Accelerated Publications

Identification of Novel Hemes Generated by Heme A Synthase: Evidence for Two Successive Monooxygenase Reactions†

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ABSTRACT: Heme A, an obligatory cofactor in eukaryotic cytochrome *c* oxidase, is produced from heme B (protoheme) via two enzymatic reactions catalyzed by heme O synthase and heme A synthase. Heme O synthase is responsible for the addition of a farnesyl moiety, while heme A synthase catalyzes the oxidation of a methyl substituent to an aldehyde. We have cloned the heme O synthase and heme A synthase genes from *Bacillus subtilis* (*ctaB* and *ctaA*) and overexpressed them in *Escherichia coli* to probe the oxidative mechanism of heme A synthase. Because *E. coli* does not naturally produce or utilize heme A, this strategy effectively decoupled heme A biosynthesis from the native electron transfer pathway and heme A transport, allowing us to observe two previously unidentified hemes. We utilized HPLC, UV/visible spectroscopy, and tandem mass spectrometry to identify these novel hemes as derivatives of heme O containing an alcohol or a carboxylate moiety at position C8 on pyrrole ring D. We interpret these derivatives to be the putative alcohol intermediate and an overoxidized byproduct of heme A synthase. Because we have shown that all hemes produced by heme A synthase require O_2 for their synthesis, we propose that heme A synthase catalyzes the oxidation of the C8 methyl to an aldehyde group via two discrete monooxygenase reactions.

Cytochrome *c* oxidase, an integral membrane and multicomponent complex, is the last enzyme in the energy-transducing, electron-transfer chain in all plants, animals, aerobic yeasts, and some bacteria (*1*, *8*−*11*). A critical component of aerobic metabolism, C*c*O,[1] catalyzes the reduction of O_2 to H_2O and harnesses the energy released from this reaction to translocate as many as four protons (eight charge equivalents) across the membrane.[2] Heme A is an obligatory cofactor in all eukaryotic and most prokaryotic C*c*Os, typically found at both an electron-transfer site and the unique copper-heme A heterobimetallic catalytic center (*12*−*18*). Interestingly, despite the importance of heme A to C*c*O and the electron transport pathway, relatively little is known about the mechanism of heme A biosynthesis.

The conversion of heme B (protoheme) to heme A requires two important modifications (Scheme 1). The first modification is the transformation of the C2 vinyl group on pyrrole ring A into a hydroxyethylfarnesyl moiety to generate heme O, while the second reaction involves the oxidation of the C8 methyl substituent on pyrrole ring D to an aldehyde. These two reactions are catalyzed by heme O synthase (HOS) and heme A synthase[3] (HAS), respectively (*12*, *19*−*25*). An intriguing question concerning the assembly of C*c*O is the mechanism by which HAS oxidizes heme O to heme A. The oxidation of a methyl group to a formyl moiety is an unusual transformation in biology, and the mechanism of this reaction is of interest to biochemists and chemists alike. A preliminary characterization of inactive bacterial HAS suggested the presence of equal quantities of heme A and heme B (although each was present in substoichiometric levels) (*26*, *27*). From these results it was proposed that heme A was unreleased product and that heme B was the site of O_2 activation. This observation led to speculation that HAS oxidizes heme O utilizing a P450-like mechanism, giving the unique situation of a heme cofactor acting on a heme substrate. However, there is currently no direct evidence to support these hypotheses (*26*, *28*).

We recently cloned the genes responsible for HOS and HAS activity in *Bacillus subtilis* (*ctaB* and *ctaA*, respectively)

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[1] Abreviations: C*c*O, cytochrome *c* oxidase; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ESI, electron spray ionization; HAS, heme A synthase; HOS, heme O synthase; HPLC, high-performance liquid chromatography; IPTG, isopropyl-*β*-D-1-thiogalactopyranoside; MS, mass spectrometry; TFA, trifluoroacetic acid.

[2] A deficiency in cytochrome *c* oxidase activity is the leading cause of respiratory chain defect in humans (*1*). Mutations in either C*c*O or any one of the numerous accessory proteins required for its assembly lead to a variety of multisystemic disorders, including hepatic failure, severe infantile myopathy, Leigh syndrome, and cardiomyopathy (*2*−*4*).

[3] HAS is sometimes referred to as heme O oxygenase in the literature. We will use the alternative nomenclature (i.e., heme A synthase) to avoid confusion with heme oxygenase (HO).

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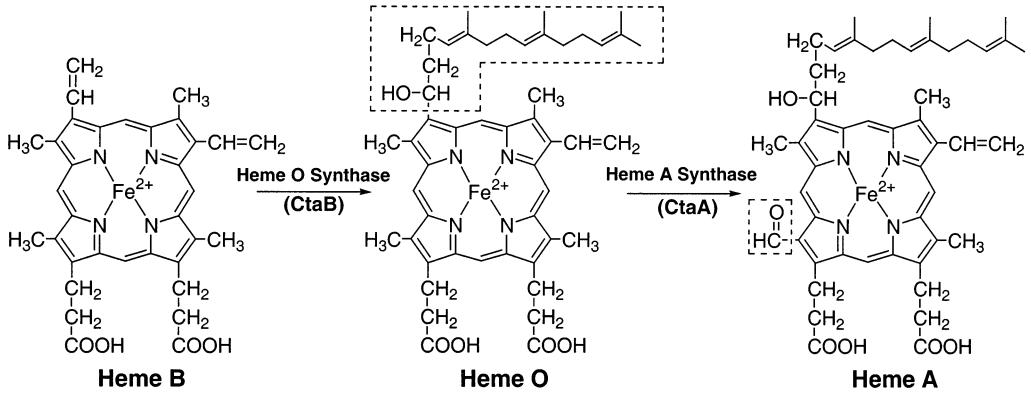
Scheme 1: Transformation of Heme B to Heme A Catalyzed by the Enzymes Heme O Synthase and Heme A Synthase[a]



