*Accelerated Publications*

# Heme A Synthase Does Not Incorporate Molecular Oxygen into the Formyl Group of Heme A†

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ABSTRACT: Heme A is an obligatory cofactor in all eukaryotic and many prokaryotic cytochrome *c* oxidases. The final step in heme A biosynthesis requires the oxidation of the C8 methyl substituent on pyrrole ring D to an aldehyde, a reaction catalyzed by heme A synthase. To effect this transformation, heme A synthase is proposed to utilize a heme B cofactor, oxidizing the substrate via successive monooxygenase reactions. Consistent with this hypothesis, the activity of heme A synthase is found to be strictly dependent on molecular oxygen. Surprisingly, when cells expressing heme A synthase were incubated with $^{18}O_2$, no significant incorporation of label was observed in heme A, the C8 alcohol intermediate, or the C8 overoxidized byproduct. Conversely, when the cells were grown in $H_2{}^{18}O$, partial labeling was observed at every heme oxygen position. These results suggest that the oxygen on the heme A aldehyde is derived from water. Although our data do not allow us to exclude the possibility of exchange with water inside of the cell, the results seem to question a mechanism utilizing successive monooxygenase reactions and support instead a mechanism of heme O oxidation via electron transfer.

Cytochrome *c* oxidase (C*c*O)[1] is the terminal oxidase in all plants, animals, aerobic yeasts, and some bacteria (*2*−*6*). Arguably the most important enzyme in aerobic metabolism, C*c*O catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen. In reducing $O_2$ to water, C*c*O also pumps up to four protons (eight charge equivalents) across the inner membrane of either mitochondria or bacteria, thus producing a protonmotive force that is ultimately utilized to synthesize ATP (*7*−*12*). In fact, the proton gradient generated by C*c*O is responsible for approximately 50% of the ATP formed during aerobic metabolism in mammals.

To accomplish this task, C*c*O utilizes a number of metal cofactors including a zinc ion, a magnesium ion, three copper ions, and two heme A molecules. The dinuclear $Cu_A$ site and one of the heme A cofactors are involved in electron transfer. The second heme A molecule interacts with the mononuclear copper ion ($Cu_B$) forming the heterobimetallic active site that catalyzes $O_2$ reduction. Thus, heme A is an obligatory cofactor in all eukaryotic and many prokaryotic C*c*Os.

The biosynthesis of heme A and its insertion into C*c*O are complex processes that are only just being elucidated at the molecular level. Heme A is derived from heme B via two enzymatic reactions. The first reaction, catalyzed by heme O synthase (HOS), results in the conversion of the vinyl group on pyrrole ring A into a 17-hydroxyethylfarnesyl moiety (Scheme 1) (*13*−*16*). In the second transformation, heme A synthase (HAS) catalyzes the oxidation of the methyl group on pyrrole ring D into an aldehyde (*16*−*19*). This second reaction is especially intriguing for a number of reasons. Not only is a formyl group an unusual end product in biosynthetic processes, presumably due the reactive nature of aldehydes, but HAS is itself proposed to be a heme-containing enzyme, utilizing a heme B cofactor at the active site (*20*).

A similar oxidation reaction occurs during chlorophyll *b* biosynthesis when chlorophyll *a* oxygenase (CAO) catalyzes the oxidation of a methyl group on pyrrole ring B to an aldehyde (*21*, *22*). Unlike HAS, however, CAO is thought to be a nonheme iron monooxygenase containing a [2Fe-2S] Rieske center (*23*). CAO is proposed to utilize successive monooxygenase steps, oxidizing the substrate first to an alcohol and then to a geminal diol that dehydrates, yielding the aldehyde product. Consistent with this hypothesis, CAO has been shown to utilize the putative alcohol intermediate as a substrate (*24*). Furthermore, careful $^{18}O_2$-labeling studies have demonstrated that the oxygen atom of the formyl group is derived from $O_2$ (*25*), just as one would predict from a nonheme iron monooxygenase.

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[1] Abbreviations: ALA, δ-aminolevulinic acid; amu, atomic mass units; ATP, adenosine triphosphate; CAO, chlorophyll *a* oxygenase; C*c*O, cytochrome *c* oxidase; DMSO, dimethyl sulfoxide; ESI, electron spray ionization; HAS, heme A synthase; HOS, heme O synthase; HSQC, heteronuclear single-quantum coherence; ITPG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria−Bertani; MS, mass spectrometry; TFA, trifluoroacetic acid.

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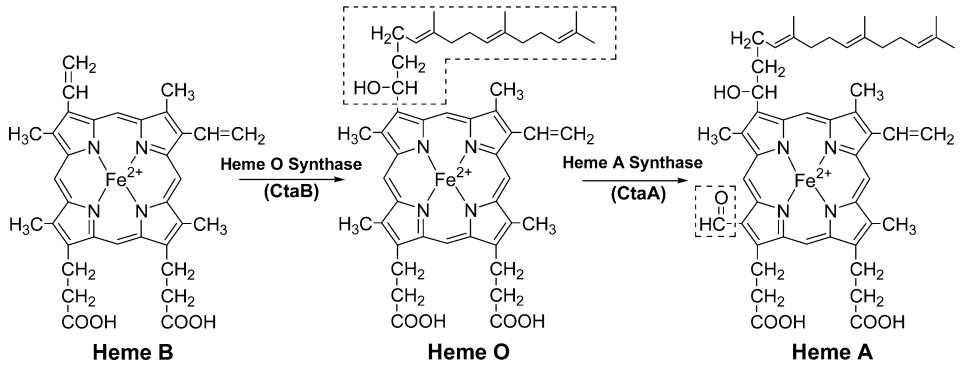
Scheme 1: Transformation of Heme B to Heme A Catalyzed by the Enzymes Heme O Synthase (HOS) and Heme A Synthase (HAS)[a]




[a] In *B. subtilis* HOS is denoted CtaB while HAS is denoted CtaA.

It has been proposed that heme A synthase utilizes a similar mechanism to catalyze the oxidation of heme O to heme A (*18*, *20*, *26*, *27*). Characterization of bacterial HAS identified both heme B and heme A in equal but substoichiometric amounts (20% each) (*20*). All of the iron present in the sample could be accounted for by the hemes, and no other redox-active metals were detected at meaningful levels. From these data, it was concluded that heme B was the cofactor and that heme A was unreleased product. Furthermore, Tzagoloff and co-workers demonstrated that HAS in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* requires a ferredoxin and a ferredoxin reductase for activity (*19*, *26*). In fact, HAS in *S. pombe* is actually fused to the ferredoxin. More recently, we observed two new heme products formed when HOS and HAS from *Bacillus subtilis* (CtaB and CtaA, respectively) are overexpressed in *Escherichia coli* (*27*). We identified these new hemes as C8 alcohol and C8 carboxylate derivatives of heme O, and we demonstrated that HAS activity is strictly dependent on molecular oxygen. All of these data are consistent with HAS utilizing successive P450-like monooxygenase steps to oxidize heme O to heme A.[2] In this scenario, the C8 alcohol derivative is the putative intermediate and the C8 carboxylate byproduct results from further oxidation of the aldehyde. The cytochromes P450 are well-known to oxidize aldehydes to the corresponding carboxylates (*28*−*30*), and in this heterologous system heme A may remain in the active site because *E. coli* does not naturally produce or utilize heme A (*5*, *31*).

If HAS utilizes successive monooxygenase steps, then the source of oxygen in the product should be derived from $O_2$ (*32*−*34*). Surprisingly, this is *not* the case. Herein we demonstrate that the dominant source of the oxygen atom in these products is derived from $H_2O$ and *not* from $O_2$. Although exchange of the label within the cell cannot be unequivocally ruled out, our results suggest that the proposed mechanism of HAS needs to be reexamined.

