Oxygen and One Reducing Equivalent Are Both Required for the Conversion of \(\alpha\)-Hydroxyhemin to Verdoheme in Heme Oxygenase*

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Heme oxygenase is a central enzyme of heme degradation and associated carbon monoxide biosynthesis. We have prepared the \(\alpha\)-hydroxyheme-heme oxygenase complex, which is the first intermediate in the catalytic reaction. The active site structure of the complex was examined by optical absorption, EPR, and resonance Raman spectroscopies. In the ferric form of the enzyme complex, the heme iron is five coordinate high spin and the \(\alpha\)-hydroxyheme group in the complex assumes a structure of an oxophlorin where the \(\alpha\)-meso hydroxy group is deprotonated. In the ferrous form, the \(\alpha\)-hydroxy group is protonated and consequently the prosthetic group assumes a porphyrin structure. The \(\alpha\)-hydroxyheme complex undergoes a redox-linked conversion between a keto and an enol form. The ferric \(\alpha\)-hydroxyheme reacts with molecular oxygen to form a radical species. Reaction of the radical species with a reducing equivalent yields the verdoheme-heme oxygenase complex. Reaction of the ferrous \(\alpha\)-hydroxyheme-heme oxygenase complex with oxygen also yields the verdoheme-enzyme complex. We conclude that the catalytic conversion of ferric \(\alpha\)-hydroxyheme to verdoheme by heme oxygenase requires molecular oxygen and one reducing equivalent.

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Heme oxygenase (HO), an amphipathic microsomal protein, catalyzes the regiospecific oxidative degradation of iron protoporphyrin IX (heme hereafter) to biliverdin, CO, and iron in the presence of NADPH-cytochrome P-450 reductase, which functions as an electron donor (Tenhunen et al., 1969; Kikuchi and Yoshida, 1980; Maines, 1988). In the catalytic cycle of HO, the enzyme first binds one equivalent of heme resulting in the formation of the heme-enzyme complex, which exhibits optical absorption spectral properties similar to those of myoglobin and hemoglobins (Yoshida and Kikuchi, 1978a, 1979). The first electron donated from the reductase reduces the ferric heme iron to the ferrous state, and a molecule of oxygen binds to form a metastable o xo form (Yoshida et al., 1980a). Electron donation to the oxid form initiates the three-stepwise oxygenase reactions during which CO and iron-biliverdin are produced, as shown in Scheme 1. Heme, therefore, participates both as a prosthetic group and as a substrate, a property unique to heme oxygenase (Yoshida and Kikuchi, 1978b).

Recent studies by us and others using recombinant HO preparations have demonstrated that in the heme-enzyme complex, a neutral form of the imidazole group of histidine (His25 in the HO isoform-1 sequence) is the axial hem ligand (Takahashi et al., 1994a, 1994b, 1995; Ito-Maki et al., 1995; Sun et al., 1993, 1994). This is different from the axial ligand coordination structures of cytochrome P-450s and peroxidases where a vectorial polar environment across the heme favors the ferryl-oxo (Fe(IV)=O) intermediates as their activated forms (Dawson, 1988). The neutral imidazole axial coordination is consistent with the proposal that a ferric hydroperoxide intermediate, Fe(III)=O, is the active species in the heme oxygenase catalysis (Noguchi et al., 1983; Wilks and Ortiz de Montellano, 1993). These studies have provided the structural information on which the molecular mechanism of the first oxygenation step has been proposed. Possible roles of the heme oxygenase protein in the regiospecific attack of the first oxygenation step have been revealed by NMR and resonance Raman scattering studies (Hernandez et al., 1994; Takahashi et al., 1995). However, our knowledge of the structure and reactivity of intermediate species, the HO complexes with \(\alpha\)-hydroxyheme and verdoheme, is severely limited. Early work by Yoshida et al. (1981) has shown that \(\alpha\)-hydroxymesozone bound to the enzyme is converted to an iron-biliverdin complex via a verdoheme species by incubation with NADPH and NADPH-cytochrome P-450 reductase. However, most of the other hydroxyheme and verdoheme studies have been carried out on the apomyoglobin complexes or model heme systems (Sano et al., 1986; Fujii, 1990; Ito-Maki et al., 1995; Morishima et al., 1995). It is important to characterize the \(\alpha\)-hydroxyheme-heme oxygenase complex so as to understand the molecular mechanism of the enzyme action of HO. The recent interest in CO as a possible physiological messenger (Verma et al., 1993; Meffert et al., 1994; Shraga-Levine et al., 1994; Suematsu et al., 1995) also warrants the elucidation of the CO biosynthesis reaction, which is asso-
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Prepared by the method described previously (Ikeda-Saito et al., 1986). Human apomyoglobin mutant, H64I, in which the distal histidine is replaced by isoleucine, was prepared by the method described previously (Ikeda-Saito et al., 1992) using the human myoglobin expression system developed by Varadarajan et al. (1989).