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

and expressed them in *Escherichia coli*. In this manuscript we report the characterization and identification of two novel hemes isolated from *E. coli* overexpressing both CtaB and CtaA. On the basis of analysis by HPLC, UV/visible spectroscopy, tandem mass spectrometry, and comparison with chemically generated authentic samples, we demonstrated that these novel hemes are the C8 alcohol and the C8 carboxylate derivatives of heme O. The O_2-dependent formation of these novel hemes provides direct evidence that HAS utilizes two discrete monooxygenase steps to convert heme O to heme A in vivo.

EXPERIMENTAL SECTION

Materials. Restriction enzymes were purchased from New England BioLabs (Beverly, MA), and *Taq* polymerase was obtained from Invitrogen (Carlsbad, CA). The pET system of vectors was acquired from Novagen (Madison, WI), while pGem was purchased from Promega (Madison, WI). The pHolly vector was a kind gift from Professor Dennis Winge (University of Utah). The porphyrin standards Fe(III) deuteroporphyrin IX chloride and ferriprotoporphyrin IX chloride were obtained from Frontier Scientific Porphyrin Products (Logan, UT) and Alfa Aesar (Ward Hill, MA), respectively. All other chemicals were purchased from Fisher Scientific. Sequencing was performed on an ABI 377 sequencer at the University of Utah's DNA Sequencing Core Facility.

Cloning of ctaB. The *ctaB* gene from *B. subtilis* strain B168 was cloned as a 0.9 kb fragment from genomic DNA by PCR using forward primer 5′-CGCCAGGATCCATGGCTAACTCCAGAATCTTAAATG-3′ and reverse primer 5′-ATACGGTGGGAATTCCATATGTTAGAAAAGCGTCAAGACAACC-3′. The PCR fragment was TA cloned into pGEM and PCR amplified from pGEM using forward primer 5′-GAGAGGATCCGGGCATATGGCTAACTCCAGAAT-3′ and reverse primer 5′-AGAACTGCGAAAAGATTGGATCCAGAG-3′ for insertion into pHolly as a *Bam*HI/*Bam*HI fragment. The sequence was confirmed using two forward primers (5′-TAATACGACTCACTATAGG-3′ and 5′-ACAATGTGGACGAAACGCCGC-3′) and two reverse primers (5′-ATTTAGGTGACACTATAG-3′ and 5′-TGTGTTAATCGTATAGCGGCG-3′). This fragment was subcloned into pET-9a and pET-3a using *Nde*I/*Bam*HI or *Bam*HI/*Bam*HI to allow us to express CtaB with an N-terminal T7 epitope tag.

Cloning of ctaA. The *ctaA* gene from *B. subtilis* strain B168 was cloned as a 0.9 kb fragment from genomic DNA by PCR using forward primer 5′-GAGAGGATCCGGGGCCATGGGGATGAATAAAGCATTAAAGC-3′ and reverse primer 5′-ACGGTACCGAATTCGGATCCTTATGATTGCCTGGATTCAT-3′. The PCR fragment was cloned into pHolly as a *Bam*HI/*Bam*HI fragment and sequenced using two forward primers (5′-TAATACGACTCACTATAGG-3′ and 5′-GTCAGAACACTGGTTAAGCCG-3′) and two reverse primers (5′-ATTTAGGTGACACTATAG-3′ and 5′-CTTTTTGCCGATTTGAAGCGG-3′). This fragment was subcloned into pET-9d and pET-3d using *Nco*I/*Bam*HI. To obtain CtaA with an N-terminal T7 epitope tag, the gene was subcloned into pET-3a and pET-9a using *Bam*HI/*Bam*HI.

Transformation and Expression of ctaB and ctaA. Plasmids were transformed into *E. coli* BL21(DE3) cells containing pLysS using standard conditions. Two-liter flasks containing 1 L of LB media and 25 mg/L chloramphenicol, 30 mg/L kanamycin, and/or 50 mg/L ampicillin were innoculated with 10 mL overnight cultures. The cells were grown to an OD_{600} of approximately 0.6 and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 75 mg/L. Cells were harvested 1 h after induction by centrifugation, washed with 0.25 M sucrose, and stored as a frozen pellet at −80 °C.

Heme Extraction. Frozen cells were resuspended in 30 mM Tris buffer (pH 8.0) containing 10 mM EDTA and 20% sucrose (approximately 20 mL of buffer was used per L of cell culture) and disrupted via sonication. Hemes were extracted with an equal volume of 5% HCl/acetone (*29*, *30*) and clarified by centrifugation. The supernatant was analyzed by reverse-phase HPLC to determine total heme composition.