## EXPERIMENTAL PROCEDURES

*Materials.* The porphyrin standard iron(III) deuteroporphyrin IX chloride was obtained from Frontier Scientific Porphyrin Products (Logan, UT). $^{18}O_2$ gas was purchased from Isotec (Miamisburg, OH) in a pressurized cylinder or received as a gift from Prof. Christine D. Keating (The Pennsylvania State University) at atmospheric pressure. $H_2{}^{18}O$ (95% purity) and NMR solvents were purchased from Cambridge Isotopes Laboratories (Cambridge, MA). Sequencing was performed on an ABI 377 sequencer at the University of Utah DNA Sequencing Core Facility. Plasmid construction, cell growth conditions, chemical heme modification, chromatographic techniques, and heme analysis are identical to those reported previously (*27*) unless noted below.

*Isolation of Heme A from Bovine Heart.* Isolation of bulk quantities of heme A was accomplished using a procedure modified from Tuppy et al. (*35*). Four bovine hearts were cleaned of excess fat, cut into small pieces, and passed twice through an electric meat grinder. The meat was kept on ice throughout these steps. The minced meat (6 kg) was suspended with frequent shaking in 6 L of an 80% acetone/5% concentrated HCl solution for at least 3 h at 4 °C. The slurry was filtered through cheesecloth and centrifuged at 5000*g* for 7 min. The filtrate was then diluted with approximately one-fifth volume of water, and the hemes were extracted using an equal volume of diethyl ether. The diethyl ether was washed three times with a 5% NaCl solution, and the hemes were then extracted into a 100 mM phosphate buffer (pH 8.0). The pH of the buffer layer was lowered to approximately 1−2 by the addition of HCl, causing some of the hemes to precipitate. The hemes were reextracted into diethyl ether, and the solution was diluted with $CH_3CN$ containing 0.1% TFA (10 mL of $CH_3CN$ per 500 mL of diethyl ether). The ether was removed via rotoevaporation, and the residual $CH_3CN$ was diluted 1:1 with water. The hemes were purified via HPLC as previously described (*27*).

*NMR Spectroscopy of Reduced and Oxidized Heme A Products.* The chemically oxidized and reduced heme A products were concentrated on a Waters Sep-Pack Vac SPE cartridge (3 $cm^3$) C18 column and eluted with a minimal amount of DMSO. To remove nondeuterated solvent, the heme solution was lyophilized, redissolved in 1−2 mL of DMSO-$d_6$, and relyophilized, and the process was repeated. The final lyophilized product was dissolved in 300 $\mu$L of degassed pyridine-$d_5$ under anaerobic conditions in a glovebox. An excess amount of sodium dithionite, dissolved in $D_2O$, was added to each sample. The sample was microfuged to pellet precipitated dithionite. To increase sensitivity, the samples were analyzed in a Shigimi NMR tube matched to

[2] The hemes in HAS are thought to be ligated by His residues, and thus HAS is presumably not a true cytochrome P450.

$D_2O$. The samples were analyzed on a Varian Inova 500 spectrometer run at 500 MHz with a 5 mm indirect detection *z*-gradient probe. The $^1H$ NMR experiments were run using a standard Varian 2 pulse sequence with a 90° pulse width of 5.275 ms. HSQC experiments were performed using a standard Varian gradient selected phase-sensitive HSQC pulse sequence with a 90° pulse width of 5.275 ms. The total experiment collection time for heme A and its derivatives was ~50 h. The $^1J_{CH}$ coupling for all three samples was 140 Hz. Data were also collected for heme A with a $^1J_{CH}$ coupling of 160 Hz in order to maximize the signal from the aldehyde proton and carbon.

*Anaerobic Expression of ctaA.* Cells were grown under rigorously anaerobic conditions using standard Schlenk line techniques as previously described (*27*). To measure the $O_2$ dependence of protein biosynthesis and activity, aliquots of anaerobic cell culture were removed at various time points after induction and probed for heme levels (HPLC) and expression of both CtaB and CtaA (western blot analysis).

*Western Blot Analysis of Anaerobic Cells.* Samples were cannula-transferred from the growth flask to a septum-sealed centrifuge tube filled with $N_2$. The media were centrifuged to harvest the cells, followed by the addition of 2× SDS sample buffer. The samples were sonicated before being loaded onto the gel. CtaB was visualized using a T7 tag monoclonal antibody while CtaA was visualized using a 6× His tag monoclonal antibody (Novagen). To keep protein loading consistent between aerobically grown and anaerobically grown cells, the amount of media harvested was adjusted on the basis of the cell culture density as determined by OD at 600 nm.

*Analysis of $^{18}O_2$ Incorporation into the Carboxylate Derivative of Heme A.* Cells expressing CtaA and CtaB were grown anaerobically to an $OD_{600}$ of approximately 0.2 (*27*). One hour after anaerobic induction with IPTG, the growth flask was evacuated until the media boiled and then backfilled with either $^{16}O_2$ or $^{18}O_2$. The flask was shaken vigorously for 10 min and placed in a $N_2$-purged glovebag for transfer into airtight 500 mL centrifuge bottles. The cells were harvested at 3000*g* for 7 min. The pellet was resuspended under $N_2$ in 1 mL of media and lysed with 2.5 mL of 4% HCl/acetone. The solution was removed from the glovebag, diluted to 5 mL with $H_2O$, and centrifuged at 17000*g* for 10 min. The supernatant was then loaded onto the HPLC to purify the carboxylate derivative for ESI-MS analysis.

*Analysis of $^{18}O_2$ Incorporation into the Alcohol Derivative of Heme A.* Cells expressing CtaA and CtaB were grown anaerobically to an $OD_{600}$ of approximately 0.2 (*27*). Immediately following anaerobic induction with IPTG the growth flask was treated with 1.0 mL of either $^{16}O_2$ or $^{18}O_2$. After 1 h of incubation, the media were poured into 500 mL of 4% HCl/acetone. The solution was centrifuged at 4000*g* for 15 min and the supernatant loaded onto a Waters Sep-Pack Vac SPE cartridge (3 $cm^3$) C18 column. The hemes were eluted with 2 mL of DMSO, diluted with 3 mL of water:acetonitrile (75:25), purified by HPLC, and analyzed via ESI-MS.

*Expression of CtaA in the Presence of $H_2{}^{18}O$.* Isotopically labeled water (15 g, 95%) was used to dissolve 0.6 g of LB powder with antibiotics and sterile filtered. An overnight culture (0.5 mL) from unlabeled water was used to inoculate the media by harvesting the cells, discarding the media, and resuspending the cells in the new $^{18}O$-labeled media. The cells were grown in a 125 mL Erlenmeyer flask at 37 °C until the culture reached an $OD_{600}$ of ~1.0. Cells were then induced for 1 h, harvested by centrifugation, and stored at −80 °C. The hemes were isolated as described elsewhere.

*Analysis of Heme Isotopic Data.* Mass spectral data were exported as XY data sets. The peak areas were integrated across 0.2 amu and normalized. Interpretation of the actual mass spectral data to determine the percent isotopic enrichment of $^{18}O$ was accomplished by fitting the data with a linear combination of actual and theoretical spectra based on natural isotopic abundances. See Supporting Information for a description, examples, and fittings.

