Ferric α-Hydroxyheme Bound to Heme Oxygenase Can Be Converted to Verdoeheme by Dioxygen in the Absence of Added Reducing Equivalents*

(Received for publication, January 13, 1999, and in revised form, March 5, 1999)

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Whether or not reducing equivalents are indispensable for the conversion of ferric α-hydroxyheme bound to heme oxygenase-1 to verdoeheme remains controversial (Matera, K. M., Takahashi, S., Fujii, H., Zhou, H., Ishikawa, K., Yoshimura, T., Rousseau, D. L., Yoshida, T., and Ikeda-Saito, M. (1996) J. Biol. Chem. 271, 6618–6624; Liu, Y., Möenne-Loccoz, P., Loehr, T. M., and Ortiz de Montellano, P. R. (1997) J. Biol. Chem. 272, 6906–6917). To resolve this controversy, we have prepared a ferric α-hydroxyheme-heme oxygenase-1 complex and titrated the complex with O₂ under strictly anaerobic conditions. The formation of verdoeheme was monitored by optical and electron spin resonance spectroscopies. Electron spin resonance spectra of the complex showed that α-hydroxyheme exists as a mixture of resonance structures composed of the iron(III) porphyrin and the iron(II) porphyrin π neutral radical. Upon addition of O₂ the latter species becomes dominant. The results obtained from these titration experiments indicate that α-hydroxyheme can be converted to verdoeheme by an approximately equimolar amount of O₂ without any requirement for exogenous electrons. The verdoeheme formed from α-hydroxyheme was shown to be in the ferrous oxidation state by the addition of CO or potassium ferricyanide to the resultant verdoeheme-heme oxygenase-1 complex.

Heme oxygenase (HO), a microsomal enzyme that oxidizes protoheme to biliverdin IXα, plays a key role in physiological heme metabolism. As shown in Scheme 1, the first step in the heme degradation catalyzed by HO is the oxidation of heme to α-hydroxyheme, a process requiring O₂ and reducing equivalents from NADPH-cytochrome P450 reductase (1). The second step is the formation of verdoeheme with the concomitant release of the hydroxylated α-meso-carbon as CO (2, 3). This step is also O₂-dependent. In the third step, biliverdin is formed from verdoeheme in a reaction that again requires O₂ and reducing equivalents from NADPH-cytochrome P450 reductase (1).

Our early stoichiometric study employing a reconstituted HO system suggested that 3 mol of O₂ and 4−5 mol of NADPH were consumed during the degradation of 1 mol of heme by HO (4). However, for the second and third steps the stoichiometry with respect to O₂ and reducing equivalent(s) from NADPH-cytochrome P450 reductase is not fully established. In particular the requirement for an exogenous electron for the conversion of α-hydroxyheme to verdoeheme (the second step) is currently controversial. On the one hand, Matera et al. (5) have claimed that the reaction requires both O₂ and one electron; on the other hand, Liu et al. (6) have reported that the reaction only needs O₂.

Recently we have established a system for expressing and purifying a truncated version of the inducible rat enzyme (HO-1) consisting of Met-1 through Pro-267 (rHO-1); the sequence (1−267) of this truncated protein is completely identical to the corresponding sequence of the rat HO-1 (7). About 130 mg of soluble and catalytically active rHO-1 can be routinely obtained from a 10-liter culture (8). In an attempt to resolve the discrepancy arising from the work of Matera et al. (5) and Liu et al. (6), we have prepared ferric α-hydroxyheme, complexed it with purified rHO-1, and characterized its oxidation to verdoeheme under various conditions by optical and ESR spectroscopies.

EXPERIMENTAL PROCEDURES

Materials—rHO-1 lacking the 22-amino acid C-terminal hydrophobic stretch was expressed in Escherichia coli and purified as described (8). The specific activity of rHO-1, 8300 units/mg protein (8), slightly exceeded that of native HO-1 from rat liver, 4500–6000 units/mg protein (9), even taking into account the reduction in molecular mass by truncation. In addition, rHO-1, when complexed with heme, catalyzed the heme degradation reaction to biliverdin in a manner spectrophotometrically the same as native HO-1 (data not shown). The purification of NADPH-cytochrome P450 reductase from rat liver was accomplished using DEAE-Sephadex and 2’,5’-ADP-Sepharose (10) column chromatographies. Gases of high purity, argon (99.999%), O₂ (99.99%), and N₂ (99.99%) were obtained from Iwatani, Fukuoka, Japan. All chemicals used were of analytical grade and were obtained commercially.

