Heme Degradation as Catalyzed by a Recombinant Bacterial Heme Oxygenase (Hmu O) from Corynebacterium diphtheriae*

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Hmu O, a heme degradation enzyme in the pathogen Corynebacterium diphtheriae, catalyzes the oxygen-dependent conversion of heme to biliverdin, carbon monoxide, and free iron. A bacterial expression system using a synthetic gene coding for the 215-amino acid, full-length Hmu O has been constructed. Expressed at very high levels in Escherichia coli BL21, the enzyme binds heme stoichiometrically to form a ferric complex. When ascorbic acid is used as the electron donor, Hmu O converts heme to biliverdin with α-hydroxymel and verdoheme as intermediates. The overall conversion rate to biliverdin is approximately 4-fold slower than that by rat heme oxygenase (HO) isofrom 1. Reaction of the hemin-Hmu O complex with hydrogen peroxide yields a verdoheme species, the recovery of which is much less compared with rat HO-1. Reaction of the hemin complex with meta-chloroperbenzoic acid generates a ferryl oxi species. Thus, the catalytically intermediate species and the nature of the active form in the first oxygenation step of Hmu O appear to be similar to those of the mammalian HO. However, the considerably slow catalytic rate and low level of verdoheme recovery in the hydrogen peroxide reaction suggest that the active-site structure of Hmu O is different from that of its mammalian counterpart.

Heme oxygenase (HO),¹ first characterized in eukaryotes, is an enzyme that catalyzes the regiospecific oxidative degradation of iron protoporphyrin IX to biliverdin IXα, iron, and CO in the presence of NADPH and NADPH-cytochrome P-450 reductase, which serves as an electron donor (1–5). Not a hemoprotein per se, HO binds heme at a 1:1 ratio and utilizes it as both a substrate and a prosthetic group (2–6).

Among the well-characterized isoforms of the mammalian HO are HO-1, which is 33 kDa, inducible, and highly expressed in the spleen and liver, and HO-2, which is 36 kDa, constitutive, and found primarily in the brain and testes (2). Both isoforms function similarly in their catalytic cycle (5). HO first binds heme stoichiometrically to form a hemin-HO complex. Then, an electron donated from NADPH-cytochrome P-450 reductase reduces the heme iron to the ferrous state (7). This allows dioxygen to bind to the ferrous iron, forming a metastable oxo complex (8). Additional electron donation to this oxo complex initiates the conversion of the heme to biliverdin IXα with α-meta-hydroxyhem and verdoheme as intermediates (Scheme 1) (3–5). The CO released during HO catalysis has been reported to act as a messenger molecule, participating in neuronal transmission and vascular regulation through the activation of soluble guanylyl cyclase (9–11). The product biliverdin is subsequently converted to bilirubin by biliverdin reductase (2, 12–13). The significant outcome of this is the removal of excess heme via the excretion of bilirubin and the recycling of iron (14).

Iron is required for the survival of most bacteria and is particularly essential for pathogens to cause diseases (15–17). To circumvent the low concentration of free extracellular iron, pathogenic bacteria have developed sophisticated systems to acquire iron from iron-containing proteins found in their hosts (17–20). One mechanism is via a bacterial heme degradation enzyme (18, 21). In contrast to the mammalian HO, whose primary purpose is to maintain iron homeostasis, that of the bacterial HO is to release iron from heme so that the iron may be utilized. Within the last few years, through genetic studies, the presence of heme-degrading enzymes has been identified in pathogenic bacteria (17, 21–22).

Hmu O, the first and only prokaryotic HO isolated to date, is expressed by the hmu O gene found in Corynebacterium diphtheriae, the causative agent of diphtheria. In comparison to the mammalian HO, Hmu O is not membrane-bound but soluble and has a smaller molecular mass of 24 kDa. It is 33% identical in sequence to the first 221 amino acids of human HO-1 (22). This enzyme has been proposed to be utilized by C. diphtheriae to release iron from the heme supplied by the pathogen’s host. Using an Escherichia coli expression system, Wilks and Schmitt (23) have purified a 24-kDa Hmu O protein that stoichiometrically binds heme and converts it to biliverdin IXα and CO upon the aerobic addition of electron donors. However, the oxygen activation mechanisms and the catalytic pathway of Hmu O are not clear. Whether or not they are similar to the mammalian system remains to be established.