*Controls To Determine the Extent of Exchange during Heme Isolation and Analysis.* To determine the extent of exchange during extraction, *E. coli* cells expressing both HOS and HAS were grown in 250 mL of selective LB media ($H_2{}^{16}O$). The cells were induced with 250 μL of IPTG (75 mg/mL) at an $OD_{600}$ of 0.6. The cells were divided into two 125 mL aliquots and collected by centrifugation for 7 min at 4000*g*. The cell pellet was resuspended in 2.5 mL of $H_2{}^{18}O$ (10%) or $H_2{}^{16}O$ followed by the addition of 2.5 mL of a 4% HCl/acetone solution. The extracted cellular debris was pelleted by centrifugation (17000*g* for 5 min) and the supernatant loaded on the HPLC. The hemes were collected and analyzed via ESI-MS. The C8 alcohol intermediate exhibited approximately 5% total exchange over all oxygen atoms while heme A showed 10% total exchange over all oxygen atoms. This suggests that approximately 5% of the aldehyde oxygen exchanged during extraction. The amount of the C8 carboxylate derivative obtained was not sufficient to analyze in this manner. However, when the purified C8 carboxylate heme derivative was incubated in the extraction mixture for 20 min (approximately two times longer than normal extraction time), ~12% of the heme showed exchange of one $^{18}O$ atom.

To determine the extent of exchange during HPLC analysis, heme A was chemically oxidized to the C8 carboxylate derivative and chemically reduced to the C8 alcohol derivative as previously described (*27*). The purified heme derivatives were dissolved in DMSO, diluted 25-fold with a 50:50 acetone:$H_2{}^{18}O$ (99%) solution containing 2% HCl, and incubated for 24 h to induce partial exchange. A comparison of the hemes by ESI-MS data before and after HPLC purification indicated that approximately 10% of the $^{18}O$ label on both the C8 carboxylate and C8 alcohol derivatives was exchanged on the HPLC column. Heme A was not analyzed in this way because essentially 100% of the aldehyde on purified heme A exchanges under these conditions; only unpurified heme A (presumably bound to lipids) is afforded some protection against aldehyde exchange.

*Heme Extraction with $H_2{}^{18}O$.* As a further control, *E. coli* cells expressing both HOS (CtaB) and HAS (CtaA) were grown in 250 mL of selective LB media ($H_2{}^{16}O$). The cells were grown to an $OD_{600}$ of 0.6 and induced with 250 μL of IPTG (75 mg/mL). The cells were divided into two 125 mL aliquots. After the cells were centrifuged at 4000*g* for 7 min, the supernatant was discarded. The cell pellet was resuspended in 2.5 mL of $H_2{}^{18}O$ (35%). Then 2.5 mL of acetone (4% concentrated HCl) was added to each aliquot. The cells

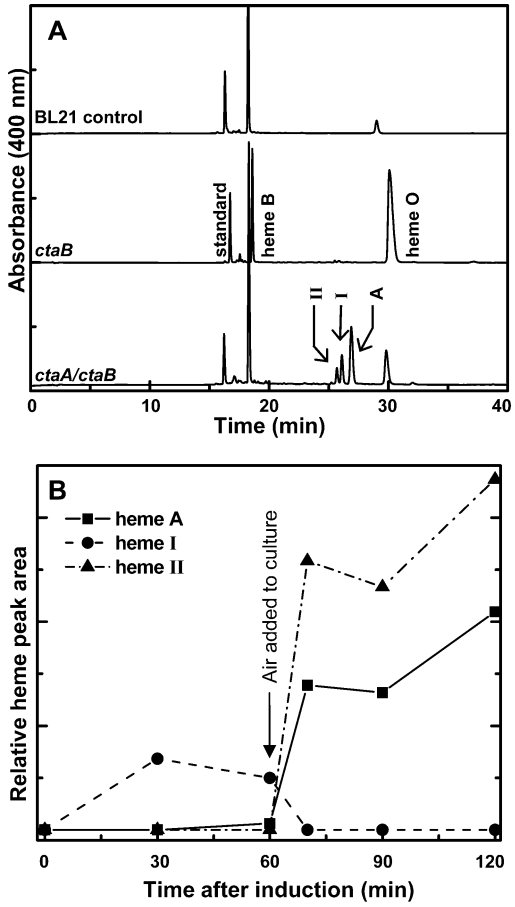



FIGURE 1: Hemes isolated from *E. coli* cells. (A) HPLC chromatograms of hemes from control cells (top), cells expressing *ctaB* (middle), or cells expressing both *ctaB* and *ctaA* (bottom) grown under aerobic conditions. Heme I was identified as the C8 alcohol derivative of heme O, and heme II was identified as the C8 carboxylate derivative of heme O. (B) Hemes isolated from *E. coli* cells expressing both *ctaB* and *ctaA* and grown under nearly anaerobic conditions.

were again centrifuged at 17000*g* for 5 min. The supernatant of each sample was loaded on the HPLC, and heme A was collected and analyzed by ESI-MS.

## RESULTS

*Overexpression of CtaB (HOS) and CtaA (HAS) and Product Analysis.* The genes responsible for HOS and HAS activity (*ctaB* and *ctaA*, respectively) were cloned from *B. subtilis* and overexpressed in *E. coli* (*27*). When CtaB is expressed singly, approximately 70% of the total extractable heme in *E. coli* is present as heme O (Figure 1A). When CtaB and CtaA are coexpressed, both heme O and heme A are produced. In addition to heme O and heme A, however, two previously unidentified hemes were also present. Neither heme derivative was observed in the absence of HAS, indicating that their presence is due to the expression of HAS. On the basis of optical data and tandem mass spectrometry, we proposed that these two new hemes were the C8 alcohol and the C8 carboxylate derivatives of heme A. Furthermore, reactions performed under nearly anaerobic conditions followed by the addition of air demonstrated that the amount of the alcohol derivative decreased as the levels of heme A and the carboxylate derivative increased (Figure 1B). From these data it was concluded that the C8 alcohol derivative is the putative intermediate while the C8 carboxylate derivative is overoxidized product.

Table 1: $^{1}$H-Correlated $^{13}$C Chemical Shifts for Heme A and Derivatives in Pyridine-$d_5$

| | $^{1}$H shifts (ppm) | $^{13}$C shifts (ppm) |
|---|---|---|
| heme A | | |
| meso H− | 10.94 | 100.75 |
| | 10.54 | 99.89 |
| | 10.39 | 101.38 |
| | 9.88 | 97.76 |
| −C(O)**H** | 11.47 | 185.83 |
| C8 alcohol | | |
| meso H− | 10.65 | 99.95 |
| | 10.35 | 99.18 |
| | 10.30 | 98.36 |
| | 10.03 | 98.04 |
| −C**H**$_2$OH | 6.27 | 55.53 |
| C8 carboxylate | | |
| meso H− | 11.43 | 101.89 |
| | 10.61 | 99.83 |
| | 10.49 | 100.55 |
| | 9.96 | 97.66 |
| −COOH | | |

A potential problem with the identification of the unknown hemes is that the NMR spectra were not straightforward to interpret. The $^{1}$H NMR spectrum of heme A contained five peaks from approximately 9.9 to 11.5 ppm. On the basis of previous assignments (*36*), four of these peaks were assigned to the bridgehead methines while the fifth (and farthest downfield at 11.47 ppm) was assigned to the aldehyde proton. The NMR spectra of the chemically generated alcohol and carboxylate derivatives, the "authentic samples", contained only four proton peaks in that region, suggesting that the reaction had indeed occurred at the aldehyde position. Unfortunately, we could not unambiguously prove from the $^{1}$H NMR spectra alone that one of the methine positions had not been altered instead of the aldehyde. This was especially problematic in the case of the carboxylate derivative where a putative methine peak at 11.43 ppm has an almost identical chemical shift to the aldehyde proton in heme A.