α-Benzoyloxyprotoporphyrin (2 mg) was dissolved in methanol (2 ml), and the methanolic solution was stirred while passing nitrogen through the solution for 20 min at room temperature. The solution was heated to 70 °C, and nitrogen-flushed 0.6 N methanolic NaOH (1 ml) was added to the benzoyloxyprotoporphyrin solution via a double tipped needle. The resulting mixture was allowed to reflux for 1.5 h under nitrogen, after which the hydroxylated heme was transferred to a mixture of ethyl acetate (2 ml) and water (2 ml) containing glacial acetic acid (0.2 ml). The precipitated product was collected by centrifugation and dried under a nitrogen stream.

Schematic 1. Intermediates in the reaction of the heme-oxygenase catalyzed conversion of iron protoporphyrin IX to the biliverdin-iron complex.

For the reconstitution with apomyoglobin or HO with α-hydroxoyheme, protein solutions in 0.1 M phosphate buffer, pH 7, were mixed with the α-hydroxyhemin in 0.1 N NaOH solution under strict anaerobic conditions until 90% of the complex formed. Ferrous forms of the α-hydroxoyheme protein complexes were prepared by titrating the α-hydroxoyhemin-protein complexes under anaerobic conditions with a solution of dithionite. The reductive titration was necessary to prepare the ferrous α-hydroxoyheme complex without excess reducing equivalent present in the sample solutions and for the preparation of the partially reduced α-hydroxoyheme complexes. The concentration of the dithionite solution was determined by titration of metmyoglobin solutions of known concentration with the dithionite solution, assuming one equivalent of dithionite will reduce one equivalent of apomyoglobin to metmyoglobin. All the titrations were done using a long stem optical cuvette with a screw top fitted for a rubber septum for evacuation and introduction of gases, designed by Dr. H. Hori of Osaka University (Osaka, Japan). O2 was removed by repeated evacuation followed by filling with nitrogen gas, and then the cuvette was filled with N2 gas to 1.2 atm. Positive inside pressure was required to prevent air penetration into the cuvette during the titration process. Absorption spectra were recorded after each addition of a dithionite or a hemin solution. The CO forms of ferrous α-hydroxoyheme complexed to HO or apomyoglobin were obtained by flushing CO over the anaerobic ferrous samples.

Verdoheme was synthesized by the method of Saito and Itano (1986). Reconstitution with apomyoglobin and HO was carried out as described by Fujii (1990).

Optical absorption spectra were recorded on a Hitachi U-3210 spectrophotometer at 20 °C. Stopped flow experiments were carried out using a Hi-Tech SF-51 MX apparatus. EPR spectra were measured by a Bruker EPR-300 spectrometer with an Oxford liquid helium cryostat. Resonance Raman spectra were obtained from the samples sealed in rotating cells at room temperature. Sample concentrations were adjusted at ~50 μM. Optical absorption spectra were recorded before and after taking Raman spectra to ensure integrity of the sample. The laser excitation wavelengths were 406.7 and 413.1 nm from a krypton laser (Spectra Physics) and 441.6 nm from a helium-cadmium laser (Liconix). The laser power was adjusted at less than 10 mW at the sample point. The scattered light was dispersed by a SpeX single spectrophotograph and detected with a cooled CCD camera (Princeton Instruments). Notch filters (Kaiser) were used to remove Rayleigh scattering. The signal to noise ratio was improved by averaging several 1-min spectra. Indene was used as a frequency standard for calibrating the Raman shifts. The spectral slit width was ~5 cm⁻¹. Measurements were carried out in 0.1 M phosphate buffer, pH 7, or 0.1 M MES buffer, pH 10.