Cellular Heme Analysis. Hemes were separated and analyzed on a Waters HPLC system equipped with a 600 Delta Pack pump and a model 996 photodiode array detector using a modified literature procedure (*6*, *30*). The solvents (buffer A = 0.1% TFA/H_2O; buffer B = 0.1% TFA/CH_3-CN) were filtered through a 0.22 μm filter and degassed with He. The hemes were loaded at 0.5 mL/min onto a C18 Waters 3.0 mm × 150 mm YMC ODS-A column (5 μm 300 Å) in 25% buffer B and resolved using a 1%/min gradient from 55% to 75% buffer B. Iron(III) deuteroporphyrin IX chloride was used as a standard. The isolated hemes were analyzed by UV/visible spectroscopy and ESI/MS. The identity of heme B, heme O, and heme A were confirmed by comparison with authentic samples. (Authentic samples of heme O were obtained from *E. coli* cells grown

under oxygenating conditions to stimulate the production of the bo_3-type oxidase. Authentic samples of heme A were isolated from bovine heart as described below.)

Measuring O_2 Dependence. A one-liter Schlenk flask containing 500 mL of anaerobic LB media with 25 mg/L chloramphenicol, 30 mg/L kanamycin, and 50 mg/L ampicillin was innoculated with a 5 mL overnight culture. The cells were grown anaerobically (using standard Schlenk line techniques) at 37 °C with stirring to an OD_{600} of approximately 0.15 (~6 h) and induced with IPTG to a final concentration of 75 mg/L. At 60 min after induction, the medium was sparged with air. As a control, cells were also grown either anaerobically or under a constant sparge of air until 2 h after induction. In all cases, 80 mL aliquots were removed (t = 0/induction, 30, 60, 70, 90, and 120 min) and lysed by cannula transfer directly into 80 mL of 5% HCl/acetone. Cells grown under "reduced oxygen" conditions were grown in a two-liter shake-flask under standard conditions except with a constant N_2 sparge. Hemes were concentrated on a 3 mL, C18 Waters Sep-Pak and eluted in 2 mL of DMSO. The DMSO samples were diluted with 2 mL of 75% H_2O/25% CH_3CN and analyzed via HPLC.

Heme A Isolation. Isolation of bulk quantities of heme A was accomplished using a procedure modified from Tuppy et al. (*31*). A 2 kg beef heart was cleaned of excess fat, cut into small pieces, and homogenized in 1 L of H_2O with a Waring blender for 3 min at 4 °C. An equal volume of 5% HCl/acetone was added and stirred for 3 h at 4 °C. The slurry was centrifuged at 5000*g* for 7 min and filtered with Whatman filter paper. The filtrate was extracted with diethyl ether until all the heme was removed (approximately 4 L). Acetonitrile containing 0.1% TFA was added to the solution (10 mL per 500 mL of diethyl ether); the ether was then removed via rotoevaporation. The residual CH_3CN was loaded at 40 mL/min onto a C18 Waters 600 Delta Pack 25 mm × 300 mm column (15 μm 300 Å) at 25% buffer B. The hemes were resolved using a 0.33%/min gradient from 40% to 80% buffer B. The heme A peak was collected and stored at 4 °C. Heme A was analyzed via UV/visible spectroscopy, ESI/MS, and ^{1}H NMR (11.58 (s, −C*H*O, 1H), 11.02 (s, bridgehead methine, 1H) 10.63 (s, bridgehead methine, 1H), 10.44 (s, bridgehead methine, 1H), 9.97 (s, bridgehead methine, 1H) in 10% D_2O and 90% pyridine-d_5 containing excess $Na_2S_2O_4$). (*32*) No degradation of heme A was observed over six months.

Chemical Oxidation of Heme A. The aldehyde on the C8 position of heme A was oxidized to a carboxylate using a modified Tollen's reagent (200 mM NaOH, 900 mM NH_4-OH, and 300 mM $AgNO_3$ in 40% pyridine). In short, heme A (in 60% CH_3CN/40% H_2O/0.1% TFA) was mixed with an equal volume of Tollen's reagent and heated overnight at 50−60 °C. The samples were centrifuged for 2 min at 17 200*g* to remove precipitate and neutralized with HCl. The oxidized product was purified by reverse-phase HPLC and analyzed via UV/visible spectroscopy, ESI/MS, and ^{1}H NMR (11.53 (s, bridgehead methine, 1H), 10.69 (s, bridgehead methine, 1H), 10.58 (s, bridgehead methine, 1H), 10.05 (s, bridgehead methine, 1H) in 10% D_2O and 90% pyridine-d_5 containing excess $Na_2S_2O_4$).

Chemical Reduction of Heme A. The aldehyde on the C8 position of heme A was reduced to an alcohol using a procedure similar to that described by Vanderkooi et al. (*33*). Heme A (in 60% CH_3CN/40% H_2O/0.1% TFA) was mixed with an equal volume of 50 mM NaOH in 20% pyridine) and deoxygenated by sparging with N_2. An excess quantity of dithionite and $NaBH_4$ was added, and the solution was allowed to stand for 20 min under N_2. The solution was then acidified with HCl and the reduced product purified by reverse-phase HPLC. Analysis of the reduced heme was accomplished by UV/visible spectroscopy, ESI/MS, and ^{1}H NMR (10.74 (s, bridgehead methine, 1H), 10.42 (s, bridgehead methine, 1H), 10.38 (s, bridgehead methine, 1H), 10.12 (s, bridgehead methine, 1H) in 10% D_2O and 90% pyridine-d_5 containing excess $Na_2S_2O_4$).