In this study, we have constructed a highly efficient bacterial expression system of Hmu O using a synthetic gene based on the hmu O gene sequence (22). We have examined the heme degradation reaction catalyzed by Hmu O using the hemin-, α-hydroxymel-, and verdoheme-complexes of the purified recombinant Hmu O. Reactions of the hemin-Hmu O complex with H2O2 and mCPBA have also been studied. We have found that the nature of the active form in the first oxygenation step and the catalytic intermediates of Hmu O are similar to those of the mammalian HO. However, the distal heme pocket structure of Hmu O appears to be different from that of its mammalian counterpart.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s)AB019621.

1 The abbreviations used are: HO, heme oxygenase; mCPBA, meta-chloroperbenzoic acid; PAGE, polyacrylamide gel electrophoresis; eq, equivalent(s).
A heme oxygenase Hmu O of pMWHmuO, the expression plasmid for the recombinant bacterial from pMW 172, was a gift from Dr. K. Nagai (MRC Laboratory of Molecular Biology, Cambridge, UK). A 48-base pair double-stranded synthetic oligonucleotide with unique restriction enzyme sites for XhoI, ClaI, and AvrII was incorporated between the Ndel and HindIII sites in the multiple cloning site of pMW 172 to make pMW A. Eight oligonucleotides and their complements, 82–91 nucleotides in length, were synthesized to prepare a 648-base pair synthetic gene coding for the entire Hmu O from the initiator ATG to the TAA stop codon. Each oligonucleotide was phosphorylated with a T4 polynucleotide kinase, then annealed with its sequences complementary to the 5′ ends of Oligos IV, VI, and VIII, and the resulting larger fragment of the digests was gel-purified. Using Tλ ligase, both Oligos I and II were ligated simultaneously to the Ndel and XhoI sites of the Ndel/XhoI fragment. The resulting plasmid, pMBW, was cleaved with XhoI and ClaI, and the large fragment was gel-purified. Both Oligos III and IV were ligated between the XhoI and ClaI sites of the larger XhoI/ClaI fragment, and pMWC was obtained. Excised by ClaI and AvrII, the larger ClaI/AvrII fragment from pMWC was gel-purified and ligated with Oligos V and VI simultaneously. The ligation of these fragments yielded pMWD, which then was cut with AvrII and HindIII. Oligos VII and VIII were ligated to the larger AvrII/HindIII fragment from pMWD. Ligation of Oligos VII and VIII resulted in the formation of pMWHmuO, the expression plasmid for the recombinant bacterial heme oxygenase Hmu O of C. diphtheriae. The nucleotide sequence was determined by an Applied Biosystems 373A DNA sequencer. Fig. 1 is a comparison of the coding sequence of the synthetic gene to that of the cloned DNA (22).

Protein Expression and Purification—A 10-ml inoculum in LB-Amp media of A500nm 1.0 was prepared with a fresh colony of E. coli BL21 (DE3) cells transformed with the expression construct pMWHmuO. 500-ml cultures were inoculated with 1 ml of the prepared inocula and grown in LB-Amp media at 37 °C until A500nm reached 1.3–1.4. The cells were grown for an additional 16 h at 25 °C before being harvested by centrifugation and stored at −80 °C until use. Typically, 2 g of cells were obtained from a 500-ml culture. Thawed on ice, the cell pellets were resuspended in 100 mM Tris buffer, pH 8, containing 100 mM NaCl and 1 mM EDTA. Lysozyme (2 mg/g cells) and phenylmethylsulfonyl fluoride of final concentration 1 mM were added to the resuspension, which was stirred continuously at 4 °C for 2 h. The cells were then sonicated (Branson 450 Sonifier) until no longer viscous and centrifuged at 39,000 × g for 1 h. The resulting supernatant was recovered for further purification.