RESULTS

Ferric α-Hydroxoyheme Complexes—The optical absorption spectrum of the ferric α-hydroxoyheme-HO complex is shown in Fig. 1 (spectrum A). The spectrum, which exhibits a broad Soret band at 406 nm with a rather featureless visible region, is quite different from that of the heme-HO complex but is similar to that of the α-hydroxoyheme-myoglobin complex reported by Sano et al. (1986). The spectrum was essentially independent of pH between pH 7 and 10. This is different from that of the heme-HO complex, which exhibits a pH-dependent transition (pKₐ 7.6) from a water-bound high spin state to a hydroxide-bound low spin state (Takahashi et al., 1994a, 1994b; Sun et al., 1993). The ferric iron in the α-hydroxoyheme-HO complex is likely to be pentacoordinate or have a sixth ligand that is not linked with an ionizable group in the distal heme pocket. In order to gain insight into the coordination state of the ferric iron, both apomyoglobin and a mutant apomyoglobin, H64I, were reconstituted with α-hydroxoyheme, and their respective optical absorption spectra were compared (data not shown). The spectrum of the α-hydroxoyheme-apomyoglobin complex is very similar to that of the HO complex (Fig. 1) and is essentially pH-independent between pH 7 and 10. The spectrum of the α-hydroxoyheme complex with the native apomyoglobin is almost identical to that of the α-hydroxoyheme complex with the H64I mutant apomyoglobin. The replacement of the distal histidine (His54) by isoleucine removes the coordinated water molecule in metmyoglobin; the hemin iron in metmyoglobin H64I...
is pentacoordinate with a vacant sixth coordination position (Bogumil et al., 1995). On the basis of similarity between the spectra of α-hydroxyheme incorporated in the apomyoglobin and the H64I mutant, we conclude that the α-hydroxyheme-apomyoglobin complex is a pentacoordinate species. We infer from these results that the ferric α-hydroxyheme-HO complex is also a pentacoordinate species.

Spectrum A in Fig. 2 is the EPR spectrum of the ferric α-hydroxyheme-HO complex in 0.1 M phosphate buffer, pH 7.0. The complex shows a typical rhombic high spin spectrum with g values at 6.08, 5.73, and −2. The spectrum is different from the axial EPR signal of the heme-HO complex but somewhat similar to those of pentacoordinate metmyoglobin mutants with the distal histidine replaced by valine, leucine, or isoleucine (Ikeda-Saito et al., 1992; Bogumil et al., 1995). The rhombic high spin signal of the α-hydroxyheme-HO complex is consistent with a pentacoordinate iron.

Fig. 3 (right panel) compares the high frequency region of the resonance Raman spectrum of the ferric α-hydroxyheme-HO complex (spectrum A) at pH 7.0. These Raman spectra are quite different from typical hemoprotein Raman spectra, which normally consist of a strong ν2 mode at ~1370 cm⁻¹ and weaker ν2 and ν4 modes at ~1500 and 1580 cm⁻¹. The spectra of both α-hydroxyheme-protein complexes are very unique: the presence of two strong lines at ~1220 and 1580 cm⁻¹ and the absence of a strong ν2 mode in the high frequency spectrum. The same comparison is made in the low frequency region as shown in the left panel of Fig. 3 (spectra A and B). The low frequency spectra too are quite different from those of the typical heme compounds. A primary difference is the presence of a strong Raman line at 677 cm⁻¹, observed for the ferric heme-HO complex attributable to the ν2 mode, which is not detected for the α-hydroxyheme-protein complexes.

The resonance Raman spectrum of the α-hydroxyheme-HO complex in buffered D₂O (pD 7) is shown in spectrum C of Fig. 3. The ferric complex has several Raman lines that show small differences when measured in H₂O and D₂O, as is seen in the H₂O minus D₂O difference spectrum (Spectrum D). However, the spectral differences at 1609, 1366, 1254, and 665 cm⁻¹ are likely caused by the presence of a trace amount of the verdoheme-HO complex in the preparation used for the D₂O measurement, as evident from the α-verdoheme-HO spectrum (Spectrum E). The difference pattern at 994 and 876 cm⁻¹ in the difference spectrum originates from nonresonance Raman lines from the phosphate buffer. The ferric form of the α-hydroxyheme-HO complex does not show any appreciable D₂O shift, and this further suggests that the ferric prosthetic group does not possess any exchangeable protons.

Ferrous α-Hydroxyheme Complexes—As shown in Fig. 1 (spectrum B), the dithionite-reduced ferrous α-hydroxyheme-HO complex shows an optical absorption spectrum with a Soret maxima at 432 nm similar to that of the ferrous heme-HO complex, which is a pentacoordinate high spin species (Takahashi et al., 1994b). Binding of exogenous CO to the reduced α-hydroxyheme-protein complex yields a spectrum with a sharp Soret maxima at 421 nm (Spectrum C), which is typical of CO derivatives of hemoproteins.