Preparation of α-Hydroxyheme-RHO-1 Complex and Titration of the α-Hydroxyheme-RHO-1 Complex with O₂—α-Benzoyloxyprotoporphyrin IX dimethyl ester was synthesized according to the method of Sano et al. (11), and its regiochemical structure was confirmed by a one- and two-dimensional ¹H NMR (300 MHz) spectra with a Bruker DSX 300 spectrometer. Insertion of iron was performed as described (12) to obtain α-benzoyloxyprotoporphrin dimethyl ester. The amount of α-hydroxyhemin dimethyl ester was determined as pyridine hemochrome using ε282 nm = 153.6 M⁻¹ cm⁻¹ in CHCl₃ (11). Alkaline hydrolysis of α-benzoyloxyprotoporphrin dimethyl ester was carried out anaerobically as described (5). A custom made anaerobic titrator illustrated in Fig. 1, which was based on a titrator reported previously (13), was used for preparation of an α-hydroxyheme-rHO-1 complex and for titration of the complex with O₂.

The following manipulations were carried out anaerobically in a glove box filled with N₂ at room temperature (25 °C) unless otherwise stated. α-Hydroxyhemin (154 nmol) was dissolved in a small amount of 0.1 M NaOH saturated with argon. The alkaline α-hydroxyhemin solu-
Observation was transferred to the titrator, which contained 2 ml of 0.1 M potassium phosphate buffer (pH 7.4). The gas phase in the titrator had been replaced with argon in advance by successive cycles of evacuation and argon flushing. The anaerobic rHO-1 solution (0.45 mM) was prepared separately by gently stirring the solution for 3 h at 5 °C under flowing argon gas moistened by passing through anaerobic water. A portion of the solution (248 nmol (1.6 eq) of rHO-1 to 154 nmol of α-hydroxyheme) was transferred into the titrator. The formation of an α-hydroxyheme-rHO-1 complex was monitored by recording the optical absorption spectra with a JASCO V-560 UV-visible spectrophotometer at 25 °C. The titrant (O2-saturated 0.1 M potassium phosphate buffer, 1.25 mM as O2 concentration) was prepared by bubbling O2 into the buffer solution for 3 h and loaded into the syringe. The syringe was quickly attached to the titrator while maintaining a positive internal pressure of argon. An aliquot of the titrant was introduced through the tip of the needle by advancing the plunger of the syringe by a calibrated and mixed with the titration solution by gentle shaking. After every addition of the titrant, optical absorption spectra were recorded and additions continued until no further absorbance changes were observed.

ESR Spectroscopy—ESR samples (500 μl) were collected during the titrations using a gas-tight Hamilton syringe equipped with a long needle via the top inlet of the three-way stopcock under continuous argon flow through the horizontal inlet. The samples were transferred from the syringe to ESR tubes that had been filled with argon. The tubes were slightly evacuated, quickly plugged with a rubber stopper, cut, sealed by fire, and frozen in liquid N2. X-band ESR spectra were recorded at 5 K using a JEOL ESR spectrometer (JES FE3X) equipped with a JEOL liquid helium cryostat (ES-LTR5X). The instrumental conditions were: modulation frequency, 100 kHz; modulation width, 1 milliteile; microwave frequency, 9.88 GHz; and microwave power, 1 μW–10 mW as indicated in the appropriatefigure legends. The microwave frequency was calibrated with a microwave frequency counter (Advantest), and the magnetic field strength was determined with a JEOL NMR counter (JEOL ES-OC1).