To resolve this ambiguity, HSQC experiments were performed on heme A and each derivative (see Supporting Information for HSQC spectra). These spectra indicate that the four protons assigned as methines in all three hemes are bound to carbons with $^{13}$C chemical shifts between 97.7 and 101.9 ppm, consistent with their assignment as methines (Table 1). Furthermore, the proton in heme A at 11.47 ppm correlates with a carbon at 185.5 ppm, indicative of an aldehyde (*37*). This peak is absent in both the chemically generated alcohol and carboxylate derivatives. All three of the hemes also contain a triplet at approximately 6.6 ppm in the $^{1}$H NMR spectrum that correlates to a carbon at ~68.5 ppm due to the alcohol on the 17-hydroxyethylfarnesyl moiety. The chemically reduced derivative of heme A, however, contains an additional benzylic-like alcohol (a singlet at 6.27 ppm correlated with a carbon at 55.53 ppm; see Figure S6), just as one would predict (*37*). Together, these results provide verification that the initial assignments of the authentic samples and the unknown hemes were correct.

*Expression of CtaB and CtaA under Anaerobic Conditions.* When CtaB and CtaA are expressed under nearly anaerobic conditions, no heme A but small quantities of the C8 alcohol

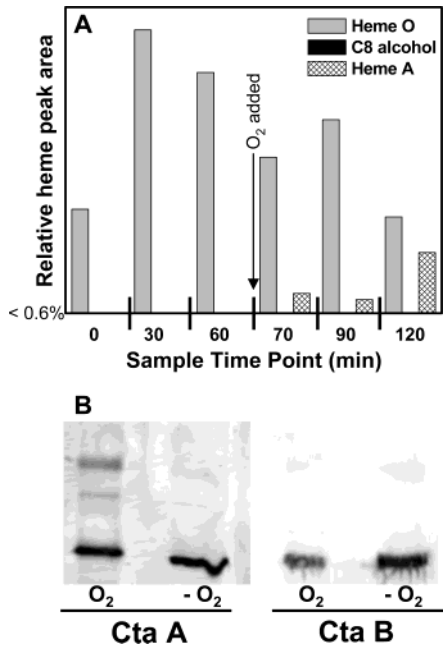



FIGURE 2: (A) Relative amounts of C8 alcohol, heme A, and heme O produced under anaerobic growth conditions. Time points were taken 0, 30, 60, 70, 90, and 120 min after induction. The culture was exposed to $O_2$ immediately after the 60 min sample was removed. No C8 alcohol was observed under these growth conditions, and no heme A was observed prior to the addition of $O_2$. Identical cells that are never exposed to $O_2$ produce no C8 alcohol intermediate or heme A even after 120 min (data not shown). (B) The protein levels of HAS and HOS were checked by western analysis as described in the Experimental Procedures after 1 h of anaerobic induction ($-O_2$) and compared to a culture grown in a shake flask ($O_2$).

derivative are observed (Figure 1b), presumably due to low levels of $O_2$ contamination. In fact, the alcohol heme derivative could be observed when less than 1 mL of $O_2$ was present in a 1 L growth flask, indicating that CtaA is exceedingly sensitive to the presence of $O_2$. Given the role of HAS in heme A biosynthesis and the maturation of cytochrome *c* oxidase, it is perhaps not surprising that CtaA has a very high affinity for $O_2$; cells cannot utilize $O_2$ for aerobic metabolism if their terminal oxidase is not assembled. When these nearly anaerobic cells are exposed to atmospheric $O_2$, the levels of heme A and the overoxidized carboxylate byproduct rise immediately as the amount of the C8 alcohol derivative drops to undetectable levels, indicating that the alcohol is a true intermediate on the pathway. While these results are consistent with the hypothesis that CtaA requires $O_2$ for activity, it could also be argued that small quantities of the alcohol derivative can be formed via an $O_2$-independent pathway and that $O_2$ is only absolutely required for the conversion of the alcohol intermediate into heme A.

To address these concerns, CtaB and CtaA were expressed under rigorously anaerobic conditions. Cells were harvested anaerobically at various time points and analyzed for heme and protein content. Results from these experiments confirm that neither heme A, nor the C8 alcohol intermediate, nor the C8 carboxylate derivative is produced in the absence of $O_2$ (Figure 2A). Under these same conditions, however, CtaB generates significant quantities of heme O, demonstrating that the lack of CtaA activity is not due to a lack of the heme O substrate.[3] Once $O_2$ is added to the system, however, heme A is rapidly produced. Western blot analysis indicates that the levels of CtaA and CtaB are similar under aerobic and anaerobic conditions (Figure 2B). Thus, the lack of oxidized products seen in anaerobic conditions is not due to low expression or CtaA instability. Together, these results conclusively demonstrate that CtaA activity is strictly dependent on the presence of $O_2$.

*Biosynthesis of Heme A in the Presence of $^{18}O_2$.* To ascertain the source of the oxygen atom in the C8 aldehyde of heme A, CtaA was expressed in the presence of $^{18}O_2$. Figure 3 shows mass spectral analysis of heme A isolated from cells incubated in either $^{16}O_2$ or $^{18}O_2$. No incorporation of $^{18}O_2$ was observed in the heme A product. This result is not particularly surprising, however, because aldehydes are well-known to exchange with $H_2O$. Thus, we also analyzed $^{18}O_2$ incorporation into the C8 alcohol intermediate and the C8 carboxylate derivative. Surprisingly, there was also negligible incorporation of $O_2$ into these heme products (Figure 3).

One possible explanation for these results is that the $^{18}O$ label is exchanging with water during heme isolation and purification. To evaluate the extent of exchange, two separate controls were performed. In the first control, hemes were extracted from cells in the presence of $H_2{}^{18}O$, HPLC purified, and analyzed by ESI-MS. In the second control, chemically generated, partially labeled hemes were HPLC purified and analyzed by ESI-MS. These controls demonstrate that the C8 alcohol and C8 carboxylate derivatives exhibit at most ~30% total exchange of a single oxygen atom over five/six possible positions during these two processes (see Supporting Information). Together, these results highlight the fact that we see negligible incorporation from $O_2$ into our products and that these observations cannot be explained by exchange during analysis.

*Biosynthesis of Heme A in the Presence of $H_2{}^{18}O$.* If $O_2$ is not incorporated into the aldehyde moiety of heme A, then presumably $H_2O$ must be the source of the oxygen atom. To test this theory, *E. coli* cells coexpressing CtaB and CtaA were grown in $H_2{}^{18}O$. Hemes from these cells were extracted, purified via HPLC, and analyzed by mass spectrometry for comparison to hemes isolated from cells grown in unlabeled water (Figure 4). Unfortunately, only small quantities of C8 alcohol and C8 carboxylate heme derivatives could be isolated in these experiments because of the scale of the reaction. Because of partial $^{18}O$ incorporation at the propionate positions, all hemes exhibited an extensive isotopic envelope. On the basis of the accepted mechanism of heme O synthase, which incorporates a hydroxide during the addition of the farnesyl moiety, we would predict that all of the heme O molecules would incorporate one additional $^{18}O$ atom compared to heme B. Clearly, this is not the case as approximately 30% of the hemes contain no $^{18}O$ atoms. A related result reported by Kreuzer-Martin et al. determined that only 24% of the cellular oxygen in *B. subtilis* is derived from water during mid-log phase (*38*). Nevertheless, under our conditions at least 40% of the farnesyl oxygen atom is derived from $H_2{}^{18}O$ (see Supporting Information).