In contrast to the results obtained for the ferric α-hydroxyheme-HO and apomyoglobin complexes, the ferrous α-hydroxyheme complexes exhibit Raman spectra characteristic of heme compounds. In the high frequency region of the 441.6-nm excitation spectra (Fig. 4, right panel), the Raman lines at 1560 and 1352 cm⁻¹ for the α-hydroxyheme-HO complex (spectrum B) and 1561 and 1352 cm⁻¹ for the α-hydroxyheme-apomyoglobin complex (spectrum A) are assignable to ν2 and ν4 modes, respectively, on the basis of the spectral similarity with the ferrous protoheme-HO complex and myoglobin. In the 413.1-nm excitation spectra, the Raman lines assignable to ν2 mode were detected at 1466 and 1469 cm⁻¹ for the HO and apomyoglobin complexes, respectively (data not shown). These frequencies demonstrate that the ferrous iron atoms in the α-hydroxyheme complexes are pentacoordinate high spin. The low frequency Raman spectra for the ferrous α-hydroxyheme complexes are shown in the left panel of Fig. 4. In this region, an iron-proximal histidine stretching line for pentacoordinate hemoproteins is expected to appear (Kitagawa, 1988). Our previous work using iron-isotopic substitution has established that the line at 218 cm⁻¹ in the ferrous heme-HO complex is the iron-proximal histidine stretching mode (Takahashi et al., 1994b). Therefore, the Raman lines found at 217 and 221 cm⁻¹ for the HO and apomyoglobin complexes of ferrous α-hydroxy-
heme, respectively, can be assigned to the iron-proximal histidine modes because of their spectral similarity with the protoheme-protein complexes. The iron-histidine stretching mode indicates that the proximal histidine in the \( \alpha \)-hydroxyheme complexes has a neutral imidazole as in the heme-HO complex (Takahashi et al., 1994a, 1994b). A line at 239 cm\(^{-1} \), which is a characteristic of deoxy myoglobin, is also present in the \( \alpha \)-hydroxyheme derivative of myoglobin. The corresponding line is not present when either heme or \( \alpha \)-hydroxyheme is complexed with HO, as expected because it originates from a myoglobin-specific interaction with the heme. Because the iron-histidine line is only observed for pentacoordinate ferrous heme derivatives, the observation of this mode supports the conclusion that the ferrous \( \alpha \)-hydroxyheme complexes are pentacoordinate species.

Unlike ferric \( \alpha \)-hydroxyheme-HO, the ferrous form of the complex shows a subtle \( D_2O \) effect in the Raman spectrum (Fig. 3). In the \( D_2O-H_2O \) difference spectrum (spectrum C), the iron-histidine line is only observed for pentacoordinate ferrous heme derivatives, the observation of this mode supports the conclusion that the ferrous \( \alpha \)-hydroxyheme complexes are pentacoordinate species.
D2O small but prominent changes are detected in the region between 595 and 607 cm⁻¹; further differences are seen at higher frequencies around 1100 and 1240 cm⁻¹. These frequencies are associated with porphyrin vibrational modes of the α-hydroxymyoglobin; hence it is likely that an exchangeable proton(s) is associated with α-electrons of the porphyrin ring. A similar D2O effect was also observed for the CO-bound form of the ferrous complex (data not shown).

Reactivity with Oxygen—When ferric α-hydroxymyoglobin-1 is exposed to oxygen, the rhombic high spin EPR spectrum is replaced by a radical type EPR signal at g = 2.004 with a peak to peak line width of ~1.7 millitesla (Fig. 2, spectrum B). This result corresponds to the radical type EPR spectrum obtained by Sano et al. (1986) when oxygen was introduced to the α-hydroxymyoglobin-apomyoglobin complex. Upon introduction of reducing reagent, the ferric α-hydroxymyoglobin radical species was rapidly converted to ferrodoxoheme complex. Reaction of ferrous α-hydroxymyoglobin with oxygen also rapidly yields a verdoxoheme complex. We conclude that there are at least two alternate pathways for the oxygen dependent conversion of α-hydroxymyoglobin to verdoxoheme in HO; one is the reaction of oxygen with the ferrous α-hydroxymyoglobin complex, and the other involves the aerobic reaction of the ferric α-hydroxymyoglobin-HO complex with the reducing agent.