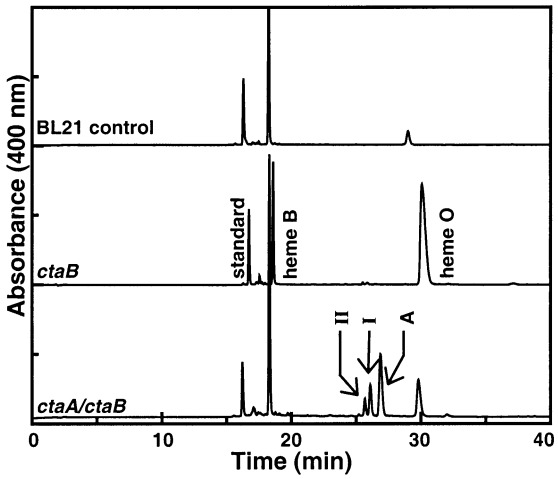


FIGURE 1: HPLC chromatograms of hemes isolated from *E. coli*. Shown are control cells (top), cells expressing *ctaB* (middle), or cells expressing both *ctaB* and *ctaA* (bottom).

Tandem Mass Spectrometry. HPLC purified heme samples were analyzed by electrospray ionization on a Micromass Quatro II mass spectrometry equipped with a Zspray API source. Ion fragments were isolated in the ion trap and successively re-fragmented for up to six cycles. The raw data can be viewed in the Supporting Information.

NMR Spectroscopy. ^{1}H NMR spectra were collected on a Varian Inova 500 instrument using a 5 mm indirect detection, pulse-field-gradient probe. In all cases the hemes were reduced with excess $Na_2S_2O_4$ and the spectra collected in 10% D_2O and 90% pyridine-d_5.

RESULTS

The genes responsible for HOS and HAS activity (*ctaB* and *ctaA*) were cloned from the bacterium *B. subtilis* into a variety of standard expression vectors and expressed in *E. coli* BL21 cells. Following induction, the gene products of both *ctaB* and *ctaA* (CtaB/HOS and CtaA/HAS) were observed by Western blot analysis using a T7 tag, indicating that HOS and HAS were being expressed both singly and in combination. As expected, both CtaB and CtaA localized to the cell membrane.

The activity of CtaB and CtaA was verified by analyzing the cellular heme content after induction. Hemes were extracted using an acid/acetone mixture followed by reverse-phase HPLC analysis as described in the Experimental section (Figure 1). Control BL21 cells (top) produced mostly heme B and a slight amount of a more hydrophobic species,

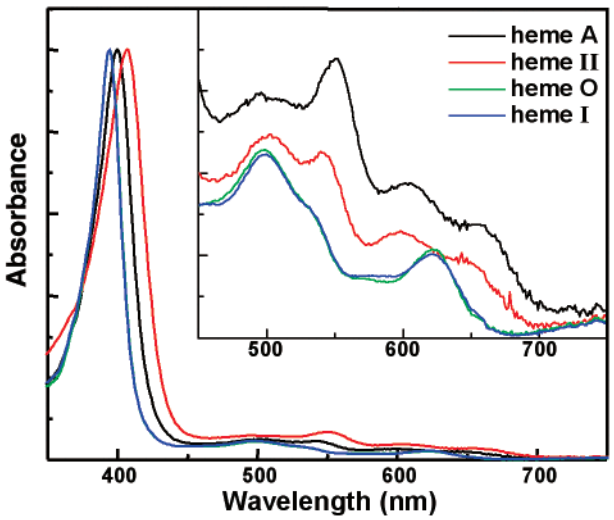


FIGURE 2: Optical spectra of four hemes isolated from *E. coli* expressing both *ctaA* and *ctaB*. Absorbance units have been normalized to the Soret peak intensity.

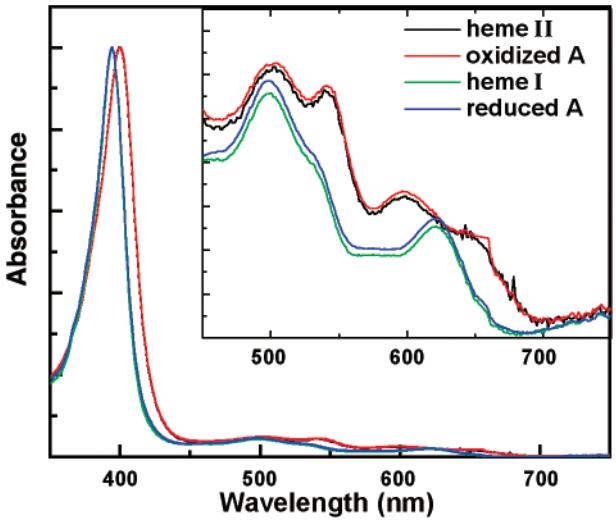


FIGURE 3: A comparison of the optical spectra of heme I, heme II, chemically oxidized heme A, and chemically reduced heme A. Absorbance units have been normalized to the Soret peak intensity.