[3] Although coproporphyrinogen III oxidase and protoporphyrinogen IX oxidase are strictly dependent on $O_2$ for activity in eukaryotes, in facultative anaerobes such as *E. coli* both coproporphyrinogen III and protoporphyrinogen IX can be oxidized using alternative electron acceptors (*1*).

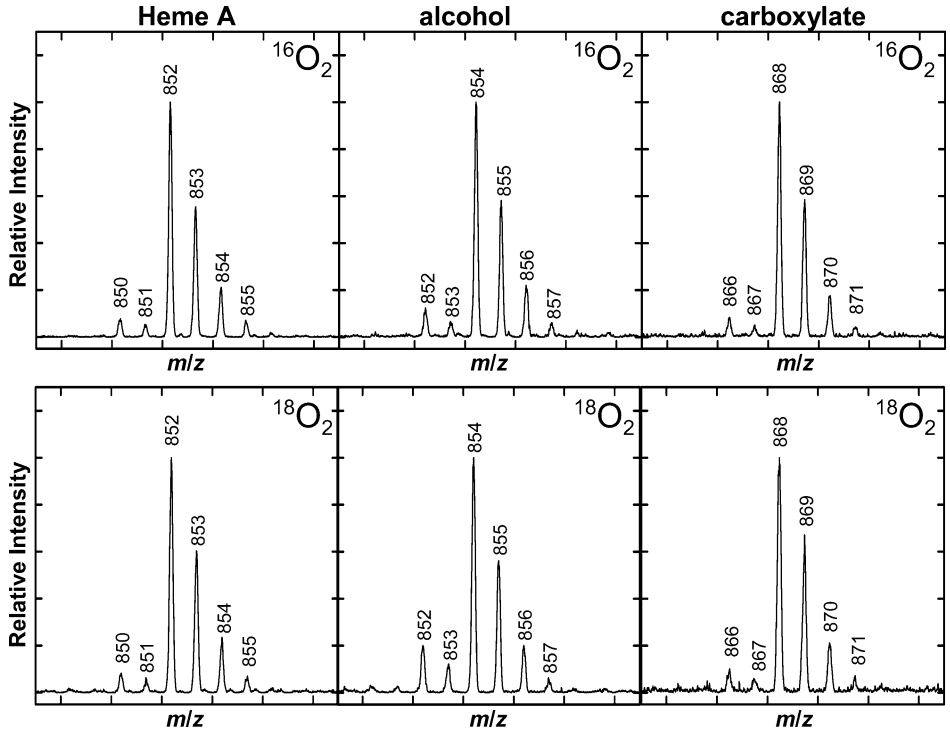



FIGURE 3: Mass spectral analysis of hemes isolated from cells grown under $^{16}O_2$ (top row) compared to hemes isolated from cells grown under $^{18}O_2$ (bottom row).

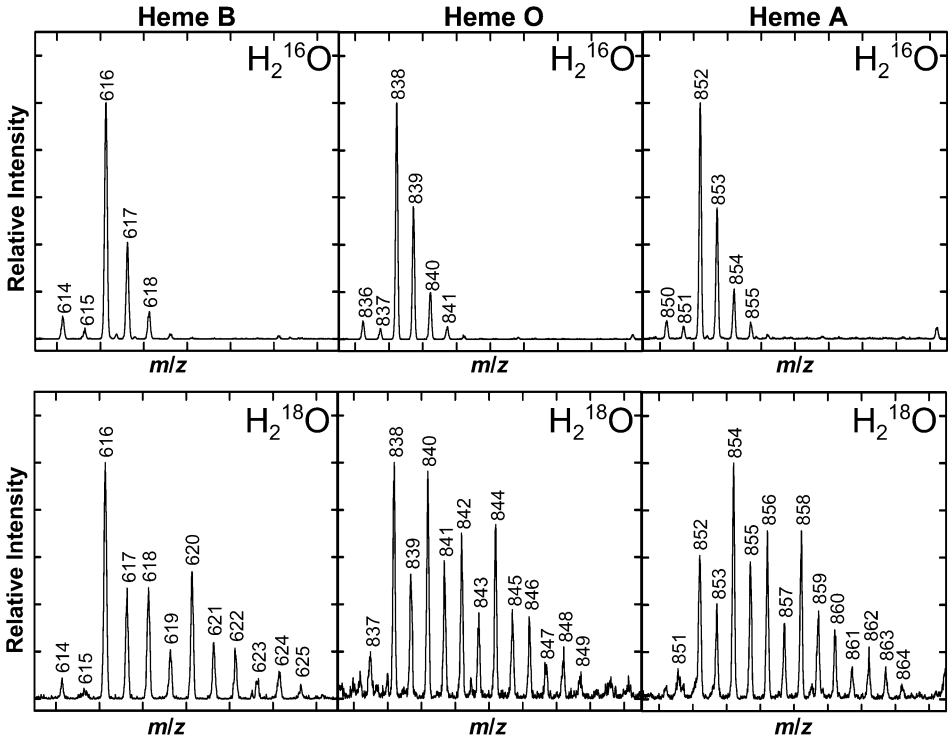
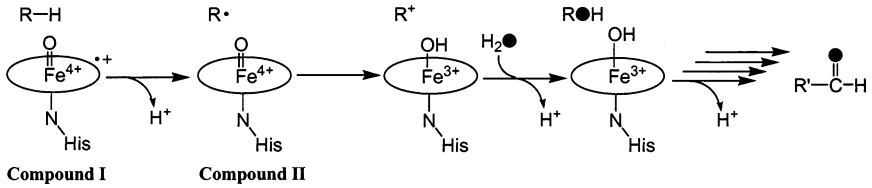



FIGURE 4: Mass spectral analysis of hemes isolated from cells grown in $H_2{}^{16}O$ media (top row) compared to hemes isolated from cells grown in $H_2{}^{18}O$-labeled media (bottom row).

Heme A was analyzed in a similar manner (Figure 4). Significantly, a portion of the heme contained $^{18}O$ at all positions, indicating that $H_2{}^{18}O$ was incorporated into the aldehyde of heme A. This can also be seen by comparing the percentage of heme molecules containing zero or one $^{18}O$ atom. In the case of heme O, the ratio of zero incorporation (838 amu) to one $^{18}O$ incorporation (840 amu) is 1:1. In the case of heme A, however, this ratio (852:854 amu) has been shifted to 1:1.33, indicating additional $^{18}O$ incorporation into heme A. Since heme A is derived directly from heme O, this result can only occur if water has been incorporated into C8 aldehyde position of heme A. Given the acidic conditions utilized to extract the hemes, it is surprising any label at all was observed at the aldehyde position. It is possible that heme A may be bound to lipids during extraction, providing partial protection from exchange. While the mechanism of incorporation and the exact percentage of $^{18}O$-label present prior to extraction/analysis can be

Scheme 2: Possible Mechanism for the Oxidation of Heme O to Heme A via Outer-Sphere Electron Transfer




debated, the key point is that no $^{18}O_2$ incorporation is observed in any heme analyzed, and $H_2O$ is the dominant source of the oxygen atom observed in the C8 aldehyde position of heme A.

## DISCUSSION

Our discovery that heme A incorporates oxygen from $H_2O$ and *not* from $O_2$ was unanticipated, given the current paradigm that HAS utilizes consecutive P450-like monooxygenase reactions. Indeed, these results inevitably reopen the question of what mechanism HAS actually utilizes. Based on our new results, two different limiting possibilities can be envisioned: (1) HAS utilizes successive monooxygenase reactions, but there is total in vivo exchange of the $^{18}O$ label with water prior to extraction, and (2) HAS utilizes a peroxidase-like mechanism, oxidizing the C8 methyl group via electron transfer. The merits of each limiting possibility are discussed below.