When oxygen was introduced to the solution of the CO form of ferrous α-hydroxymyoglobin-1, the original optical absorption spectrum of the CO form of α-hydroxymyoglobin was replaced by the spectrum of the CO complex of verdoxoheme, which has absorption maxima at 402 and 638 nm (Yoshida et al., 1980b; Yoshida and Noguchi, 1984). Conversion to CO verdoxoheme-HO from the CO complex of α-hydroxymyoglobin by oxygen is rapid. Indeed, when the CO complex of α-hydroxymyoglobin-HO in CO-saturated buffer was mixed with air-saturated phosphate buffer (pH 7) in the stopped flow apparatus, CO-verdoxoheme species was formed within the dead-time of the mixing apparatus (~2 ms), too fast to determine the reaction rates. We infer that either the CO affinity of α-hydroxymyoglobin-HO is extremely low due to very rapid CO dissociation from the iron or that oxygen is activated on the edge of the porphyrin macrocycle, as reported for phagocytic cytochrome b558, rather than by binding to the iron (Iosogai et al., 1995).

The requirement of the reducing equivalent for the conversion to the verdoxoheme complex from the ferric α-hydroxymyoglobin-HO complex was evaluated by observing the reaction of oxygen with a mixture of ferric and ferrous α-hydroxymyoglobin-HO complexes anaerobically generated by adding various amounts of dithionite between zero and one equivalent to the complex. The experiments were conducted in the presence of CO, because the CO form of the verdoxoheme-HO complex is stable, and the HO enzyme reaction thus does not proceed further (Yoshida et al., 1982). This makes it possible to spectrophotometrically estimate the verdoxoheme species formed by the reaction with oxygen. After confirming that there was no changes in the optical spectrum of the ferric α-hydroxymyoglobin under an N2 or a CO atmosphere, varying amounts of dithionite were added. Oxygen was introduced to the mixture of ferric and ferrous CO forms of the α-hydroxymyoglobin-HO complex while maintaining a partial CO atmosphere. Three experiments using 1.0, 0.84, and 0.42 equivalents of dithionite yielded 106, 85, and 47% verdoxohem-CO species. The higher than predicted yield of verdoxoheme is attributable to the small amount of verdoxoheme that forms in the presence of oxygen without the benefit of a reducing agent. Less than 10% verdoxoheme-CO formed if no reducing agent was added. The experiment therefore shows that the amount of verdoxoheme-CO that formed was proportional to the equivalents of dithionite added. We conclude that oxygen and one reducing equivalent are required for the conversion of the ferric α-hydroxymyoglobin to verdoxoheme in HO.

DISCUSSION

Active Site Structure of the α-Hydroxymyoglobin Complexes—The present resonance Raman and optical absorption results have established that the ferrous α-hydroxymyoglobin and α-porphyrin complexes are pentacoordinate high spin species with a neutral imidazole axial ligand as determined for deoxy myoglobin and ferrous ligand-free heme-HO complexes (Takahashi et al., 1994a, 1994b). Although atypical resonance Raman spectra of the ferric α-hydroxymyoglobin complexes preclude the determination of the coordination state of the ferric iron using ν2, ν5, and ν4 frequencies, the optical absorption results on the mutant H64I myoglobin are indicative of a pentacoordinate high spin iron in the ferric α-hydroxymyoglobin and α-porphyrin complexes. Pentacoordinate ferric high spin species are observed in the mutant myoglobins in which the distal histidine is replaced by Val, Leu, and Ile residues; the replacement of distal histidine by apolar residues precludes water penetration to the heme iron (Ikeda-Saito et al., 1992; Bogumil et al., 1995). The ferrous heme-HO complex and metmyoglobin are established as water-bound hexacoordinate compounds with an ionizable distal residue; hence, the distal pocket amino acids are not responsible for the vacant sixth position of the ferric iron in the α-hydroxymyoglobin complexes; unique characteristics of the prosthetic macrocyle, as discussed below, might cause the altered coordination structure.