Table 1: Wavelength of Absorbance Maxima of Hemes in HPLC Elution Solvent[a]

	heme B	heme O	heme I	heme A	heme II
Soret	398	394	394	406	400
α/β bands	501	494	494	494	496
	626	621	621	551	542
				604	598
shoulder	535	532	532	655	655

[a] Heme B was obtained in HPLC buffers at approximately 40:60 (A:B), and heme I, heme II, heme A, and heme O were acquired in approximately 25:75 (A:B).

identified as heme O.[4] When cells contained the plasmid pET-3a:*ctaB*, however, over 70% of the total heme content was converted to heme O. As expected, cells coexpressing *ctaA* and *ctaB* (pET-3a:*ctaB* and pET-9d:*ctaA*) were found to contain heme O and heme A (bottom), verifying that both CtaB and CtaA are active when overexpressed in *E. coli*.

In addition to heme B, heme O, and heme A, cells coexpressing *ctaA* and *ctaB* also contained two additional hemes (denoted heme I and heme II). These hemes were not observed in cells lacking CtaA, indicating that heme I and heme II are due to the presence of HAS. The optical spectra of these unknown hemes (Figure 2) show the expected characteristics, a Soret peak near 400 nm and various local absorbance maxima between 475 and 700 nm (Table 1). Interestingly, the optical spectrum of heme I is nearly identical to that of heme O, while the spectrum of heme II is very similar to heme A, suggesting that heme I is related to heme O and that heme II is related to heme A. Mass spectrometry revealed that heme I has a mass of 854 amu (heme O + 16 amu) and that heme II has a mass of 868 amu (heme A + 16 amu). Together, these results indicate that heme I and heme II are generated by the addition of a single oxygen atom to heme O and heme A, respectively.

To determine the exact location of the extra oxygen atom, hemes I and II were further analyzed by tandem mass spectrometry in which ion fragments were isolated and successively refragmented for up to six cycles. These results were then compared with the fragments obtained from heme O and heme A (Table 2). Following fragmentation of the parent ion, fragments with the following masses were sequentially removed from all hemes: 248, 59, and 59 amu. These masses are consistent with the sequential loss of the 17-hydroxyethylfarnesyl tail and two acetate ions from the propionates (Scheme 2).[5] The mass of the next fragment that was lost, however, depended on the heme being analyzed. For instance, fragment "X" in heme O was 15 amu (consistent with the loss of a methyl substituent), while heme A lost 28 amu (suggesting the loss of the aldehyde). In the case of heme I and heme II, fragment "X" was 29 and 44 amu, respectively. Significantly, the final mass that was observed after the loss of fragment "X" was nearly identical for all four hemes (Table 2), indicating that they had lost the fragment that differentiated them from one another. These data strongly suggest that the extra oxygen atom is located on C8, i.e., the carbon that is oxidized to the aldehyde in heme A.

On the basis of the results described above, one possibility is that heme I is an alcohol derivative of heme O while heme II is a carboxylate derivative of heme O. Such a model would explain the mass spectral data, indicating that heme I and heme II contain an additional oxygen atom at C8 relative to heme O and heme A, and it would be consistent with the optical data demonstrating that heme I is electronically similar to heme O and that heme II is electronically similar to heme A. (Like heme A, a carboxylate derivative of heme O would contain an electron-withdrawing sp^2-hyridized carbon at position C8, while heme O and the alcohol derivative would not.) To test this hypothesis, the alcohol and carboxylate derivatives of heme O were chemically generated via the reduction and oxidation of heme A for comparison to heme I and heme II. The optical spectra of the chemically generated species exactly match heme I and heme II (Figure 3), as do the HPLC retention times (data

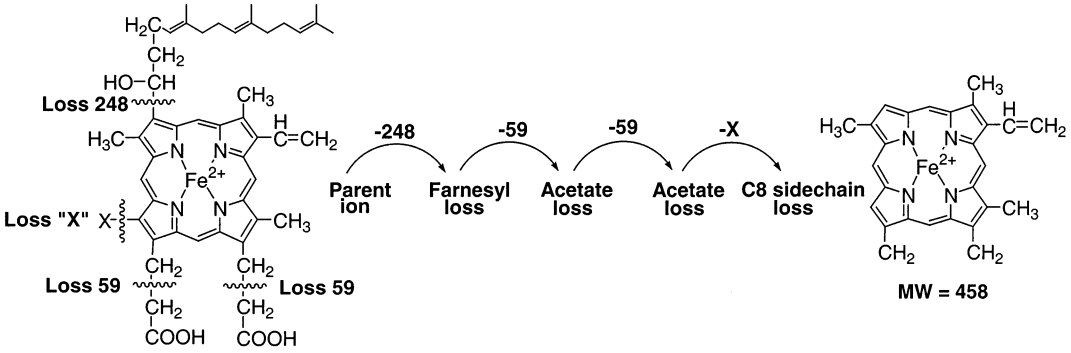
[4] The identity of heme B, heme O, and heme A were confirmed by comparison with authentic samples as described in the Experimental section.

[5] Loss of an acetate group has been observed previously in the analysis of various hemes and is a common segment to lose in mass spectrometry (*5*−*7*).