RESULTS

Analysis of α-Hydroxyheme-rHO-1 Complex by Optical and ESR Spectroscopies—The α-hydroxyheme-rHO-1 complex exhibited an optical spectrum with a broad Soret band at 407 nm and featureless absorption in the visible region with shoulders at 535, 575, and 630 nm (Fig. 2, spectrum a). The spectrum was almost identical to those reported earlier for the α-hydroxyheme-HO complex (5, 6). The ESR spectra of the complex were measured at different microwave powers ranging from 1 μW to 10 mW at 5 K. At the higher microwave powers a rhombic signal with g values of 6.06, 5.79, and 2.00 was observed (Fig. 3, D and E). Upon decreasing the power, however, a radical signal at g = 2.004 became evident (Fig. 3, A–C). Recent optical, ESR, and resonance Raman spectroscopic studies have demonstrated that α-hydroxyheme bound to HO is in a five-coordinate high spin state and assumes a resonance structure comprised of a ferric enolate form (Scheme 2, J), a ferric keto or oxophlorin form (2), and a ferrous π neutral radical (3) (5, 6, 14, 15). The absorption spectrum with the Soret band at 407 nm as well as the charge transfer shoulder at 630 nm, the rhombic ESR spectrum at g = 6 and g = 2, and the radical signal with g = 2.004 are consistent with the spin state and the resonance structure assigned to the complex. It should be noted that the g = 2.004 radical signal is almost completely saturated at microwave powers greater than 1 mW, which is probably the reason why Matera et al. (5) failed to observe the radical signal at an operating power of 1 mW. Interestingly, the addition of CO to the complex almost completely eliminates the rhombic signal of the complex with a very large increase in the radical signal (Fig. 3, F as compared with B). A similar result was previously obtained with a sample of the α-hydroxyheme-HO complex generated by anaerobic addition of H2O2 to the heme-HO complex (6). These observations suggest that CO displaces the resonance equilibrium state in favor of the ferrous π neutral radical.
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Stepwise additions of O$_2$ to the α-hydroxyheme-rHO-1 complex caused a marked loss of the Soret absorption and a small decrease in absorbance at 560–800 nm, whereas new absorbance features appeared around 640 and 685 nm (Fig. 2, spectra represented by dotted lines). The spectral changes were almost complete with the addition of 1.0 eq of O$_2$, and further addition of 0.3 eq of O$_2$ caused only marginal spectral change (Fig. 2, spectrum b). The absorbance maxima at 640 and 685 nm are consistent with the formation of a mixture of ferrous CO-verdoheme and CO-free ferrous verdoheme, respectively, as previously reported (16, 17). After standing for 2 h under flowing argon, the absorption at 640 nm clearly decreased, whereas that at 685 nm increased (Fig. 2, inset, spectral change in the 560–800 nm region of spectrum b to spectrum c) indicating that CO slowly dissociated from the ferrous verdoheme. The absence of a clear isosbestic point at around 620 nm during the titration is probably because of the generation of both the CO-bound and CO-free verdoheme species.

That the verdoheme formed was in the ferrous oxidation state was confirmed spectrophotometrically. The CO-free verdoheme-rHO-1 complex obtained after standing under argon flow (Fig. 4, spectrum a) could be completely converted to the CO-bound form upon the addition of CO (Fig. 4, spectrum b; $\lambda_{\text{max}}$ = 409, 541, and 640 nm). If ferric verdoheme had been the titration product, no decrease should have been detected at 685 nm and no corresponding increase at 640 nm. Furthermore, when the CO-free verdoheme-rHO-1 complex was treated with a stoichiometric amount of ferricyanide, a spectrum exhibiting absorption maxima at 350, 404, 530, and 683 nm was obtained (Fig. 4, spectrum c); this spectrum is very similar to that reported by Liu et al. (6) for the ferric verdoheme-HO complex. The spectrum of the ferric verdoheme remained unchanged by further addition of CO (data not shown).

The absorption around 820 nm first increased and then decreased during the titration with O$_2$ (Fig. 2). This behavior suggests the existence of an intermediate between α-hydroxyheme and verdoheme. In this connection, it is of interest that Liu et al. (6) reported that an 820-nm absorption band appeared in the product of the anaerobic reaction of the heme-HO complex with 1 eq of H$_2$O$_2$ and disappeared when the product was exposed to O$_2$.

Fig. 3. ESR spectra of α-hydroxyheme-rHO-1 complex recorded at different microwave powers. Traces A, B, C, D, and E represent the spectra of the α-hydroxyheme-rHO-1 complex measured at 1 mW, 10 mW, 100 mW, 1 mW, and 10 mW, respectively. Trace F, the spectrum after addition of CO to the complex measured at 10 mW. Other instrumental conditions are described under “Experimental Procedures.” mT, millitesla.

Conversion of α-Hydroxyheme to Verdoheme by Addition of O$_2$—Stepwise additions of O$_2$ to the α-hydroxyheme-rHO-1 complex caused a marked loss of the Soret absorption and a small decrease in absorbance at 550–600 nm, whereas new absorbance features appeared around 640 and 685 nm (Fig. 2, spectra represented by dotted lines). The spectral changes were almost complete with the addition of 1.0 eq of O$_2$, and further addition of 0.3 eq of O$_2$ caused only marginal spectral change (Fig. 2, spectrum b). The absorbance maxima at 640 and 685 nm are consistent with the formation of a mixture of ferrous CO-verdoheme and CO-free ferrous verdoheme, respectively, as previously reported (16, 17). After standing for 2 h under flowing argon, the absorption at 640 nm clearly decreased, whereas that at 685 nm increased (Fig. 2, inset, spectral change in the 560–800 nm region of spectrum b to spectrum c) indicating that CO slowly dissociated from the ferrous verdoheme. The absence of a clear isosbestic point at around 620 nm during the titration is probably because of the generation of both the CO-bound and CO-free verdoheme species.