Solid ammonium sulfate was added to the supernatant to a concentration of 60% saturation, and the solution was stirred for 30 min at 4 °C. After centrifugation at 14,000 × g for 30 min, the supernatant was increased to 85% ammonium sulfate saturation and centrifuged. The subsequent precipitates, which contained the Hmu O protein, were dissolved in 20 mM phosphate buffer, pH 7. The dissolved solution was gel-filtered through a Sephadex G75 column, which had been equilibrated with 20 mM phosphate buffer, pH 7. Fractions with A280 nm greater than 0.35 were pooled and loaded onto a column of DEAE-cellulose equilibrated with 20 mM phosphate buffer, pH 7. Eluted in 20 mM phosphate buffer, pH 7, with a linear gradient of 0 to 0.4 M KCl, fractions with A280 nm values greater than 0.3 were pooled and concentrated by ultrafiltration. The enzyme was stored at 77 K until use.

Reconstitution of Hmu O with Hemin—250 μM hemin in 5-μl increments was added to 10 μl of Hmu O in 800 μl of 0.1 M phosphate buffer, pH 7, at 20 °C. After each addition of hemin, the absorption spectrum was recorded. By plotting the absorbance at 404 nm against the amount of hemin added, titration curves were constructed, and the amount of hemin required to form the hemin-Hmu O complex was determined. To ensure that all Hmu O were hemin-bound, hemin of concentration twice that of Hmu O was added to the purified protein solution. After incubation at 4 °C, the excess hemin was removed by a DEAE-cellulose column. Fractions with A280 nm/A250 nm greater than or equal to 3 were collected, concentrated by ultrafiltration, and stored at 77 K until use.

Formation of the a-meso-Hydroxyhemin- and Verdoheheme-Hmu O Complexes—Verdoheheme (protoverdoheheme-IIXα) was synthesized as described by Saito and Itano (25). a-Meso-hydroxyhemin (a-meso-hydroxyprotophohem) was made by hydrolyzing a-meso benzoyloxyprotophohem as described previously (26). The ferric a-meso-hydroxyheme- and ferrous verdoheheme-Hmu O complexes were prepared as described by Mansfield Matera et al. (26) and Fujii (27), respectively.

Reaction of the Hemin-Hmu O Complex with Ascorbic Acid—Heme degradation catalyzed by Hmu O was studied using ascorbic acid as the electron donor (12). Ascorbic acid of final concentration 17.5 mM was added to an optical cuvette containing 10 μM hemin-Hmu O in 2.3 ml of 0.1 M phosphate buffer, pH 7, at 20 °C. Spectral changes between 300–800 nm were recorded until the reaction was complete, as indicated by the maximum loss of the Soret band (A404 nm) and the formation of biliverdin.

For experiments performed with CO, the hemin-Hmu O complex was prepared as described above but injected into a sealed cuvette filled with phosphate buffer that had been presaturated with 50% CO and 50% O2. Ascorbic acid of final concentration 17.5 mM was added to induce hemin degradation. The formation of the verdoheheme-CO complex was monitored spectrophotometrically. When there was no further change in the spectrum, pyridine (20% final concentration) was added to the mixture (28), and the formation of the pyridine-verdoheheme complex was recorded.

In other experiments, after the reaction had been arrested at the verdoheheme-CO stage, the verdoheheme that had accumulated in the presence of CO was allowed to continue to the fully oxidized product by displacing the CO with 100% O2.

Reaction of the Heme-Hmu O Complex with H2O2 and mCPBA—Preparation for the assays of the hemin-Hmu O complex with H2O2 and mCPBA was similar to that described previously (29). Reagent grade H2O2 was added to separate solutions containing the hemin-Hmu O complex in 0.1 mM phosphate buffer, pH 7, to yield final H2O2:hemin-Hmu O ratios of 1:1, 2:1, 3:1, and 5:1.