*Does the Label from $^{18}O_2$ Exchange with Water?* As discussed above, no $O_2$ incorporation is observed in the biosynthesis of the C8 alcohol intermediate, heme A, or the overoxidized C8 carboxylate byproduct. At the same time, however, incorporation from $H_2O$ is observed in heme A. One possibility that could explain the data is that HAS utilizes a P450-like mechanism but that compound I exchanges with water prior to reacting with the substrate. "Compound I" generated from model systems is well-known to exchange with solvent (*39*, *40*), and compound II (the one electron reduced form of compound I) has been shown to exchange in a variety of peroxidases (*41*−*44*). To our knowledge, however, compound I has never been shown to exchange with solvent *in a native protein* (*33*). For these reasons, we do not think it likely that the ferryl oxygen is exchanging with solvent during the reaction.

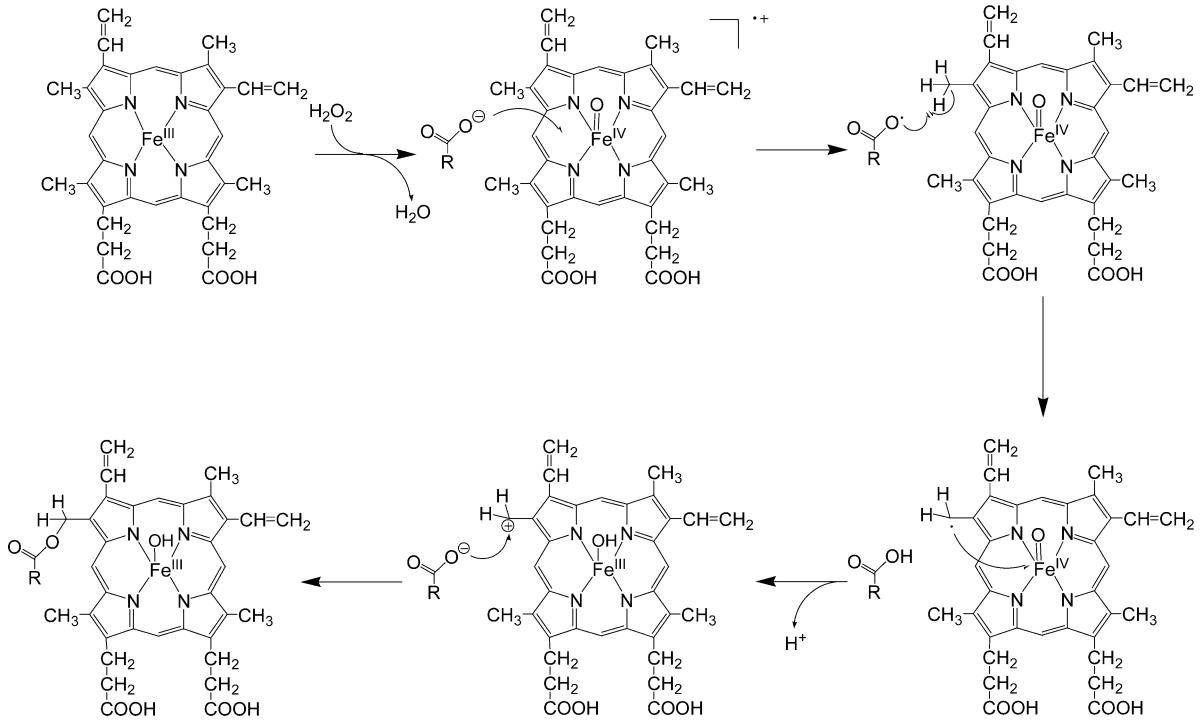
A second possibility is that the hemes are exchanging in the cells. Aldehydes are known to exchange with water, and it is conceivable that heme A diffuses out of the active site of HAS and exchanges prior to heme analysis. While certainly feasible, this seems unlikely for a couple of reasons. First, the aldehyde in chlorophyll *b* does not exchange in vivo (*25*), indicating that, at least in chlorophyll *b*, the aldehyde is protected from exchange during biosynthesis, transport, and insertion. Second, our controls indicate that even under acidic extraction conditions only a portion of the heme A aldehyde exchanges, presumably because it is protected by bound lipids. Thus, although the hemes will exchange their oxygen label slowly with $H_2O$, this does not appear to be sufficient to explain the lack of $^{18}O$ label observed in hemes isolated from cells grown in $^{18}O_2$. Furthermore, even if in vivo exchange of the aldehyde were occurring, it still would not explain the lack of $O_2$ incorporation into the C8 alcohol intermediate.

*Does HAS Utilize Outer-Sphere Electron Transfer?* An intriguing possibility is that HAS utilizes $O_2$ to catalyze heme O oxidation via outer-sphere electron transfer. In this scenario (Scheme 2), compound I extracts an electron from heme O followed by loss of a proton to generate compound II and a carbon-based radical. A second electron transfer to compound II results in a carbocation that is trapped by water to generate the alcohol. Repeating the process would lead to the aldehyde, similar to the oxidation of veratryl alcohol to veratraldehyde catalyzed by lignin peroxidase (*45*, *46*). The distinguishing feature in this mechanism is that the oxygen atom in the alcohol intermediate and the aldehyde product is derived from $H_2O$ and *not* $O_2$. This reaction is essentially the reverse of the "cooxidation" mechanism sometimes seen in heme-containing peroxidases, whereby a substrate radical is trapped by $O_2$ (*47*, *48*).

If HAS utilizes outer-sphere electron transfer like peroxidases, it is reasonable to ask if HAS uses $H_2O_2$ as the oxidant. A number of experimental observations argue strongly against this scenario. The activity of HAS is strictly dependent on $O_2$, and while it is theoretically possible that HAS utilizes $H_2O_2$ derived from $O_2$, the sensitivity of HAS toward $O_2$ and the speed with which heme A is generated upon the addition of $O_2$ make this scenario exceedingly unlikely. In addition, it would not be consistent with the observation that HAS in *S. pombe* is fused to a required ferredoxin (*19*). Thus, it appears that HAS utilizes $O_2$ in the catalytic cycle.

A question that remains unanswered, however, is whether HAS utilizes a heme B cofactor to activate $O_2$ and generate compound I or whether HAS actually catalyzes the autoxidation of heme O, with the heme B cofactor acting solely as an electron transfer agent. Precedent exists for both possibilities. Peroxidases are obviously well-known to oxidize substrates via outer-sphere electron transfer (*45*, *49*−*51*), and thus a heme B cofactor may very well be the site of $O_2$ binding. At the same time, however, many mammalian peroxidases and members of the CYP4A, CYP4B, and CYPF classes of the cytochromes P450 undergo autoxidation, ultimately leading to a heme-protein cross-link (*52*). In a mechanism proposed by Ortiz de Montellano and co-workers, the decay of compound I leads to the formation of a carbocation on a methyl substituent of the porphyrin ring. This carbocation is trapped by a nearby glutamate residue, producing an ester cross-link (Scheme 3). If the glutamate residue is mutated to an aspartate, the shorter side chain is no longer able to cross-link to the porphyrin, and an alcohol derivative of the heme is produced; experiments with $H_2{}^{18}O$ confirmed that this oxygen atom is derived from water (*53*). If HAS utilizes a similar mechanism to oxidize heme O to heme A, then heme O is the site of $O_2$ binding, and the role of the heme B cofactor is limited to electron transfer. Interestingly, this hypothesis would be consistent with the

Scheme 3: Proposed Mechanism for the Autoxidation of Hemes Forming an Ester Cross-Link[a]



[a] Adapted from Colas and Ortiz de Montellano (*52*). Note that if Glu310 is mutated to an Asp in CYP4B1, the carbocation is trapped by $H_2O$ and an alcohol derivative is obtained (*53*).

observation that the heme B cofactor appears to be low spin with bis-His axial ligation. Currently, however, our data do not allow us to distinguish between these two different mechanistic possibilities.