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Analysis of Verdoheme Formation by ESR Spectroscopy—ESR spectroscopy provided additional evidence that α-hydroxyheme was converted to ferrous verdoheme. Fig. 5 shows the ESR spectral changes during the titration described in the previous section. Addition of a small amount of O$_2$ (0.1 eq) to the anaerobic α-hydroxyheme-rHO-1 complex caused a clear decrease of the signal intensities in the $g = 6$ and $g = 2$ regions (Fig. 5, B as compared with A). By the addition of O$_2$ up to an equimolar amount these signals almost completely disappeared (Fig. 5C), implying that α-hydroxyheme was nearly quantitatively converted to the ESR-silent ferrous verdoheme.
The sum of the squares of the low-spin g values is 14.7. Using Taylor’s technique for extracting the ligand field parameters of low-spin ferric hemes (18) the axial and rhombic field are calculated to be 6.9 and 6.5 with a rhombicity of 0.94 (using the coordinate system of Blumberg and Peisach (19)). The sum of the squares of the orbital coefficients is 1.0. With these parameters the ferric low-spin verdoheme, if this is an accurate characterization, falls unequivocally within the O-family of the low-spin hemoprotein groups categorized by Blumberg and Peisach (19) and suggests that the ferric verdoheme has hydroxide as the sixth ligand to the heme iron.

DISCUSSION

We conclude from the results obtained by the titration experiments that the conversion of $\alpha$-hydroxyheme to verdoheme can be accomplished with an almost equimolar amount of $O_2$ in the absence of added reducing equivalents and that the verdoheme so formed is in the ferrous oxidation state. The ferric $\alpha$-hydroxyheme bound to HO-1 is five-coordinate (5) wherein the fifth ligand is His-25 (20, 21). The $\alpha$-hydroxyheme-HO complex exists in three resonance forms: a ferric enolate ion (Scheme 2), a ferric keto anion (2), and a ferrous π neutral radical (3) (5, 6, 14, 15). In light of several lines of evidence accumulated thus far and in line with our present finding, it is likely that among the three resonance forms only the ferrous radical species (3), because of its radical nature, is able to interact with oxygen. Here we propose a possible mechanism for the conversion of $\alpha$-hydroxyheme to verdoheme (Scheme 2).

First, dioxygen electrophilically attacks the ring carbon adjacent to the $\alpha$-oxy group without oxygenation of iron. In fact, the $\alpha$-hydroxyheme-HO-1 complex in the presence of CO (Fig. 3F) was converted into the ferrous verdoheme-HO-1 complex by $O_2$ alone, in a manner similar to that depicted in Fig. 2 (data not shown). Second, rearrangement of a dioxygen adduct (4) occurs through an unknown mechanism producing CO and ferrous verdoheme (5). A similar mechanism has been proposed by Liu et al. (6), and indeed our findings agree with their report with respect to the requirement for $O_2$ but not for electrons. However, there is a discrepancy concerning the oxidation state of the verdoheme iron. Liu et al. (6) claimed that only ferric verdoheme was formed in the anaerobic reaction of hemin HO-1 complex with 1 eq of $H_2O_2$ followed by introduction of 100% $O_2$ and also in the aerobic reaction of the complex with 1 eq of $H_2O_2$. The obvious experimental difference between Liu et al. (6) and our work resides in the gas atmosphere surrounding the verdoheme-HO complex, i.e. strictly anaerobic conditions were maintained during the formation of verdoheme in our experiments, whereas in the study of Liu et al. (6) the verdoheme formed was finally exposed to 100% $O_2$ or air. Hence, it is rather likely that the ferrous verdoheme generated in their system was spontaneously oxidized by atmospheric $O_2$.

