A rat heme oxygenase (HO-1) gene without the sequence coding for the last 23 amino acids has been constructed and expressed behind the pho A promoter in Escherichia coli. The enzyme is expressed at high levels therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Heme oxygenase catalyzes the NADPH- and cytochrome P450 reductase-dependent oxidation of heme to biliverdin (1).

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The abbreviations used are: heme, iron protoporphyrin IX regardless of the oxidation and ligation state of the iron; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; mCPBA, meta-chloroperbenzoic acid; guaiacol, 2-methoxyphenol.
Expression and Purification of the Truncated Heme Oxygenase—A 3-ml inoculum in low phosphate induction media was set up on plates with fresh colonies of transformed E. coli DH5αF’. From the fresh mid-log phase cultures 100 ml was used to inoculate 1-liter cultures of the same media. The cells were grown at 30 °C for 18 h or until the media became green. Expression of the protein for periods over 24 h results in partial proteolysis of the 30-kDa protein. The harvested cells were lysed in 50 mM Tris buffer (pH 8.0) containing 6 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, and 1 mM phenylmethylene-sulfonyl fluoride in a Bio-Spec Bead Beater. The cells were then spun at 27,000 × g for 60 min. Ammonium sulfate was added to the resulting supernatant to a final concentration of 30% of saturation and the solution was stirred for 60 min. Following centrifugation (27,000 × g for 20 min), the ammonium sulfate concentration was raised to 60% of saturation. The 30–60% pellets were collected and resuspended in 10 mM potassium phosphate buffer (pH 7.4) containing 252 mM KCl (buffer B). The gradient was increased linearly from 0 to 25% buffer B, held for 10 min at 25% buffer B, and then increased linearly to 100% buffer B. The fractions in the first half of the gradient containing heme oxygenase activity were pooled and dialyzed against buffer A.

Absorption and EPR Spectroscopy—The spectra of the heme-heme oxygenase complex were recorded on a Hewlett-Packard 8450 A spectrophotometer. The reduced ferrous-CO complex was formed by addition of dithionite to a carbon monoxide-saturated solution of the ferric complex. The ferrous-O₂ complex was obtained by one-electron oxidation (2), by passing the carbon monoxide complex down a Sephadex G25 column pre-equilibrated with 10 mM potassium phosphate (pH 7.4) buffer.

The heme-heme oxygenase complex was frozen in liquid nitrogen immediately after addition of 1–5 eq of mCPBA. EPR spectra were recorded using the following conditions: gain, 4.0 × 10⁷; microwave power, 2 milliwatts; modulation intensity, 8 G; time constant, 0.5 s; scan time, 4 min. The magnetic field was set at 3259 G, and the spectra were recorded over a 500-G range.

Reactions of Heme Oxygenase with Peroxides—One equivalent of 1
Peroxide Reactions of Truncated Heme Oxygenase

Peroxide Reactions of Truncated Heme Oxygenase

H_{2}O_{2} (10 μl) in 0.1 M potassium phosphate buffer (pH 7.4) was added to a solution of the heme-heme oxygenase complex (10 μm) in the same buffer. The reaction was monitored spectrophotometrically. Following maximum loss of the Soret band and maximum increase in the absorption at approximately 680 nm, pyridine was added to the solution to a final concentration of 20%. The product was extracted into chloroform (1 ml) and the solvent was removed under a stream of nitrogen. In a separate reaction, pyridine was added to half of the solution obtained from reaction of the heme-heme oxygenase complex with the remainder of the solution was allowed to react with cytochrome P450 reductase and NADPH as described below. The product formed in the reaction with cytochrome P450 reductase, and NADPH was extracted for analysis by UV and HPLC as described above.

The heme-heme oxygenase complex (7.5 μm) in 0.1 M potassium phosphate (pH 7.4) buffer was allowed to react with 5 eq of mCPBA, cumene hydroperoxide, or tert-butylhydroperoxide. The oxidation of guaiacol was measured in incubations containing 10 μm heme-heme oxygenase complex, 2.73 μm guaiacol, and 50 μm H_{2}O_{2} in a total of 1 ml of 0.1 M potassium phosphate (pH 7.4) buffer.