Analytical Methods—Protein expression and purity were analyzed by SDSPAGE on 10–20% gradient gels. The isoelectric point of the purified Hmu O was determined by a Pharmacia FastSystem following the manufacturer’s instruction. Optical absorption spectra were recorded on a Hewlett-Packard 8453 spectrophotometer at 20 °C in 0.1 mM phosphate buffer, pH 7.
RESULTS AND DISCUSSION

Expression and Purification of Hmu O——Sligar and co-workers (31) have pioneered the use of synthetic genes for bacterial expression of hemoproteins. The advent of efficient and inexpensive synthesis has made it feasible to obtain longer (80–90-mer) oligonucleotides than those (20 mer) used earlier (31). In our approach here, using longer oligonucleotides has enabled us to ligate the eight double-stranded oligonucleotides (Oligos I to VIII) to the expression vector in minimal ligation steps. The ligation products generated during the plasmid preparation were conveniently purified by standard agarose gel electrophoresis, and the final expression plasmid was readily obtained. Furthermore, the synthetic gene has allowed for the incorporation of unique restriction enzyme sites convenient for cassette mutagenesis.

Using pMWHmuO, we have expressed successfully a recombinant Hmu O protein, as depicted in the SDS-PAGE (Fig. 2, lane 2). The initial expression of Hmu O by culturing the transformed BL21 cells at 37 °C resulted in an accumulation of the expressed protein mostly in inclusion bodies. Solubilization of the inclusion bodies, following the methods used for mammalian HO mutants (32), successfully yielded an active Hmu O. However, our current method of culturing the E. coli then 25 °C, instead of 37 °C continuously, has increased significantly the yield of the protein in the soluble form. Both the purified Hmu O in the soluble form and from inclusion bodies have the same enzymatic properties and optical absorption spectra. Our expression of Hmu O mostly in the soluble form has facilitated the purification process and yields 100–120 mg of purified Hmu O/liter of bacterial culture, 5-fold greater than that reported by Wilks and Schmitt (23). As indicated by SDS-PAGE, the purified Hmu O we obtain is homogeneous and has a single band at 24 kDa (Fig. 2, lane 3), the size as expected from the deduced Hmu O amino acid sequence (24.1 kDa) (22). The pI value estimated by isoelectricphoresis is 5.4.

We have observed that the transformed E. coli cultured at 37 °C are pale yellow, whereas those cultured at 37 °C then 25 °C are green. The former is because of the expressed protein have the same enzymatic properties and optical absorption spectra. Our expression of Hmu O mostly in the soluble form has facilitated the purification process and yields 100–120 mg of purified Hmu O/liter of bacterial culture, 5-fold greater than that reported by Wilks and Schmitt (23). As indicated by SDS-PAGE, the purified Hmu O we obtain is homogeneous and has a single band at 24 kDa (Fig. 2, lane 3), the size as expected from the deduced Hmu O amino acid sequence (24.1 kDa) (22). The pI value estimated by isoelectricphoresis is 5.4. We have observed that the transformed E. coli cultured at 37 °C are pale yellow, whereas those cultured at 37 °C then 25 °C are green. The former is because of the expressed protein

FIG. 1. Nucleotide and amino acid sequences of the synthesized pMWHmuO as compared with hmu O. The nucleotide sequence of the cloned hmu O is shown below the amino acid sequence of the synthetic Hmu O enzyme. The arabic numerals above the sequence indicate the numbering of the nucleotide and amino acid sequences.

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FIG. 2. SDS-PAGE analysis of the recombinant Hmu O protein. Lane 1, molecular mass markers; lane 2, Hmu O expressed in E. coli; lane 3, purified Hmu O.
being in mostly inclusion bodies, whereas the latter is in the soluble form. The green E. coli cells indicate that biliverdin has been formed from the heme degradation catalyzed by the expressed Hmu O and a reductase system in the E. coli, a phenomenon observed also for the E. coli expression of mammalian HO isoforms (29, 32–33). During the purification of Hmu O, even after DEAE-cellulose column chromatography, the fractions with the heme oxygenase enzyme activity have a broad Soret band between 380 and 400 nm, indicating that biliverdin is bound to Hmu O. In the case of rat HO-1, most of the biliverdin is removed by the DEAE-cellulose. Thus, the biliverdin affinity of Hmu O appears to be higher than that of rat HO-1. However, despite biliverdin being attached to the enzyme, hemin still binds to Hmu O readily, as described below. This indicates that biliverdin bound to Hmu O can be easily displaced by hemin, contrary to that claimed by Wilks and Schmitt (23).