The D2O effects on the porphyrin modes of the resonance Raman lines are detected for the ferrous α-hydroxymyoglobin-HO complex and its CO adduct but not for the ferrous form. Because the α-meso-hydroxymyoglobin complex on the porphyrin ring has the only exchangeable proton that can affect the resonance Raman spectrum, we attribute this isotope effect to the deuteration of the α-meso-hydroxymyoglobin-HO. The absence of D2O effects in the ferric Raman spectrum suggests that the α-meso-hydroxymyoglobin is likely to be deprotonated. This leads us to propose an iron redox state-linked transition between the keto and the enol forms of the α-hydroxymyoglobin (Scheme 2). In this scheme, the ferric α-hydroxymyoglobin consists primarily of the keto conformation, or oxophlorin, of which one of the resonance structures includes the

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2. Our recent Raman results have shown that the Fe–CO and Fe3C–O stretching frequencies of the CO forms of the α-hydroxymyoglobin-HO and α-hydroxymyoglobin-apomyoglobin complexes are essentially the same as those in the CO derivatives of the heme-HO complex and of myoglobin, respectively. This indicates that the Fe–CO bonding and the electrostatic interaction between the bound CO and the surrounding heme pocket in the α-hydroxymyoglobin are very similar to those in the heme-HO complex. The CO dissociation rate of α-hydroxymyoglobin-HO is thereby expected to be similar to that of the heme-HO complex (0.01 s⁻¹; J. S. Olson, T. Yoshida, and M. Ikeda-Saito, unpublished observations). This slow dissociation rate suggests that oxygen activation in the presence of CO proceeds by a heme edge mechanism without oxygen binding to the iron.
deprotonated meso α-hydroxyheme functional group (Masuoka and Itano, 1987; Sano et al., 1986). The protonated hydroxy structure is the predominant form for the ferrous complex. We think that the proposed oxoporphyrin structure, which has a reduced macrocycle symmetry, is responsible for the atypical resonance Raman and optical absorption spectral properties of the ferric α-hydroxyheme complexes. The protonated hydroxy form assumed in the ferrous α-hydroxyheme complex holds the normal porphyrin macrocycle symmetry, hence its resonance Raman and optical absorption spectral properties are typical of hemoproteins. A neutral overall charge of the α-hydroxyheme complex is presumed to be the driving force for the keto-enol transition between the ferric and ferrous states. In the ferric state, the iron(III) is countered with the combined negative two charge from the porphyrin ring and negative one charge from the oxy anion at the α-meso position. Iron(II) of the ferrous α-hydroxyheme-protein complex is neutralized by the negative two anion of the porphyrin ring; additional negative charge is not needed.

Fujii (1990) and Morishima et al. (1995) showed that ferric α-hydroxyheme exists in a resonance structure of a keto form (1 of Scheme 3), a phenolate form (2), and a ferrous neutral radical form (3). The ferrous neutral radical form is generated by an intramolecular electron transfer between the iron and the macrocycle, and this radical form was assigned as the reacting species with molecular oxygen (Fujii, 1990; Morishima et al., 1995). In heme oxygenase, the keto form (1) appears to be the major species of the resonance structure of the α-hydroxyheme as judged from the EPR and resonance Raman spectra. However, the ferrous neutral radical species (3) is likely to be present, albeit at too low concentration to be detected, because the ferric α-hydroxyheme-HO reacts with molecular oxygen to yield a radical species.

Possible Mechanisms of Verdoheme Formation from α-Hydroxyheme in HO—The present results show that verdoheme can be formed by either the reaction of the ferrous α-hydroxyheme-HO complex with oxygen or that of the ferric α-hydroxyheme-HO complex with reducing agent in the presence of molecular oxygen. In order to explain the two alternate pathways for the verdoheme formation, we propose the reaction scheme depicted in Scheme 3. In this scheme, both pathways are initiated by the ferric oxoporphyrin resonance structure (Fujii, 1990; Morishima et al., 1995). The first pathway utilizes an intramolecular electron transfer from the enolate anion (2) to the ferric iron to yield a ferrous iron and a neutral radical on the porphyrin ring (3). The reaction of 3 with molecular oxygen forms an intermediate species that exhibits a radical EPR signal at g = 2.004. The absence of a ferric EPR signal suggests a ferrous oxidation state. Detection of a radical species is an indication of the absence of strong magnetic interactions between the radical spin and the paramagnetic ferrous iron. Possibility of a π-cation porphyrin radical often seen in peroxidase (Dawson, 1988) enzymes is very remote, and the radical spin is likely located at a distant site from the metal. Previously, Sano et al. (1986) proposed a ferrous peroxy radical form (4) as a possible structure of the radical intermediate with the g = 2.004 EPR signal. Our EPR result is not proof of but is consistent with the peroxy radical form (4). However, other possible structures, such as that resulting from coordination of the terminal end of the dioxygen radical to the iron, cannot be ruled out. The structure of the intermediate is currently being investigated.

