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A kinetic study of the mechanism of conversion of α-hydroxyheme to verdoheme while bound to heme oxygenase

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Abstract

O_2-dependent reactions of the ferric and ferrous forms of α-hydroxyheme complexed with water-soluble rat heme oxygenase-1 were examined by rapid-scan stopped-flow measurements. Ferric α-hydroxyheme reacted with O_2 to form ferric verdoheme with an O_2-dependent rate constant of 4×10^5 M^{-1} s^{-1} at pH 7.4 and 9.0. A decrease of the rate constant to 2.8×10^5 M^{-1} s^{-1} at pH 6.5 indicates that the reaction proceeds by direct attack of O_2 on the π-neutral radical form of α-hydroxyheme, which is generated by deprotonation of the α-hydroxy group. The reaction of ferrous α-hydroxyheme with O_2 yielded ferrous verdoheme in a biphasic fashion involving a new intermediate having absorption maxima at 415 and 815 nm. The rate constants for this two-step reaction were 68 and 145 s^{-1}. These results show that conversion of α-hydroxyheme to verdoheme is much faster than the reduction of coordinated iron (<1 s^{-1}) under physiological conditions [Y. Liu, P.R. Ortiz de Montellano, Reaction intermediates and single turnover rate constants for the oxidation of heme by human heme oxygenase-1, J. Biol. Chem. 275 (2000) 5297–5307], suggesting that, in vivo, the conversion of ferric α-hydroxyheme to ferric verdoheme precedes the reduction of ferric α-hydroxyheme.

Keywords: Heme oxygenase; α-Hydroxyheme; Verdoheme; Stopped-flow spectrophotometry

Heme oxygenase (HO) is a microsomal enzyme that catalyzes the O_2-dependent degradation of heme using reducing equivalents from NADPH-cytochrome P450 reductase and produces biliverdin, CO, and iron [1–3]. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. The degradation of heme to biliverdin by HO is a three-step process [4,5]. In the first step, O_2 bound to the reduced heme iron is activated to ferric hydroperoxide, and electrophilic addition of its terminal oxygen to the α-*meso* carbon produces α-hydroxyheme in the ferric state. In the second step, α-hydroxyheme is converted to verdoheme with concomitant release of the α-*meso* carbon as CO [6]. Lastly, the oxygen bridge of verdoheme is cleaved to yield iron and biliverdin.

Major progress has been made in understanding the nature of the first reaction, α-hydroxylation of heme, but the mechanisms for the subsequent steps are still not clear. In particular, whether or not α-hydroxyheme requires an electron before reacting with O_2 to yield verdoheme in the second step remains unsettled. Liu et al. [7,8] reported that the oxygen molecule alone converts α-hydroxyheme to ferric verdoheme and that one electron is used to reduce the ferric verdoheme to the ferrous species. Their results were partly supported by our stoichiometric experiment that showed the conversion of the ferric α-hydroxyheme bound to HO to the ferrous verdoheme occurs solely in the presence of oxygen and an electron was not required [9,10]. Although there is a difference in the final oxidation states of the verdoheme obtained by Liu et al. and by us, the two studies agreed that the only requirement for the conversion of

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α-hydroxyheme to verdoheme is an oxygen molecule. In contrast, Mansfield Matera et al. [11,12] insisted that the ferric α-hydroxyheme bound to HO must be reduced to the ferrous form before it reacts with O_2 to yield the ferrous verdoheme, thus indicating that this reaction consumed both O_2 and an electron quasi-simultaneously.

To further explore the mechanism of conversion of α-hydroxyheme to verdoheme, we have examined the O_2-dependent reactions of the ferric and ferrous forms of α-hydroxyheme in complex with water-soluble recombinant rat HO-1 using the rapid-scan stopped-flow technique under strictly anaerobic conditions. This is the first kinetic study of the conversion of α-hydroxyheme to verdoheme.