Table 2: Assignment of Fragments Lost in Tandem Mass Spectral Analysis of Hemes

	heme O	heme A	heme I	chemically reduced	heme II	chemically oxidized
parent ion	838	852	854	854	868	868
−248 farnesyl	590	604	606	606	620	620
−59 acetate	531	545	547	547	561	561
−59 acetate	472	486	488	488	502	502
−X variable	457	458	459	459	458	458
−15 methyl	442	443	445	445	443	443

Scheme 2: Sequential Loss of Heme Fragments by Tandem Mass Spectrometry



not shown). Significantly, the fragments obtained from tandem mass spectrometry also match heme I and heme II (Table 2), as do the relative peak intensities (Supporting Information), confirming the hypothesis that heme I and heme II are the alcohol and carboxylate derivatives of heme O.

To ascertain if O_2 is an important reactant in the formation of heme A, heme I, and heme II, *E. coli* cells coexpressing *ctaA* and *ctaB* (pET-3a:*ctaB* and pET-9d:*ctaA*) were grown under both reduced oxygen and anaerobic conditions. Induction under low O_2 concentrations resulted in relatively low levels of heme A and the alcohol derivative (Figure 4a). Under these same conditions, no carboxylate derivative was observed. Addition of O_2 by sparging air into the cell culture led to a rapid drop in the quantity of the alcohol derivative as it was converted into more highly oxidized species. Concomitantly, the amount of heme A and the overoxidized carboxylate byproduct both rose. When cells were grown under nearly anaerobic conditions (Figure 4b), no heme A or heme II was observed until the addition of air at 60 min following induction. Interestingly, under these conditions more heme II than heme A is produced. While there is a slight quantity of the alcohol derivative (heme I) prior to the addition of air, presumably due to O_2 contamination, it is approximately 3 times lower than that found in cells grown under reduced oxygen conditions (Figure 4a) or cells grown under a constant sparge of air (data not shown). In addition, the fact that cells grown anaerobically are able to produce heme A within 10 min after exposure to air (Figure 5) demonstrates that the cells are viable and that CtaA is active. (In all cases, the results have been normalized to take into account the differences in cell densities under the different growth conditions.) These results demonstrate that the formation of heme A, the alcohol derivative, and the carboxylate derivative all require O_2, and they support the notion that the alcohol derivative is an intermediate in the conversion of heme O to heme A.

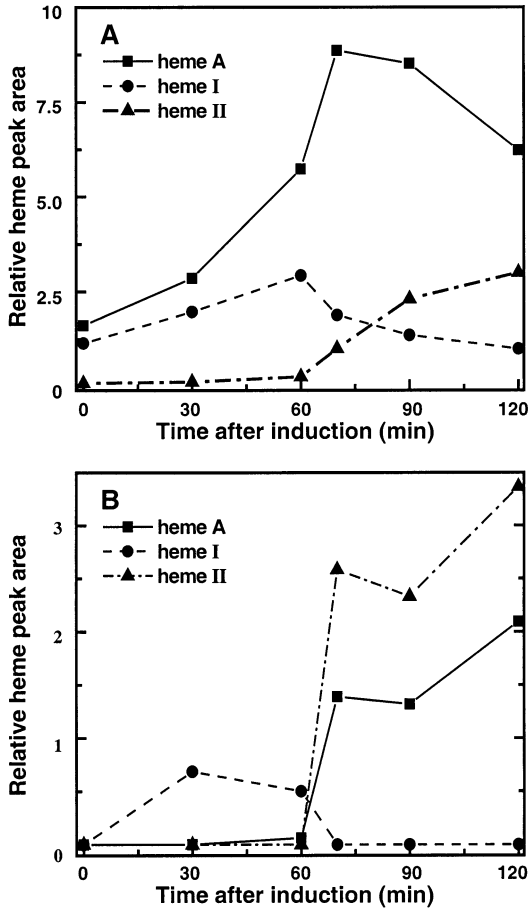


FIGURE 4: Relative heme production under reduced O_2 (A) and nearly anaerobic (B) growth conditions. Both cultures were sparged with air at 60 min.

DISCUSSION

How might a heme-containing enzyme catalyze the oxidation of heme O to heme A? One obvious possibility (Scheme 3) is that HAS may utilize two successive monooxygenase

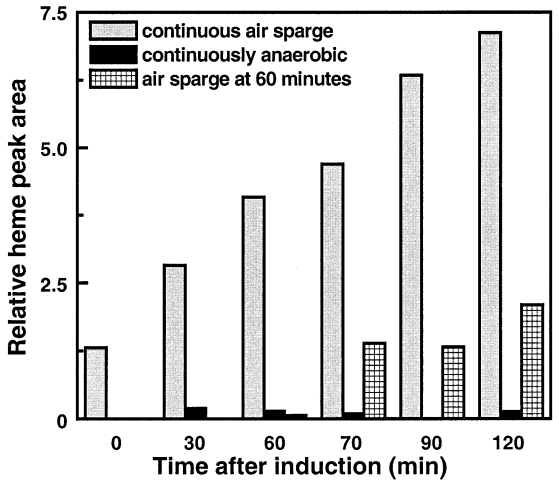
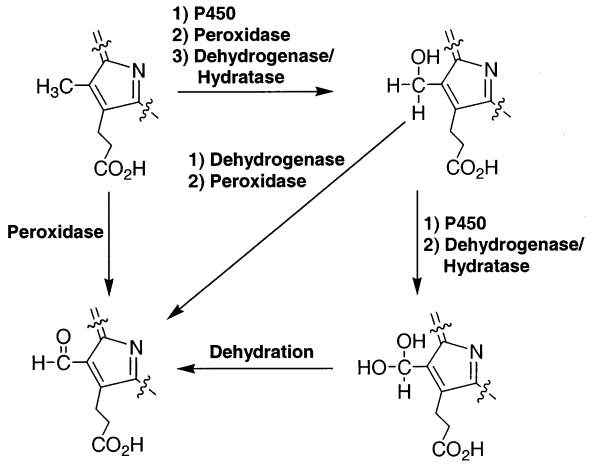