Properties of the Hemin-Hmu O Complex—Fig. 3 shows the optical absorption spectra of the purified recombinant Hmu O (broken line) and its stoichiometric complex with hemin (solid line). The optical absorption spectrum of the hemin-Hmu O complex at pH 7 has a Soret maxima at 404 nm and peaks at 500 and 630 nm in the visible region (Fig. 3, solid line). The absorption spectrum is similar to those of the mammalian hemin-HO complexes and indicates that the hemin-Hmu O complex is ferric hexacoordinate high spin protoheme complexes including the hemin complex of rat HO-1 (165 mM$^{-1}$cm$^{-1}$) (34). The spectra were recorded immediately before the addition of ascorbic acid.

Because the Soret region of the optical absorption spectrum of the hemin-Hmu O complex is different from that of free hemin in pH 7 buffer, spectrophotometric titration of Hmu O with hemin was carried out utilizing this difference. Illustrated in the inset of Fig. 3, the titration curve of Hmu O with hemin has a well defined inflection point. From this, the molar stoichiometry of their binding has been established to be 1:1, which is in agreement with that reported by Wilks and Schmitt (23).

By the pyridine hemochrome method (35), the extinction coefficients for hexacoordinate high spin protoheme complexes including the hemin complex of rat HO-1 (165 mM$^{-1}$cm$^{-1}$) (34).

Degradation of the Hemin Bound to Hmu O with Ascorbic Acid—Ascorbic acid can serve as the electron donor in the oxidative degradation of hemin by mammalian HO (12). In this study, ascorbic acid is shown to support the Hmu O-catalyzed conversion of heme to biliverdin. As depicted in Fig. 4, the addition of ascorbic acid to the hemin-Hmu O complex initiates heme degradation. In a period of 2 h, the Soret peak at 404 nm disappears, and broad absorption bands centered near 380 and 680 nm appear, indicating that heme is converted to biliverdin. Ascorbic acid is shown to support the Hmu O-catalyzed oxidation of heme by mammalian HO (12).

Formation of the Verdohe CO Complex Using Ascorbic Acid—Heme catalysis by mammalian HO yields biliverdin with verdohe as a precursor (28). When the heme-HO complex is incubated with reducing equivalents under an atmosphere of O$_2$ and CO, heme catalysis can be arrested by CO at the verdohe stage, resulting in an accumulation of the ferrous verdohe-CO complex and thus inhibiting biliverdin formation (28–29). In our current study of Hmu O catalysis, we have conducted the reaction of the hemin-Hmu O complex with ascorbic acid in an atmosphere of approximately 50% CO and 50% O$_2$. The product formed exhibits an

absorption spectrum with distinct peaks at 351, 404, 538, and 636 nm, as indicated in Fig. 5A, spectrum a. The spectrum is similar to that of the CO-bound ferrous verdoheme-rat HO-1 complex (36). With the addition of 20% pyridine, a new spectrum with peaks at 398, 417, 537, and 679 nm appears (data not shown). This spectrum resembles that of the pyridine complex of verdoheme-HO-1 (28).

Prolonged incubation of the bacterial verdoheme-CO complex in the 50% CO and 50% O_2 environment does not change its spectrum. When the CO and O_2 mixture is replaced with 100% O_2, the optical absorption spectrum of the reaction product (Fig. 5A, spectrum b) becomes similar to that obtained by the ascorbic acid reaction of the hemin-Hmu O complex in the absence of CO (Fig. 5A, spectrum c). This likeness of the two spectra indicates that the originally CO-bound verdoheme-Hmu O complex has converted to biliverdin and that verdoheme is a precursor to biliverdin in HO catalysis.

The formation of the verdoheme intermediate during the Hmu O-catalyzed heme degradation reaction is further corroborated by the optical absorption spectrum of Hmu O complexed with a chemically synthesized verdoheme. The spectrum of the CO-bound ferrous verdoheme IXα-Hmu O, shown in Fig. 5B, spectrum a, has peaks at 352, 405, 541, and 634 nm, similar to those aforementioned of the verdoheme-CO intermediate formed during Hmu O catalysis (Fig. 5A, spectrum a). The removal of the bound CO by evacuation results in the ferrous deoxy verdoheme IXα-Hmu O complex, whose optical absorption spectrum (Fig. 5B, spectrum b) has a Soret peak at 400 nm and bands at 532 and 685 nm. The spectrum is similar to those of the mammalian HO verdoheme complexes (28, 36). Subsequent exposure of this ferrous verdoheme IXα-Hmu O complex to O_2 and ascorbic acid leads to its conversion to biliverdin (data not shown). These results unequivocally demonstrate verdoheme as the precursor to biliverdin.