Materials and methods

Preparation of α-hydroxyheme–HO-1 complex. α-Hydroxyheme was synthesized as described [10,11,13]. A soluble form of rat HO-1 (denoted HO-1) lacking the 22-amino acid C-terminal hydrophobic segment was expressed in *Escherichia coli* and purified as described previously [14]. Anaerobic reconstitution of the ferric α-hydroxyheme–HO-1 complex in 100 mM potassium phosphate buffer at pH 7.4 was carried out as described [9,10]. The solutions of the ferric α-hydroxyheme–HO-1 complex at pH 6.5 or 9.0 were prepared by repeated anaerobic cycles of mixing the neutral solution of the ferric α-hydroxyheme–HO-1 complex with 100 mM phosphate buffer at pH 6.5 or 9.0 followed by concentration with a centrifugal filter device. The ferrous form of the α-hydroxyheme complex was prepared by reducing the ferric form with a stoichiometric amount of sodium dithionite under anaerobic conditions.

Stopped-flow spectroscopy and kinetic measurements. Single turnover reactions of the reconstituted α-hydroxyheme–HO-1 complex with O_2 were monitored using an Applied Photophysics SX.18MV-R stopped-flow apparatus with a diode array detector. To make the experiment as anaerobic as possible, the apparatus was placed in a glove box filled with N_2 at room temperature (25 °C). To remove traces of O_2 from the stopped-flow system, the flow system of the apparatus was flushed with 10 mM sodium dithionite solution and then thoroughly washed with anaerobic water and buffer. Typically, syringe A contained the ferric or ferrous α-hydroxyheme–HO-1 complex (30 μM) in 100 mM potassium phosphate buffer at pH 7.4. Syringe B contained various amounts of O_2 in the same buffer ($[O_2] = 100$–800 μM). The O_2-dissolved buffers were prepared by mixing anaerobic and air-saturated buffers or by mixing air-saturated and O_2-saturated buffers in appropriate ratios. The concentration of O_2 was measured using a YSI 5300A Biological Oxygen Monitor System immediately prior to the stopped-flow measurements. After the solutions in syringes A and B were mixed in a ratio of 1:1, the spectra were recorded every 2.5 ms between 300 and 1100 nm. The observed first-order rate constants for the absorption changes were obtained by extraction of the data at selected wavelengths followed by least squares exponential fitting using KaleidaGraph Version 3.6 (Synergy Software).

ESR spectroscopy. X-band ESR spectra were recorded at 10 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 amplitude, 1 mT; microwave frequency, 9.93 GHz; and microwave power, 1–1000 μW. The microwave frequency was measured with a microwave frequency counter (Advantest) and the magnetic field strength determined with a JEOL NMR counter (JEOL ES-OC1).

Results and discussion

Conversion of ferric α-hydroxyheme to ferric verdoheme

Fig. 1 shows changes in absorption spectra during the reaction of ferric α-hydroxyheme–HO-1 complex with O_2. Upon mixing, the Soret absorption at 405 nm decreased and the absorption between 640 and 720 nm increased. The appearance of the broad absorption centered at 680 nm indicates the formation of ferric verdoheme [7,9,10], and the well-defined isosbestic points at 460, 555, and 612 nm suggest that no spectral intermediates are present in this reaction. These spectral changes are similar to those of a previous study, in which ferric α-hydroxyheme complexed to human HO, obtained by anaerobic reaction of the heme–HO complex with one equivalent of H_2O_2, was converted to ferric verdoheme upon exposure to oxy-