The radical intermediate (4) is reduced with one electron to form a peroxy species. Heterolytic cleavage of the peroxy anion with the aid of proton forms hydroxide anion and the verdoheme species (6) is generated with a release of CO.

In the second pathway, one-electron reduction reduces the ferric α-hydroxyheme-HO complex (1) to the ferrous (5) form, followed by a reaction with molecular oxygen to form a verdoheme species (6). There are several possible mechanisms. The oxygen molecule could bind to the ferrous iron and a ferric peroxy species, Fe3+ OOH, would be generated by an intramolecular electron transfer. The hydroperoxide would then shift to a ring carbon to form the peroxy species, which is the same as the reduction product of 4. Alternatively, the reaction of oxygen with the ferrous α-hydroxyheme could generate a superoxide and the ferric α-hydroxyheme, the latter of which is in an equilibrium of 1, 2, and 3. 3 would react with the superoxide to form the peroxy species of the first pathway, and then the verdoheme and CO would be generated. Unfortunately, distinction between these mechanisms cannot be made due to the lack of information on intermediates between 5 and 6.

Sano et al. (1986) reported that the reaction of oxygen with...
the ferric α-hydroxyheme-myoglobin complex yielded a biliverdin-iron complex through a verdoxenase species without the addition of reducing agents. Our results on the heme oxygenase complex is different from those reported of Sano et al. (1986) in that a reducing equivalent is required for the formation of verdoxenase from the ferric α-hydroxyheme-HO complex. We have also observed a very slow gradual conversion to a mixture of verdoxenase and an iron-biliverdin complex after prolonged (4–5 h) incubation of the ferric α-hydroxyheme-HO complex with air. Because verdoxenase is “instantaneously” generated upon the addition of reducing agents to the complex in the presence of air, we disregard this slow spontaneous conversion as a nonphysiological reaction. It is possible that this is what Sano et al. observed in their myoglobin studies.

In a model system, ferric mesohydroxyheme bispyridine complex was shown to form ferrous verdoxenase bispyridine complex upon reacting with oxygen (Fujii, 1990; Morishima et al., 1995). Different from the heme oxygenase system, this reaction does not require an electron. Instead, the reaction proceeds via a ferrous neutral radical intermediate (similar to 3), where an electron may likely be provided from the solvent pyridine. Balch et al. (1993) have shown the reduction of ferric to ferrous verdoxenase in the presence of pyridine, indicating that pyridine can behave as a reductant.

Conclusion—Our present work unequivocally demonstrates that one reducing equivalent and molecular oxygen are required to convert ferric α-hydroxyheme to verdoxenase by the heme oxygenase enzyme catalysis through two different pathways. One pathway is the reaction of oxygen with ferrous α-hydroxyheme in which ferric iron is reduced by the reducing equivalent. The other pathway is the reduction of the radical species generated by the reaction of ferric α-hydroxyheme with molecular oxygen. A unique characteristic of ferric α-hydroxyheme is that it exists as a resonance structure including a ferrous neutral radical species. The resonance structure is a very significant property of α-hydroxyheme, because this makes it possible for the ferric α-hydroxyheme-HO complex to react with molecular oxygen and initiate verdoxenase formation.

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REFERENCES

Balch, A. L., Latos-Grazynski, L., Noll, B. C., Olmstead, M. M., Szterenberg, L. M., and Safari, N. (1993) J. Am. Chem. Soc. 115, 1422–1429

Bogumil, R., Maurus, R., Hildebrand, D. P., Brayer, G. D., and Mauk, G. (1995) Biochemistry 34, 10483–10490

Dawson, J. H. (1988) Science 240, 433–439

Fujii, H. (1990) Studies on the Electronic Structure and Reactivities of Catalytic Intermediates in Heme Enzymes. Ph.D. thesis, Kyoto University

Hernandez, G., Wilks, A., Paless, R., Smith, K. M., Ortiz de Montellano, P. R., and La Mar, G. N. (1994) Biochemistry 33, 6631–6641

Ikeda-Saito, M., Horii, H., Anderson, L. A., Prince, R. C., Pickering, I. J., George, G. N., Sanders, C. R., Il, Lutz, L., McKeevay, E. J., and Mattera, R. (1992) J. Biol. Chem. 267, 22843–22852