Reaction of the \( \alpha \)-meso-Hydroxyhemin-Hmu O Complex with \( O_2 \) — In HO catalysis, \( \alpha \)-meso-hydroxyheme is the precursor to verdoheme (3–5, 26). To determine whether or not it is an intermediate as well as a precursor to verdoheme in Hmu O-mediated heme catabolism, we have prepared \( \alpha \)-meso-hydroxyhemin-Hmu O using chemically synthesized \( \alpha \)-meso-hydroxyhemin and studied its reactivity with O_2. As depicted in Fig. 6, spectrum a, the optical absorption spectrum of the \( \alpha \)-meso-hydroxyhemin-Hmu O complex exhibits a broad Soret peak at 404 nm with a relatively featureless visible region, similar to that of the \( \alpha \)-meso-hydroxyhemin-HO catalysis (26). Upon addition of dithionite, the heme iron is reduced, thus forming a ferrous \( \alpha \)-hydroxyheme-Hmu O complex (Fig. 6, spectrum b) with a Soret maxima at 430 nm. The binding of exogenous CO to this complex yields the CO form of the ferrous \( \alpha \)-hydroxyheme-Hmu O complex, whose spectrum has a distinct Soret peak at 418 nm (Fig. 6, spectrum c). The optical absorption spectra of the ferric, ferrous, and ferrous CO forms of the \( \alpha \)-meso-hydroxyhemin-Hmu O complex are similar to those of the respective forms of the \( \alpha \)-meso-hydroxyheme complex of HO-1 (26). Based on these results, we infer that the coordination structure and electronic states of the \( \alpha \)-hydroxyheme-Hmu O complexes are pentacoordinate high spin for the ferric and ferrous forms and hexacoordinate low spin for the ferrous CO form, concurring with those established for the \( \alpha \)-hydroxyheme complexes of HO-1 (26).

Reaction of the ferrous CO-bound \( \alpha \)-hydroxyheme-Hmu O with O_2 causes an immediate shift of the Soret band to 404 nm, and a defined peak at 634 nm emerges (Fig. 6, spectrum d). This is similar to HO-1 (26). The optical absorption spectrum of
the newly formed species is identical to that we have observed for CO-bound verdoxheme-Hmu O.4 Prolonged exposure of the aforementioned species to O2 in the presence of ascorbic acid produces an optical spectrum similar to that of a biliverdin complex (data not shown). The results described above un-
deniably demonstrate that a-hydroxheme is an intermediate of the Hmu O-mediated heme degradation, specifically as the
precursor to verdoxheme.

Reactions of the Hemin-Hmu O Complex with mCPBA and H2O2—In a previous study, to identify the active intermediate in the first oxygenation step of HO catalysis, which is the conversion of hemin to a-hydroxhemin, the hemo-HO-1 complex was reacted with mCPBA (29). This resulted in the forma-
tion of the ferryl oxo species (Fe4+ = O). In our current Hmu O study, reaction of the hemin-Hmu O complex with mCPBA causes a decrease and a shift in the Soret peak from 404 to 418 nm and the concomitant appearance of absorption bands at 527 and 549 nm (Fig. 7). The spectrum is characteristic of the ferryl oxo-heme species (29, 33). No further heme degradation oc-
urred hereafter. Hence, ferryl oxo is not an active intermedi-
ate in Hmu O catalysis.

In the case of the mammalian HO, a ferric hydroperoxide (Fe3+ -OOH) species has been proposed to be an active intermediate in the first oxygenation step of HO catalysis (29). Unlike mCPBA, H2O2 hydroxylates the alpha-meso position of heme to form alpha-meso-
hydroxhemin, which then is converted to verdoxheme in the presence of O2 (29). Here we have reacted the hemo-Hmu O complex with H2O2 and found that a verdoxheme complex, as indicated by the absorption band at 688 nm (Fig. 8A), is formed. This verdoxhemin formation was further corroborated by the optical absorption spectrum of its pyridine complex (data not shown). Based on these results, a ferric hydroperoxide species must be an active intermediate in the first oxygenation step of Hmu O catalysis as it is in mammalian HO.