## CONCLUSION

The results presented here demonstrate that the source of the oxygen atom in the heme A aldehyde is derived largely from $H_2O$ and *not* from $O_2$ despite the fact that HAS activity is strictly dependent on $O_2$. While we cannot conclusively rule out the possibility of in vivo oxygen exchange, it would appear that the standard mechanism proposed for heme A biosynthesis, whereby HAS utilizes successive monooxygenase reactions, needs to be reexamined. Our working model is that HAS generates heme A from heme O via successive electron transfer steps. Whether this mechanism entails generation of compound I at the heme B cofactor followed by outer-sphere electron transfer or whether it entails heme O autoxidation remains to be determined. Research is currently underway to elucidate further the details of this intriguing mechanism.

## SUPPORTING INFORMATION AVAILABLE

Figure S1 shows the basic structure of heme O, heme A, the C8 alcohol derivative, and the C8 carboxylate derivative as well as the labeling system for the carbon atoms. Figures S2−S8 provide the raw data of the $^1$H NMR and HSQC spectra for heme A (S2−S4), the C8 alcohol intermediate (S5, S6), and the overoxidized C8 carboxylate byproduct (S7, S8). Also included are experimental details concerning the control reactions, a thorough explanation of the calculations utilized to determine the percentage of $^{18}$O incorporation into the various products, and key raw data. This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES


1. Akhtar, M. (2003) Coproporphyrinogen III and Protoporphyrinogen IX Oxidases, in *The Iron and Cobalt Pigments: Biosynthesis, Structure, and Degradation* (Kadish, K. M., Smith, K. M., and Guilard, R., Eds.) pp 75−92, Academic Press, Amsterdam.
2. Malmström, B. G. (2001) Respiration: Cytochrome Oxidase, in *Biological Systems and Artificial Supramolecular Systems* (Balzani, V., Ed.) pp 39−55, Wiley-VCH, New York.
3. Ferguson-Miller, S., and Babcock, G. T. (1996) Heme/Copper Terminal Oxidases, *Chem. Rev. 96*, 2889−2907.
4. García-Horsman, J. A., Barquera, B., Rumbley, J., Ma, J., and Gennis, R. B. (1994) The Superfamily of Heme-Copper Respiratory Oxidases, *J. Bacteriol. 176*, 5587−5600.
5. Thöny-Meyer, L. (1997) Biogenesis of Respiratory Cytochromes in Bacteria, *Microbiol. Mol. Biol. Rev. 61*, 337−376.
6. Kim, E., Chufán, E. E., Kamaraj, K., and Karlin, K. D. (2004) Synthetic Models for Heme-Copper Oxidases, *Chem. Rev. 104*, 1077−1133.
7. Trumpower, B. L., and Gennis, R. B. (1994) Energy Transduction by Cytochrome Complexes in Mitochondrial and Bacterial Respiration: The Enzymology of Coupling Electron-Transfer Reactions to Transmembrane Proton Translocation, *Annu. Rev. Biochem. 63*, 675−716.
8. Michel, H. (1999) Cytochrome *c* Oxidase: Catalytic Cycle and Mechanisms of Proton Pumping−A Discussion, *Biochemistry 38*, 15129−15140.
9. Wikström, M. (2000) Proton Translocation by Cytochrome *c* Oxidase: A Rejoinder to Recent Criticism, *Biochemistry 39*, 3515−3519.
10. Schmidt, B., McCracken, J., and Ferguson-Miller, S. (2003) A Discrete Water Exit Pathway in the Membrane Protein Cytochrome *c* Oxidase, *Proc. Natl. Acad. Sci. U.S.A. 100*, 15539−15542.
11. Bloch, D., Belevich, I., Jasaitis, A., Ribacka, C., Puustinen, A., Verkhovsky, M. I., and Wikström, M. (2004) The Catalytic Cycle of Cytochrome *c* Oxidase is Not the Sum of Its Two Halves, *Proc. Natl. Acad. Sci. U.S.A. 101*, 529−533.
12. Kashket, E. R. (1985) The Proton Motive Force in Bacteria: A Critical Assessment of Methods, *Annu. Rev. Microbiol. 39*, 219−242.
13. Puustinen, A., and Wikström, M. (1991) The Heme Groups of Cytochrome *O* from *Escherichia coli*, *Proc. Natl. Acad. Sci. U.S.A. 88*, 6122−6126.