Reaction of Heme Oxygenase with Cytochrome P450 Reductase and NADPH—Cytochrome P450 reductase (42 μM; 3:1, reductase:heme oxygenase) and NADPH (14 μM) were added to a cuvette containing a solution of the heme-heme oxygenase complex (14 μM) in 0.1 M potassium phosphate buffer (pH 7.4) that had been presaturated with carbon monoxide by bubbling with the gas. The progress of the reaction was monitored by UV spectroscopy. After approximately 10 min, at which point no further change occurred in the spectrum, pyridine (20% final concentration) was added. The mixture was extracted with chloroform and the product isolated by removing the organic solvent under a stream of nitrogen. Under these conditions the reaction is arrested at the verdoheme stage. In some experiments, the verdoheme intermediate accumulated in the presence of carbon monoxide was not extracted but was allowed to continue to the fully oxidized product by displacing the carbon monoxide with oxygen in the presence of NADPH before isolating the product for HPLC analysis.

RESULTS

Expression and Purification of Truncated Heme Oxygenase

A truncated heme oxygenase gene without the bases coding for the terminal 23 amino acids was constructed in pBAce (Fig. 1) and expressed in E. coli. The soluble truncated form of the protein thus obtained in excellent yield was fully active (see below). Expression of the truncated heme oxygenase gene turns the medium green due to the accumulation of biliverdin, as shown in the inset.

Fig. 1. Construction of the heme oxygenase gene in the pBAce vector (pBAH030). The truncated heme oxygenase gene (803 base pairs) was cloned into the NdeI and SalI sites behind the pho A promoter.

Table 1

| Fraction | Protein | Specific activity | Purification | Activity yield |
|----------|---------|------------------|--------------|---------------|
| Cell supernatant | 3750 | 136 | 1.0 | 100 |
| 40% (NH_{4})_{2}SO_{4} | 540 | 227 | 1.7 | 24 |
| Mono Q chromatography | 104 | 6522 | 48 | 134 |

Fig. 2. SDS-PAGE analysis of the purified heme oxygenase protein and absorption spectrum of the heme-heme oxygenase complex. The spectra are of the ferric heme complex (a (--)), the ferrous dioxoheme complex (b (-- -- --)), and the ferrous carbon monoxide complex (c ---). SDS-PAGE analysis of the purified protein, with the positions of the molecular weight markers indicated on the edge, is shown in the inset.

Fig. 3. Conversion of the ferric heme-heme oxygenase complex to biliverdin IXα in the presence of cytochrome P450 reductase. (--), ferric complex and cytochrome P450 reductase; --- -- --, immediately following the addition of NADPH; ---, 10 min after addition of NADPH.

Purification of the truncated heme-heme oxygenase complex by ammonium sulfate fractionation and FPLC (Table I) yielded a protein that gave a single band at 30 kDa on SDS-PAGE (Fig. 2). NH_{2}-terminal sequencing of the 30-kDa band showed that the first 5 amino acids (MERPQ) were identical to those of the rat liver enzyme. The amount of protein purified from 3 liters of cells was estimated to be 104 mg, based on an extinction coefficient of 405 nm of 140 μmol cm⁻¹ cm⁻¹ (3). The purification stages and specific activities are shown in Table I.

Properties of the Truncated Heme-Heme Oxygenase Complex

The Soret maximum of the ferric complex is relieved by Mono Q chromatography.
is added to purified heme oxygenase and the excess heme is removed by passage through a hydroxylapatite column is at 404 nm (Fig. 2). Reduction of the ferric complex with dithionite under an atmosphere of carbon monoxide yields the ferrous-carbon monoxide complex with a Soret band at 418 nm and well defined \( \alpha \) and \( \beta \) bands at 568 and 538 nm, respectively (Fig. 2). Passage of the carbon monoxide complex through a Sephadex G25 column causes the Soret band to shift to 410 nm, and the \( \alpha \) and \( \beta \) bands to shift to 574 and 540 nm, respectively, as expected for conversion of the carbon monoxide complex to the ferrous dioxygen complex (Fig. 2). These values are comparable with those reported for the ferrous dioxygen complex of native rat liver heme oxygenase (2).

**Catalytic Turnover of Truncated Heme Oxygenase**—The heme in the ferric heme-heme oxygenase complex is quantitatively converted to biliverdin by addition of NADPH and cytochrome P450 reductase (Fig. 3). Residual heme was not detected by HPLC after the reaction and the product formed had a retention time and absorption spectrum identical to those of authentic biliverdin IXa (not shown). The specific activity of the enzyme, determined from the increase in absorbance at 408 nm, was 6000 nmol h\(^{-1}\) mg\(^{-1}\), a value identical to that reported for purified rat liver heme oxygenase (2). Turnover of the truncated heme-heme oxygenase complex in the presence of carbon monoxide was examined to determine if the reaction, as in the case of the native enzyme, can be arrested at an intermediate stage by complexation with carbon monoxide. In fact, carbon monoxide causes accumulation of a biliverdin precursor (\( \lambda_{\text{max}} = 640 \) nm) spectroscopically identical to that obtained with the native enzyme (Fig. 4a) (21, 23). Furthermore, the spectrum obtained when the intermediate accumulated in the presence of carbon monoxide is treated with pyridine and extracted into chloroform (Fig. 4c) is similar to that reported for similar treatment of the native enzyme (21, 23).