FIGURE 5: Effect of O_2 on heme A production. Cells were grown under continuous air sparge (grey), continuously anaerobic (black), or anaerobically until 60 min after induction and then continuously sparged with air (checked). Data have been corrected to account for differences in cell density.

Scheme 3: Possible Mechanisms for the Oxidation of Heme O to Heme A



reactions to generate a geminal diol (which could then spontaneously dehydrate). Literature precedent for a P450-type mechanism is afforded by aromatase (*34*−*36*) and cytochrome $P450_{LTB\omega}$ (*37*, *38*), both of which are proposed to utilize consecutive monooxygenase steps at a single carbon in their reaction mechanism.[6] Furthermore, $P450_{cam}$ has been used to catalyze the oxidation of 2-ethyl hexanol to 2-ethyl-hexanoic acid in a "benign" chemical synthesis (*39*). Another possible mechanism by which heme O may be converted to heme A involves a single monooxygenase reaction to generate the alcohol intermediate followed by a dehydrogenase step, analogous to the mechanism proposed for the conversion of chlorophyll *a* to chlorophyll *b* (*40*).[7] These are but two possibilities, and other, perhaps less likely, alternatives can easily be envisioned (Scheme 3). For example, a peroxidase-type mechanism can be drawn to generate an aldehyde directly from a methyl group, or a peroxidase could oxidize an alcohol intermediate, as exemplified by the nonproductive oxidation of veratryl alcohol to veratryl aldehyde by lignin peroxidase (*41*−*43*). Finally, a series of dehydrogenase/hydratase mechanisms can also be imagined (*40*). Currently, there is no direct evidence to support any of these possible mechanisms in the biosynthesis of heme A.

To probe the mechanism of heme A biosynthesis, heme O synthase (HOS) and heme A synthase (HAS) from *B. subtilis* have been overexpressed in *E. coli*. Because *E. coli* do not naturally contain heme A (*44*), any heme A that is observed must be due to the activity of CtaA. Heme analysis of these cells revealed the expected hemes B, O, and A (Figure 1). Interestingly, two additional and previously unidentified hemes were also present, though they appear to be present in genetically modified yeast as well (*45*). Significantly, these two hemes were not observed in either control cells or cells overexpressing only *ctaB*, demonstrating that the appearance of these new hemes must be due to the presence of CtaA.[8] In addition, we have also identified O_2 as a key reactant in the formation of heme I, the conversion of heme I to heme A, and the conversion of heme A to heme II.

The structures of heme I and heme II were probed using a variety of techniques, including HPLC, UV/visible spectroscopy, and tandem mass spectrometry. The mass spectral data revealed that heme I and heme II contain an additional oxygen atom relative to heme O and heme A, respectively, and that this extra oxygen atom is located on carbon 8 of pyrrole ring D. One intriguing possibility is that heme I is the putative alcohol intermediate (Scheme 3) while heme II is an overoxidized carboxylate byproduct. This interpretation would be consistent with the optical data which demonstrated that heme I is electronically similar to heme O while heme II resembles heme A. Unfortunately, the mass of fragment "X" that was lost during tandem mass spectrometry from heme A (28 amu), heme II (29 amu), and heme I (44 amu) did not exactly match $-C(O)H$, $-CH_2OH$, or $-CO_2H$; the fragments lost were 1, 2, and 1 amu too low, respectively. To address these issues, the putative alcohol and carboxylate derivatives of heme O were generated chemically from the reduction and oxidation of HPLC purified heme A. The chemically generated species were found to match heme I and heme II in all aspects, confirming that heme I is the putative alcohol intermediate and that heme II is an over-oxidized byproduct of heme A synthase.[9]

[6] The biosynthesis of estrogens from androgens requires the removal of the 14α-methyl group and the aromatization of the A-ring in a reaction catalyzed by the cytochrome P450 aromatase. The first two steps of this reaction involve successive monooxygenase reactions to give the aldehyde intermediate. The final oxidation step involves C−C bond cleavage to generate formate concomitant with the aromatization of the A-ring. Cytochrome $P450_{LTB\omega}$ oxidizes leukotriene B_4 (LTB_4) via three successive steps to 20-COOH-LTB_4, presumably as a way to mediate the activity of this potent neutrophil chemoattractant.

[7] The conversion of chlorophyll *a* to chlorophyll *b* in higher plants requires the oxidation of the methyl substituent on ring B to a formyl group, identical to the reaction required in the biosynthesis of heme A. For an alternative proposed mechanism, please see ref *49*.