14. Saiki, K., Mogi, T., Ogura, K., and Ankaru, Y. (1993) *In Vitro* Heme O Synthesis by the *cyoE* Gene Product from *Escherichia coli*, *J. Biol. Chem. 268*, 26041−26045.
15. Glerum, D. M., and Tzagoloff, A. (1994) Isolation of a Human cDNA for Heme A: Farnesyltransferase by Functional Complementation of a Yeast COX10 Mutant, *Proc. Natl. Acad. Sci. U.S.A. 91*, 8452−8456.
16. Mogi, T. (2003) Biosynthesis and Role of Heme O and Heme A, in *The Iron and Cobalt Pigments: Biosynthesis, Structure, and Degradation* (Kadish, K. M., Smith, K. M., and Guilard, R., Eds.) pp 157−181, Academic Press, Amsterdam.
17. Svensson, B., Lübben, M., and Hederstedt, L. (1993) *Bacillus subtilis* CtaA and CtaB Function in Haem A Biosynthesis, *Mol. Microbiol. 10*, 193−201.
18. Sakamoto, J., Hayakawa, A., Uehara, T., Noguchi, S., and Sone, N. (1999) Cloning of *Bacillus stearothermophilus ctaA* and Heme A Synthesis with the CtaA Protein Produced in *Escherichia coli*, *Biosci., Biotechnol., Biochem. 63*, 96−103.
19. Barros, M. H., Carlson, C. G., Glerum, D. M., and Tzagoloff, A. (2001) Involvement of Mitochrondrial Ferredoxin and Cox15p in Hydroxylation of Heme O, *FEBS Lett. 492*, 133−138.
20. Svensson, B., Anderson, K. K., and Hederstedt, L. (1996) Low-Spin Heme A in the Heme Biosynthetic Protein CtaA from *Bacillus subtilis*, *Eur. J. Biochem. 238*, 287−295.
21. Rüdiger, W. (2003) in *The Iron and Cobalt Pigments: Biosynthesis, Structure, and Degradation* (Kadish, K. M., Smith, K. M., and Guilard, R., Eds.) pp 71−108, Academic Press, Amsterdam.
22. Schneegurt, M. A., and Beale, S. I. (1992) Origin of the Chlorophyll *b* Formyl Oxygen in *Chlorella vulgaris*, *Biochemistry 31*, 11677−11683.
23. Tanaka, A., Ito, H., Tanaka, R., Tanaka, N. K., Yoshida, K., and Okada, K. (1998) Chlorophyll *a* Oxygenase (*CAO*) is Involved in Chlorophyll *b* Formation from Chlorophyll *a*, *Proc. Natl. Acad. Sci. U.S.A. 95*, 12719−12723.
24. Oster, U., Tanaka, R., Tanaka, A., and Rüdiger, W. (2000) Cloning and Functional Expression of the Gene Encoding the Key Enzyme for Chlorophyll *b* Biosynthesis (CAO) from *Arabidopsis thaliana*, *Plant J. 21*, 305−310.
25. Porra, R. J., Schäfer, W., Cmiel, E., Katheder, I., and Scheer, H. (1994) The Derivation of The Formyl-Group Oxygen of Chlorophyll *b* in Higher Plants From Molecular Oxygen, *Eur. J. Biochem. 219*, 671−679.
26. Barros, M. H., Nobrega, F. G., and Tzagoloff, A. (2002) Mitochondrial Ferredoxin Is Required for Heme A Synthesis in *Saccharomyces cerevisiae*, *J. Biol. Chem. 277*, 9997−10002.
27. Brown, K. R., Allan, B. M., Do, P., and Hegg, E. L. (2002) Identification of Novel Hemes Generated by Heme A Synthase: Evidence for Two Successive Monooxygenase Reactions, *Biochemistry 41*, 10906−10913.
28. Watanabe, K., Narimatsu, S., Yamamoto, I., and Yoshimura, H. (1991) Oxygenation Mechanism in Conversion of Aldehyde to Carboxylic Acid Catalyzed by a Cytochrome P-450 Isozyme, *J. Biol. Chem. 266*, 2709−2711.
29. Kuo, C.-L., Raner, G. M., Vaz, A. D. N., and Coon, M. J. (1999) Discrete Species of Activated Oxygen Yield Different Cytochrome P450 Heme Adducts from Aldehydes, *Biochemistry 38*, 10511−10518.
30. French, K. J., Strickler, M. D., Rock, D. A., Rock, D. A., Bennett, G. A., Wahlstrom, J. L., Goldstein, B. M., and Jones, J. P. (2001) Benign Synthesis of 2-Ethylhexanoic Acid by Cytochrome P450cam: Enzymatic, Crystallographic, and Theoretical Studies, *Biochemistry 40*, 9532−9538.
31. Ingledew, W. J., and Poole, R. K. (1984) The Respitory Chains of *Escherichia coli*, *Microbiol. Rev. 48*, 222−271.
32. Sono, M., Roach, M. P., Coulter, E. D., and Dawson, J. H. (1996) Heme-Containing Oxygenases, *Chem. Rev. 96*, 2841−2887.
33. Ortiz de Montellano, P. R. (1995) Oxygen Activation and Reactivity, in *Cytochrome P450, Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 245−303, Plenum Press, New York.
34. Groves, J. T., and Han, Y.-Z. (1995) Models and Mechanisms of Cytochrome P450 Action, in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., Ed.) pp 3−49, Plenum Press, New York.
35. Tuppy, H., and Birkmayer, G. D. (1969) Cytochrome Oxidase Apoprotein in "Petite" Mutant Yeast Mitochondria. Reconstitution of Cytochrome Oxidase by Combining Apoprotein with Cytohemin, *Eur. J. Biochem. 8*, 237−243.
36. Caughey, W. S., Smythe, G. A., O'Keeffe, D. H., Maskasky, J. E., and Smith, M. L. (1975) Heme A of Cytochrome *c* Oxidase. Structure and Properties: Comparisons with Hemes B, C, and S and Derivatives, *J. Biol. Chem. 250*, 7602−7622.
37. Wehrli, F. W., and Wirthlin, T. (1976) *Interpretation of Carbon-13 NMR Spectra*, Heyden & Son, London.
38. Kreuzer-Martin, H. W., Lott, M. J., Dorigan, J., and Ehleringer, J. R. (2003) Microbe Forensics: Oxygen and Hydrogen Stable Isotope Ratios in *Bacillus subtilis* Cells and Spores, *Proc. Natl. Acad. Sci. U.S.A. 100*, 815−819.
39. McLain, J. L., Lee, J., and Groves, J. T. (2000) Biometric Oxygenations Related to Cytochrome P450: Metal-Oxo and Metal-Peroxo Intermediates, in *Biometric Oxidations Catlyzed by Transition Metal Complexes* (Meunier, B., Ed.) pp 91−169, Imperial College Press, London.
40. Meunier, B., and Bernadou, J. (2000) Active Iron-Oxo and Iron-Peroxo Species in Cytochromes P450 and Peroxidases; Oxo-Hydroxo Tautomerism with Water-Soluble metalloporphyrins, *Met. Ions Biol. Syst. 97*, 1−35.
41. Hosten, C. M., Sullivan, A. M., Palaniappan, V., Fitzgerald, M. M., and Terner, J. (1994) Resonance Raman Spectroscopy of the Catalytic Intermediates and Derivatives of Chloroperoxidase from *Caldariomyces fumago*, *J. Biol. Chem. 269*, 13966−13978.
42. Hashimoto, S., Teraoka, J., Inubushi, T., Yonetani, T., and Kitagawa, T. (1986) Resonance Raman Study on Cytochrome*c* Peroxidase and Its Intermediate, *J. Biol. Chem. 261*, 11110−11118.
43. Reczek, C. M., Sitter, A. J., and Terner, J. (1989) Resonance Raman Characterization of Heme Fe(IV)═O Groups of Intermediates of Yeast Cytochrome *c* Peroxidase and Lactoperoxidase, *J. Mol. Struct. 214*, 27−41.
44. Sitter, A. J., Reczek, C. M., and Terner, J. (1985) Heme-Linked Ionization of Horseradish Peroxidase Compound II Monitored by the Resonance Raman Fe(IV)═O Stretching Vibration, *J. Biol. Chem. 260*, 7515−7522.
45. ten Have, R., and Teunissen, P. J. M. (2001) Oxidative Mechanisms Involved in Lignin Degradation by White-Rot Fungi, *Chem. Rev. 101*, 3397−3413.
46. Khindaria, A., Yamazaki, I., and Aust, S. D. (1995) Veratryl Alcohol Oxidation by Lignin Peroxidase, *Biochemistry 34*, 16860−16869.
47. Ortiz de Montellano, P. R., and Grab, L. A. (1987) Cooxidation of Styrene by Horseradish Peroxidase and Phenols: A Biochemical Model for Protein-Mediated Cooxidation, *Biochemistry 26*, 5310−5314.
48. Miller, V. P., DePillis, G. D., Ferrer, J. C., Mauk, A. G., and Ortiz de Montellano, P. R. (1992) Monooxygenase Activity of Cytochrome *c* Peroxidase, *J. Biol. Chem. 267*, 8936−8942.
49. Bosshard, H. R., Anni, H., and Yonetani, T. (1991) Yeast Cytochrome *c* Peroxidase, in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., and Grisham, M. B., Eds.) pp 51−84, CRC Press, Boca Raton, FL.
50. Veitch, N. C., and Smith, A. T. (2001) Horseradish Peroxidase, *Adv. Inorg. Chem. 51*, 107−162.
51. Dunford, H. B. (1999) *Heme Peroxidases*, Wiley-VCH, New York.
52. Colas, C., and Ortiz de Montellano, P. R. (2003) Autocatalytic Radical Reactions in Physiological Prosthetic Heme Modification, *Chem. Rev. 103*, 2305−2332.
53. Zheng, Y.-M., Baer, B. R., Kneller, M. B., Henne, K. R., Kunze, K. L., and Rettie, A. E. (2003) Covalent Heme Binding to CYP4B1 via Glu310 and a Carbocation Porphyrin Intermediate, *Biochemistry 42*, 4601−4606.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