**Reaction of Truncated Heme Oxygenase with \( H_2O_2 \)**—Reaction of the heme-heme oxygenase complex with \( H_2O_2 \) was investigated to determine if the peroxide can replace cytochrome P450 reductase, NADPH, and \( O_2 \) in the formation of biliverdin. Addition of 1 eq of \( H_2O_2 \) to the heme-heme oxygenase complex resulted in virtual loss of the Soret absorbance and an increase in absorbance at 680 nm (Fig. 4b). At no time did the spectrum suggest formation of a ferryl species. Acidification and extraction with chloroform resulted in loss of the green color from biliverdin but formation of a pink residue tentatively attributed to dipyrrole ("propentdyopent") and monopyrrole porphyrin degradation products (31, 32). However, addition of pyridine to the complex prior to acidification and extraction results in the observation of a normal verdoheme-like spectrum with maxima at 400, 504, 536, and 680 nm (Fig. 4c). Confirmation that the intermediate formed with \( H_2O_2 \) is enzyme-bound ferric verdoheme is provided by the observation that addition of cytochrome P450 reductase and NADPH to the intermediate formed with \( H_2O_2 \) results in its conversion to biliverdin IXa (Fig. 5). The product was identified by direct comparison of its absorption spectrum and HPLC retention time with those of an authentic sample of biliverdin IXa (not shown).

**Reaction of Truncated Heme Oxygenase with Alkyl- and Acyl-hydroperoxides**—The ability of alkyl and acylhydroperoxides to replace cytochrome P450 reductase and NADPH in the heme oxygenase reaction was investigated to determine whether the enzyme would tolerate the presence of a substituent on one of the peroxide oxygen atoms. Reaction of the heme-heme oxygenase complex with 1 eq of cumene hydroperoxide, tert-butylhydroperoxide, or mCPBA was found to decrease the intensity of the Soret band without increasing the absorption at 640–680 nm. Reaction with 5 eq of cumene hydroperoxide caused little shift in the Soret maximum, but reaction with 5 eq of mCPBA shifted the maximum from 404 to 408 nm (Fig. 6). All the acyl- and alkylhydroperoxides cause the Soret absorption to diminish with an isosbestic point between 416–420 nm, spectroscopic changes consistent with the formation of a ferryl intermediate. Support for a ferryl intermediate is provided by the observation that addition of phenol or ascorbic acid regenerates the spectrum of the ferric heme-heme oxygenase complex. Furthermore, the formation of a verdoheme-like biliverdin precursor was not observed with the acyl- and alkylhydroperoxides. Thus, the 680-nm verdoheme-pyridine complex observed when pyridine is added after reaction with \( H_2O_2 \) is not detected when pyridine is added after reaction with the alkylhydroperoxides.
Peroxide Reactions of Truncated Heme Oxygenase

Heme Oxygenase as Peroxidase—The ability of phenol to regenerate the heme-heme oxygenase complex from the ferryl species produced by reaction with alkylhydroperoxides suggests that the ferryl species catalyzes the oxidation of phenols. In fact, heme-heme oxygenase complex promotes the peroxidation of guaiacol in the presence of 5 eq of mCPBA (rate = 6.9 nmol s⁻¹ mg⁻¹) or cumene hydroperoxide (rate = 2.2 nmol s⁻¹ mg⁻¹), but does not detectably catalyze guaiacol oxidation in the presence of NADPH and cytochrome P450 reductase.³

Studied of the structure and mechanism of heme oxygenase have been hindered by the intractability of the microsomal protein. Efforts to circumvent these problems by expressing a cDNA coding for the full-length rat HO-1 enzyme in E. coli have met with limited success, providing a low yield of the enzyme that remains associated with bacterial membranes and is proteolytically digested to smaller fragments. Similar results were reported during the course of this work by Ishikawa et al. (13). We have overcome these problems by expressing a truncated form of heme oxygenase without the 23 COOH-terminal amino acids that serve as a membrane anchor. A soluble 30-kDa protein was thus obtained that was fully active at a level of approximately 30 mg/liter after purification (Table I).

