrrole complexes of the coproheme decarboxylase

A) High frequency window of the 413.1-nm excited rR spectra of (a) ferrous coproheme-, (b) ferrous harderoheme-, and (c) ferric coproheme-decarboxylase complexes reacted with eight equivalents of H_2O_2 to generate enzyme-heme *b* followed by reduction with sodium dithionite. B) The 441.6 nm excited low frequency rR spectra of (a) ferrous enzyme-coproheme complex, (b) ferrous enzyme-harderoheme complex, (c) product of ferric enzyme-coproheme with H_2O_2 followed by reduction with dithionite, and (d) ferrous enzyme-heme *b* complex prepared by reduction of constituted enzyme-ferric heme *b* complex. Note the change in the intensities of the propionate and vinyl bends relative to one another. All samples were prepared in 0.1 M sodium phosphate buffer pH 6.8 and reduced with sodium dithionite.

Figure 7. The Fe(III)/(II) redox potential for decarboxylase-bound coproheme is peroxidase-like, and becomes more positive as each propionate is converted to a vinyl

Titration of the ferric coproheme-decarboxylase complex with dithionite in the presence of oxidized indigo disulphonate (red) results in the concurrent reduction of both substrate and dye (blue). (Inset) Plotting the $\ln(\text{Enz}_{\text{ox}}/\text{Enz}_{\text{red}})$ versus $\ln(\text{Dye}_{\text{ox}}/\text{Dye}_{\text{red}})$ allowed for the determination of redox potential of -190 mV from the slope (versus SHE, pH 7, 20°C). Analogous data measured for the complexes of the decarboxylase with harderoheme and heme *b*, yielded -170 mV, and -160 mV, respectively (see Supplementary Information).

Scheme 1. Possible coproheme decarboxylase pathways starting from Fe(II)coproheme[‡]

[‡]The ferrous coproheme III substrate is shown with sites of pyrrole ring substituents (methyl or propyl) labeled 1–8. The tetrapyrrole is depicted as an oval and the propionates as “P”.

Only the reacting propionate 2 is shown, for clarity. A) Oxidase, B) oxygenase and C) peroxide-dependent mechanisms are shown. A mechanism for the conversion of the harderoheme III intermediate to heme *b* is not shown but could occur by an analogous mechanism to A–C.

Scheme 2. Possible pathways for oxidative decarboxylation.[‡]

[‡]Only the first oxidative decarboxylation is shown. O₂-mediated oxidation of the coproheme iron is followed by reaction of the ferric coproheme with H₂O₂ to form a hypothetical Fe(III)-OOH adduct. Two Compound I-dependent pathways are shown. (A) Net H-atom (H⁺ + e⁻) transfer from the propionate β-carbon to Compound I. (B) Net H-atom transfer followed by hydroxyl rebound to yield a hydroxylated intermediate. Direct hydroxylation from the ferric-hydroperoxy species (C) could likewise occur, analogous to heme oxygenase chemistry.

Table 1

UV/vis bands for complexes of HemQ coproheme decarboxylase with substrate, intermediate, or product

Complex	Soret	Visible
Fe(III)		
coproheme	395	497, 533, 630
harderoheme	396	495, 533, 610
heme <i>b</i>	406	510, 527, 630
Fe(II)		
coproheme	424	548
harderoheme	418	~520sh, 551
heme <i>b</i>	427	560
Fe(II) reaction intermediates		
coproheme + O ₂	408	539, 573
coproheme + H ₂ O ₂	408	539, 572
coproheme + peracetic acid	408	539, 572
harderoheme + H ₂ O ₂	408	537, 572
harderoheme + peracetic acid	408	537, 572

Table 2

Observed oxidation products following addition of defined amounts of H₂O₂ to decarboxylase complexes with ferrous/ferric coproheme or harderoheme, expressed on a per-substrate basis^a

heme substrate		Fe(II) coproheme		Fe(II) harderoheme		Fe(III) coproheme		Fe(III) harderoheme	
H ₂ O ₂ (eq) added	vinyls after reaction	Fe(III) after reaction ^c	vinyls after reaction	Fe(III) after reaction	vinyls after reaction ^c	Fe(III) after reaction ^c	vinyls after reaction	Fe(III) after reaction ^c	vinyls after reaction
0	0	0	0	0	0	0	0	0	0
0.5	0.32±0.05	0.05	0.07±0.02	ND ^d	0.14±0.02	0.07±0.02	0.11±0.03	0.13±0.02	0.14±0.02
1	0.51±0.07	0.70	0.13±0.02	ND	0.27±0.04	0.14±0.04	0.45±0.07	0.13±0.02	0.14±0.02
1.5	0.58±0.06	0.98	0.15±0.03	ND	0.54±0.06	0.15±0.03	0.54±0.06	0.13±0.02	0.14±0.02
2	0.60±0.1	0.91	0.15±0.03	ND	0.54±0.06	0.15±0.03	0.54±0.06	0.13±0.02	0.14±0.02

^aReactions were carried out at 20 °C in pH 7.4 KPi.

^bConversion of propionates to vinyl groups was determined via HPLC quantification of the total harderoheme and/or heme *b* products of each reaction. Each reaction was carried out in triplicate with the error reported as the standard deviation of the three measurements.

^cFe(III) was determined by double integration of the EPR spectra and spin quantification, using horse heart myoglobin as a standard (Figure S6).

^dNot determined.

Table 3

Rate constants for reactions of decarboxylase complexes with oxidants (pH 7.4, 20 °C)

Substrate	oxidant	k (M ⁻¹ s ⁻¹) ^a	k_{decay} (s ⁻¹)
coprohemeFe(II)	O ₂	1.2×10^6	0.20 ± 0.02 s ⁻¹
	H ₂ O ₂	32 ± 2 s ⁻¹ ^b	0.48 ± 0.02 s ⁻¹
	PAA	30 ± 3 s ⁻¹ ^b	0.47 ± 0.04 s ⁻¹
harderohemeFe(II)	H ₂ O ₂	41 ± 5 s ⁻¹ ^b	0.74 ± 0.01 s ⁻¹
coprohemeFe(III)	H ₂ O ₂	210	0.00060 M ⁻¹ s ⁻¹
	PAA	3600	0.0050 M ⁻¹ s ⁻¹
harderohemeFe(III)	H ₂ O ₂	270	0.00090 M ⁻¹ s ⁻¹

^aThe second order rate constants in each case describe the conversion of starting material to the first observable species, which is either an intermediate (for Fe(II)/O₂) or heme (for reactions starting with ferric hemes). Formation of ferric coproheme as the final product (in the case of the ferrous starting materials) or heme decomposition (ferric starting materials) is described by k_{decay} . This rate constant is first order for the ferrous substrates and second order for the ferric.

^bThese values were insensitive to changes in oxidant concentration and are reported as first-order rate constants. Errors for k_{obs} are standard deviations from 3 measurements. Second order plots constructed from similarly measured k_{obs} had linear correlation values >95%.