[8] Heme I is also observed in cells only expressing *ctaA*, providing additional evidence that the formation of heme I is due to CtaA (data not shown). While heme II is not observed in these cells, this is presumably due to the very low levels of oxidized products in the absence of a readily available supply of the substrate heme O.

[9] Cell lysate from nontransformed *E. coli* cells were incubated separately with heme I and heme A to determine if either can be further oxidized in the absence of CtaA by a bacterial enzyme or during heme extraction. No production of heme A or heme II was observed under these conditions.

The identification of heme I as the putative alcohol intermediate and heme II as an overoxidized carboxylate byproduct has important mechanistic implications. Obviously the ability to trap the alcohol intermediate precludes any proposed mechanism in which heme A is formed directly from heme O. (Although multiple pathways could exist, this is uncommon in key biosynthetic pathways.) Second, a proposed mechanism must also account for the further oxidation of heme A to the carboxylate. And finally, because heme I, heme A, and heme II formation requires dioxygen, each step ($-CH_3 \rightarrow -CH_2OH \rightarrow -C(O)H \rightarrow -CO_2H$) in the proposed mechanism must utilize O_2. Given these constraints, the most likely pathway is a series of P450-like monooxygenase reactions (Scheme 3). The first step produces the alcohol intermediate (heme I), while the second step forms the corresponding geminal diol. Spontaneous dehydration of the diol leads to heme A, while the overoxidized carboxylate byproduct (heme II) arises from a third reaction cycle. Our results therefore provide compelling evidence that CtaA oxidizes heme O to heme A via successive monooxygenase reactions.[9]

The theory that HAS is a P450-like monooxygenase is also supported by recent data from Hederstedt and Tzagoloff. As mentioned previously, Hederstedt and co-workers observed equimolar (albeit substoichiometric) quantities of heme B and heme A in inactive CtaA (*26*). In addition to a heme cofactor, P450-like monooxygenases also require an external electron source to supply additional electrons to complete the reaction cycle. Tzagoloff and co-workers have determined that Cox15p (the *S. cerevisiae* ortholog to CtaA) requires a mitochondrial ferredoxin for activity (*19, 46*). Because CtaA and Cox15p exhibit functional homology, it is reasonable to infer that Cox15p is a heme-containing monooxygenase and that CtaA requires an external electron source. The existence of heme A in *E. coli* overexpressing CtaA clearly suggests that a native *E. coli* protein is able to function as a CtaA reductase in vivo. In this heterologous system, intermolecular electron transfer may be limiting under most growth conditions, allowing us to isolate a product that has undergone only a single monooxygenase step. When cells are grown under near anaerobic conditions, however, the alcohol intermediate fails to accumulate to substantial levels (Figure 4b) as O_2 becomes the limiting reagent. Upon the addition of air to near anaerobic cells, heme A and the carboxylate derivative appear concomitant with the rapid disappearance of the alcohol intermediate. We interpret the failure of the alcohol intermediate to accumulate either under these conditions or in natural systems as an indication that the second oxidation event (i.e., heme I to heme A) is kinetically favored to the oxidation of heme O to heme I.

Another intriguing aspect of HAS is the nature of the axial ligand. While heme-containing monooxygenases typically utilize cysteine as the axial ligand, preliminary spectroscopic studies suggest that both the unreleased product heme A and the active site heme B in CtaA are axially ligated by histidines (*26*), a notion supported by the lack of a conserved Cys residue between bacterial and eukaryotic HAS. To our knowledge, secondary amine monooxygenase is the only other heme-containing monooxygenase reported to utilize His instead of Cys axial ligation (*47*, *48*). Thus, CtaA provides an ideal opportunity to address the role of the axial heme ligand.

CONCLUSION

Heme O synthase (HOS) and heme A synthase (HAS) from *B. subtilis* have been overexpressed in *E. coli* BL21 cells. Heme analysis of cells overexpressing both CtaA and CtaB revealed that in addition to heme B, heme O, and heme A, these cells also produce two previously unidentified hemes (Figure 1). We have unambiguously identified these unknown hemes as the C8 alcohol and C8 carboxylate derivatives of heme O. These derivatives were not observed in either untransformed cells or cells overexpressing only CtaB, demonstrating that the formation of these new hemes is due to CtaA. We have also identified O_2 as a key reactant in the production of heme A and both heme O derivatives. Furthermore, our data demonstrate that the alcohol derivative, which is not observed under natural conditions, is an intermediate in the formation of heme A. These results provide the first direct evidence that CtaA utilizes two successive monooxygenase reactions in the conversion of heme O to heme A and illustrate the potential advantage of using heterologous systems to uncouple complex reaction processes.

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SUPPORTING INFORMATION AVAILABLE

Figures S1−S7 provide the raw data obtained from heme analysis via tandem mass spectrometry for heme B (S1), heme A (S2), the carboxylate derivative generated chemically (S3), the carboxylate derivative generated in vivo, i.e., heme II (S4), heme O (S5), the alcohol derivative generated in vivo, heme I (S6), and the alcohol derivative generated chemically (S7). Figure S8 illustrates the conversion of heme O to oxidized products under conditions of reduced O_2 and the chromatograms used to generate Figure 4a. This material is available free of charge via the Internet at http://pubs.acs.org.

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