Heme binds to the truncated heme oxygenase to give a complex with the same spectroscopic properties as the complex of the native protein in the ferric, ferrous dioxy, and ferrous carboxy states (Fig. 2). The truncated enzyme, when reconstituted with rat liver cytochrome P450 reductase and NADPH, converts heme to biliverdin (Fig. 3) at the same rate as the native enzyme (Table I). As also reported for the native enzyme (21, 22), incubation of the truncated heme-heme oxygenase complex with cytochrome P450 reductase and NADPH under an atmosphere of carbon monoxide results in the accumulation of an intermediate with an absorption maximum at 638 nm (Fig. 4A). This putative verdoheme-enzyme complex is converted to biliverdin in the presence of excess NADPH if the carbon monoxide is replaced by oxygen. The truncated enzyme is thus catalytically indistinguishable from the native enzyme.

Reaction of the heme-heme oxygenase complex with limited amounts of H₂O₂ yields an intermediate which, if directly extracted from the complex, decomposes to unidentified heme degradation products. However, if the intermediate is reduced under an atmosphere of carbon monoxide, a species is obtained with the same spectroscopic properties as the verdoheme complex produced by incubation of the heme-heme oxygenase complex with cytochrome P450 reductase and NADPH under an atmosphere of oxygen and carbon monoxide (Fig. 4). Furthermore, if cytochrome P450 reductase and NADPH are added to the enzyme-bound intermediate produced with H₂O₂, biliverdin is formed as the principal reaction product (Fig. 5). H₂O₂ can thus substitute for catalytically reduced molecular oxygen in meso-hydroxylation of the heme. Meso-Hydroxylation, in effect, yields verdoheme because the conversion of α-meso-hydroxyheme to verdoheme requires molecular oxygen but no additional reducing equivalents. In contrast, the conversion of verdoheme to biliverdin clearly requires reduction of the verdoheme-enzyme complex by cytochrome P450 reductase. H₂O₂ is not a viable substitute for catalytically activated dioxygen in this step of the transformation.

The finding that H₂O₂ supports heme α-meso-hydroxylation suggests that the hydroxylating species may be a ferryl (fer-
nally Fe\(^{IV}=O\) complex generated by heterolysis of the peroxide. This mechanism is suggested by the formation of ferryl complexes in the reactions of \(H_2O_2\) with iron porphyrins (33), peroxidases (34), globins (35, 36), catalases (37), and monooxygenases (38). A possible mechanism for transfer of the ferryl oxygen to the \(alpha\)-meso-carbon is a stepwise "walk" of the oxygen from the iron via a pyrrole nitrogen to the meso position (39). To explore this aspect of the catalytic process, we examined the reaction of the heme-heme oxygenase complex with \(mCPBA\), cumene hydroperoxide, and tert-butylhydroperoxide. These peroxides react with hemoproteins to give the same ferryl complex as are obtained with \(H_2O_2\) and should therefore support biliverdin formation if a ferryl species is responsible for the reaction (40). However, reaction of the heme-heme oxygenase complex with \(mCPBA\) or the alkylhydroperoxides produces a relatively stable species with a spectrum similar to that of the Fe\(^{IV}=O\) ferryl complex of myoglobin (Fig. 6) (41). This ferryl complex is one oxidation equivalent above the 

![Scheme 2](image_url)

**Scheme 2. Hypothetical nucleophilic and electrophilic mechanisms for heme oxygenase-catalyzed hydroxylation of the porphyrin ligand.** A, nucleophilic addition of the unprotonated iron dioxygen complex to the porphyrin; B, electrophilic reaction of the protonated iron dioxygen complex with the porphyrin. Part of the iron protoporphyrin IX ring and an active site base (\(\mathbf{B}\)) are shown.

(a) nucleophilic addition of the terminal oxygen of the unprotonated [Fe-OO\(^{-}\)] to the meso-carbon to give a peroxo-bridged (Fe-O-O-\(\mathbf{B}\)) intermediate or (b) electrophilic addition of the terminal oxygen of the protonated complex [Fe-OOH] with concomitant cleavage of the dioxygen bond (Scheme 2). The two latter mechanisms rationalize the ability of \(H_2O_2\) but not alkylhydroperoxides to replace cytochrome P450 reductase and NADPH in heme hydroxylation, the absence of a ferryl species from the reaction trajectory that inserts the oxygen atom, and the ability of the protein to control the regiospecificity of heme hydroxylation.

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