Ishikawa, K., Sato, M., Ito, M., and Yoshida, T. (1992) Biochem. Biophys. Res. Commun. 182, 981–986

Isogai, Y., Iizuka, T., and Shiro, Y. (1995) J. Biol. Chem. 270, 7853–7857

Ito-Maki, M., Ishikawa, K., Materia, K. M., Sato, M., Ikeda-Saito, M., and Yoshida, T. (1995) Arch. Biochem. Biophys. 317, 253–258

Kikuchi, G., and Yoshida, T. (1986) Trends Biochem. Sci. 5, 323–325

Kitagawa, T. (1988) in Biological Applications of Raman Spectroscopy (Spiro, T. G., ed) Vol. 3, pp. 97–131, John Wiley & Sons, Inc., New York

Maines, M. D. (1988) FASEB J. 2, 2557–2560

Masuko, N., and Itano, H. A. (1987) Biochemistry 26, 3672–3680

Meffert, M. K., Haley, J. E., Schuman, E. M., Schulman, H., and Madison, D. V. (1994) Neuron 13, 1225–1233

Morishima, I., Fujii, H., Shiro, Y., and Sano, S. (1995) J. Biol. Chem. 34, 1528–1535

Noguchi, M., Yoshida, T., and Kikuchi, G. (1983) J. Biochem. (Tokyo) 93, 1027–1036

Saito, S., and Itano, H. A. (1986) J. Chem. Soc. Perkin Trans. 1 –7

Sano, S., Sano, T., Morishima, I., Shiro, Y., and Maeda, Y. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 531–535

Shirage-Levine, Z., Galron, R., and Sokolovsky, M. (1994) Biochemistry 33, 14656–14659

Suematsu, M., Goda, N., Sano, T., Kashiwagi, S., Egawa, T., Shinoda, Y., and Ishimura, Y. (1995) J. Clin. Invest. 96, 2431–2437

Sun, J., Wilks, A., Ortiz de Montellano, P. R., and Leehr, T. M. (1993) J. Biol. Chem. 268, 14151–14157

Sun, J., Leehr, T. M., Wilks, A., and Ortiz de Montellano, P. R. (1994) Biochemistry 33, 13734–13740

Takahashi, S., Wang, J., Rousseau, D. L., Ishikawa, K., Yoshida, T., Host, J. R., and Ikeda-Saito, M. (1994a) J. Biol. Chem. 269, 1010–1014

Takahashi, S., Wang, J., Rousseau, D. L., Ishikawa, K., Yoshida, T., Takeuchi, N., and Ikeda-Saito, M. (1994b) Biochemistry 33, 5531–5538

Takahashi, S., Ishikawa, K., Takeuchi, N., Ikeda-Saito, M., Yoshida, T., and Rousseau, D. L. (1995) Am. Chem. Soc. 117, 6002–6006

Teale, F. W. J. (1959) Biochim. Biophys. Acta 35, 543

Teale, F. W. J. (1959) Biochim. Biophys. Acta 35, 543

Vanadaranjan, R., Laframboise, D. G., and Boxer, S. G. (1989) Biochemistry 28, 3771–3781

Wera, A., Hirsch, D. J., Giotti, G. V., and Snyder, S. H. (1993) Science 259, 381–385

Wilks, A., and Ortiz de Montellano, P. R. (1993) J. Biol. Chem. 268, 22357–22362

Yoshida, T., and Kikuchi, G. (1978a) J. Biol. Chem. 253, 4224–4229

Yoshida, T., and Kikuchi, G. (1978b) J. Biol. Chem. 253, 4230–4236

Yoshida, T., and Kikuchi, G. (1979) J. Biol. Chem. 254, 4487–4491

Yoshida, T., and Noguchi, M. (1984) Biochem. (Tokyo) 96, 563–570

Yoshida, T., Noguchi, M., and Kikuchi, G. (1986a) J. Biol. Chem. 261, 4418–4420

Yoshida, T., Noguchi, M., and Kikuchi, G. (1986b) J. Biol. Chem. 261, 4567–4569

Yoshida, T., Noguchi, M., and Kikuchi, G. (1987) Biochem. (Tokyo) 26, 557–563

Yoshida, T., Noguchi, M., Kikuchi, G., and Sano, S. (1981) J. Biochem. (Tokyo) 90, 125–131

Yoshida, T., Noguchi, M., and Kikuchi, G. (1982) J. Biol. Chem. 257, 9345–9348
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