We have also found that the extent of conversion to verdoxhemin is dependent on the H2O2 concentration, with 5 equivalents of H2O2 inducing the most change, and 1 equivalent, the least. In comparison to the H2O2 reactions with rat HO-1 (Fig. 8B), those with Hmu O are less efficient and do not result in the virtual loss of the Soret band. Even with 1 equivalent of H2O2,

verdoxheme formation by rat HO-1 is much greater than that by Hmu O.

In the H2O2 reaction with the hemo-HO complex, deproto-
nation of the peroxide by a distal group in the heme pocket facilitates its binding to the hemo iron (38). If the deprotonation is deficient, formation of the active intermediate ferric hydroperoxide is hindered, and consequently, verdoxheme re-
cover is reduced. Based on this, one possible reason for the decreased verdoxhemin recovery in Hmu O is that the active-site structure, namely the nature of the distal group, of Hmu O differs from that of rat HO-1. Structural differences in the distal heme pocket between Hmu O and rat HO-1 might also explain our observation of the slow ascorbic acid-supported heme degradation by Hmu O. In the first segment of the proposed Hmu O catalytic pathway, ascorbic acid reduces the hemo iron to the ferrous state, thus permitting O2 to bind to the iron to form a metastable ferrous oxy Hmu O complex. The bound O2 in HO-1 forms a hydrogen bond with a distal group (39). This hydrogen bond facilitates the formation of the ferric hydroperoxide active species by decreasing the reduction potential of the oxy form. The structure of the distal heme pocket of Hmu O might perturb the hydrogen bond interaction between the bound O2 and a distal group so that the reduction potential of the ferrous oxy Hmu O complex is altered, thereby reducing the rate of ferric hydroper-
oxide formation. Consequently, the turnover of Hmu O is re-
tarded when ascorbic acid is used as the source of the reducing equivalents.

Conclusion—Utilizing a full-length synthetic hmu O gene, we have expressed in high yield a recombinant bacterial heme oxygenase that is catalytically identical to the cloned Hmu O from C. diphtheriae. We have found similarities between Hmu

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4 CO does not inhibit the conversion of a-hydroxheme to verdoxheme in Hmu O catalysis. As reported for HO-1, this conversion appears to proceed without the dissociation of CO from the a-hydroxheme iron. Direct oxygen attack of the porphyrin ring may be an alternative mechanism (26).

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O and the mammalian HO in terms of enzymatic properties. As with the mammalian HO, under aerobic conditions and in the presence of an electron donor, Hmu O catalyzes heme degradation and yields biliverdin as the final product. Verdoheme is demonstrated to be the immediate precursor to the biliverdin complex in both the degradation of the heme-Hmu O complex and the oxidation of a verdoheme IXa-Hmu O complex prepared from a chemically synthesized verdoheme and Hmu O. When the α-hydroxyheme-Hmu O complex we have prepared is oxidized in the presence of a reductant, verdoheme and subsequently a biliverdin complex, are formed. In addition, we have found that a ferric hydroperoxide species is an active intermediate of the first oxygenation step in Hmu O catalysis. These results and that of a previous study, which showed CO being concomitantly released during Hmu O catalysis (23), suggest that the Hmu O catalytic pathway is similar to that of mammalian HO.

However, despite these similarities, there are differences between Hmu O and mammalian HO. Among them are the higher affinity of Hmu O for biliverdin, the formation of biliverdin instead of an Fe(III)-biliverdin complex in the reaction with ascorbic acid, and the different rate of heme catabolism by Hmu O as compared with rat HO-1. We propose that the distal site structure and the mechanisms by which heme is catabolized in the presence of an electron donor, Hmu O catalyzes heme degradation and the low verdoheme formation in the H₂O₂ reaction. Further investigation of the Hmu O active site structure and the mechanisms by which heme is catabolized in Hmu O catalysis is needed to explain these differences.

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