Matera et al. (5) argued that both reducing agent and $O_2$ are required for the conversion. According to their description, the addition of $O_2$ to the ferric $\alpha$-hydroxyheme-HO complex resulted in the generation of a radical adduct analogous to $\cdot \dot{4}$ in Scheme 2, but to generate verdoheme from this radical adduct the addition of an electron was required. Thus, they conclude that the catalytic conversion of ferric $\alpha$-hydroxyheme to verdoheme by HO requires $O_2$ plus one electron and proposed two alternative pathways for this conversion; one is the reaction of oxygen with ferrous $\alpha$-hydroxyheme-HO complex and the other involves the aerobic reaction of the ferric complex with the reducing agent (refer to Scheme 3 in Ref. 5). In fact, we also observed that $\alpha$-hydroxyheme bound to rHO-1 in the presence of 1 eq of NADPH and a catalytic amount of NADPH-cytochrome P450 reductase was converted to verdoheme by the addition of $O_2$ (data not shown). This observation, however,
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does not necessarily imply that both oxygen and its reducing equivalent are required for conversion of α-hydroxyheme to verdoheme. Why they were unable to obtain verdoheme from ferric α-hydroxyheme by adding O₂ alone is not clear. Nevertheless the pathways requiring an exogenous electron suggested by Matera et al. (5) still deserve to be examined, because under physiological conditions HO should be always ready to accept electrons from NADPH-cytochrome P450 reductase. In this context, direct comparison of the reaction rates between electron transfer from NADPH-cytochrome P450 reductase to the ferric iron in α-hydroxyheme bound to HO and electronegative attack of O₂ to porphyrin radical should be valuable. For this purpose, an anaerobic multiwavelength stopped-flow apparatus is under construction.

It should be noted that the ESR signal of the ferrous π radical is accurately isotropic and has a g value essentially identical with the free electron, that its peak-to-trough width is 1.6 milliteslas, and that it is readily saturated (Fig. 3). This requires that the unpaired electron be substantially delocalized over the macrocycle, thus allowing hyperfine coupling to protons and/or nitrogen to contribute to the line width, which is substantially larger than that observed with porphyrin π cation radicals (22). Furthermore the heme iron must be in the low-spin ferrous state, a circumstance that implies the heme is six-coordinate. This is naturally the case in the presence of CO but raises the question of the identity of this sixth ligand in the absence of this molecule.

We should consider two possibilities. First, although we conducted the series of titration experiments under conditions as anaerobic as possible, we cannot completely exclude the presence of a residual amount of O₂ and thus the production of CO by oxidation of α-hydroxyheme because of this contaminating O₂. Thus the π neutral radical seen before starting titration (Fig. 3, A-C) might be liganded with O₂ or CO at the sixth position. Relevant to this, Sano et al. (11) observed that when anaerobic solution of the ferric α-hydroxyheme-myoglobin complex was allowed to come into contact with a trace amount of air (much less than 1 eq of oxygen), the rhombic signal (g = 6.3) decreased and the major free radical signal (g = 2.004) appeared, although they assigned the signal to an oxygen adduct of α-hydroxyheme analogous to 4 in Scheme 2. Second, the hydrogen-bonding network formed between the oxygen bound to the heme-HO complex and the distal amino acid residues seems important for oxygen activation and for regiospecific oxidation of the a-meso-carbon (23, 24). In the case of the α-hydroxyheme-HO complex, the heme moiety of a portion of the complex may interact with one of the distal residues, leading to the low-spin ferrous π neutral radical.

Although there are several discrepancies among studies using HO systems, our conclusion is consistent with chemical model studies such as the coupled oxidation of pyridine hemin by ascorbate and oxygen. It has been established that the resonance hybrid of hydroxyheme in the complex with pyridine is converted to ferrous verdoheme with O₂ alone (11, 14, 15). The stoichiometry of the reaction, however, requires expulsion of an oxidizing equivalent. Fuhrhop et al. (25) postulated that an oxidizing species with the formal stoichiometry of (½)H₂O₂ would be expelled in the coupled oxidation system. The difference between the chemical and enzymic systems is the coordination state of heme iron; six-coordinate in the model system and five-coordinate in the enzyme system. The half-time of verdoheme formation was 8 min for the α-hydroxyheme-pyridine complex, whereas it was only 100–120 ms for the α-hydroxyheme-myoglobin complex (five-coordinate model) (11). The mechanism of the relatively higher reactivity of the five-coordinate model, although the low-spin ferrous π neutral radical (six-coordinate) is considered the immediate precursor for verdoheme, is of great interest for the HO reaction.

Acknowledgment—1H NMR spectra were obtained through the auspices of the Kyushu National Industrial Research Institute.

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