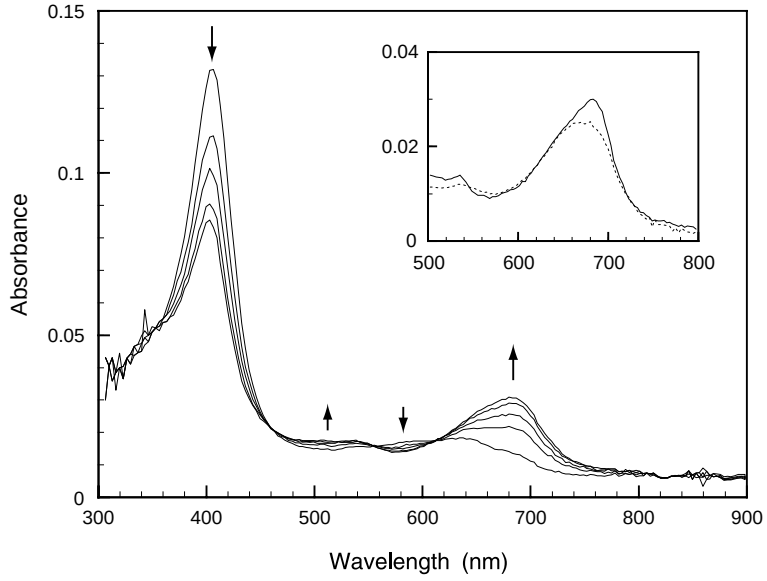


Fig. 1. Spectral changes of the ferric α-hydroxyheme–HO-1 complex (15 μM) in the reaction with O_2 (123 μM) at 25 °C and pH 7.4. Rapid-scan absorption spectra were recorded at 0, 10, 20, 40, and 80 ms after mixing. Inset: absorption spectra between 500 and 800 nm of the reaction product of the ferric α-hydroxyheme–HO-1 complex (15 μM) with O_2 (62 μM) recorded at 1 and 60 s after mixing.

gen [7]. When the ferric α-hydroxyheme–HO-1 complex was mixed with low [O_2] (30–70 μM), the spectrum of the resultant ferric verdoheme changed slowly over 30 s to produce a new spectrum with absorbance maxima at 400, 535, and 685 nm (Fig. 1, inset); these wavelengths are characteristic of the ferrous form of verdoheme [7,9,10]. This is consistent with our early observation that anaerobic titration of the ferric α-hydroxyheme–HO-1 complex with O_2 yields ferrous verdoheme [10]. The reducing equivalent required for reduction of the ferric verdoheme to the ferrous form may be provided from the environment of the verdoheme iron atom, for example, from the enzyme protein or from buffer.

The increase in absorbance at 680 nm that occurs 0.2 s after mixing was well fitted to a single exponential and the calculated first-order rate constant ($k_{1,obs}$) for the formation of the ferric verdoheme at an oxygen concentration of 123 μM was 49 s^{-1} at 25 °C and pH 7.4. A plot of $k_{1,obs}$ values vs. O_2 concentration was linear up to an oxygen concentration of 400 μM and yielded a second-order rate constant (k_1) of 3.9×10^5 M^{-1} s^{-1} (Fig. 2). The $k_{1,obs}$ values calculated from the decrease in the Soret absorption of the ferric α-hydroxyheme–HO-1 complex were also linearly dependent on O_2 concentration with a similar k_1 of 4.0×10^5 M^{-1} s^{-1} (data not shown).

On the basis of ESR and resonance Raman spectroscopic studies of α-hydroxyheme in complex with HO [7,10,11] or apomyoglobin [13], it is believed that the ferric form of α-hydroxyheme is deprotonated at neutral pH and is in equilibrium with a ferrous π-neutral radical form that is generated by an intramolecular electron transfer between the iron and the porphyrin ring. This radical form is thought to be the reacting species with O_2; direct binding of O_2 to the ferrous radical at the porphyrin edge triggers CO extrusion and verdoheme formation. The linear dependency of the $k_{1,obs}$ values on O_2 concentration should reflect the reaction of the radical form of α-hydroxyheme with O_2.

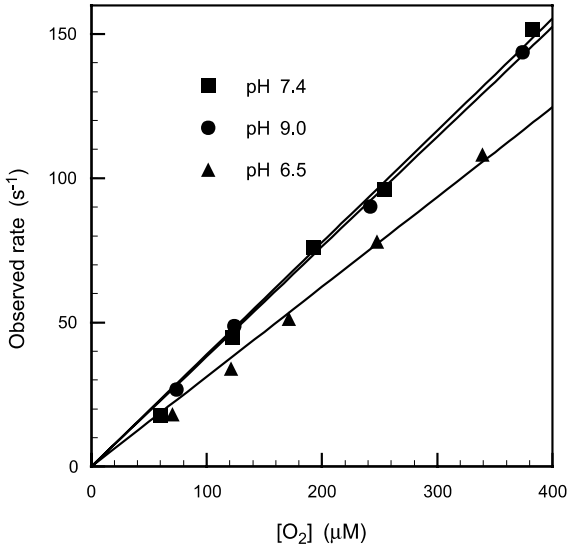


Fig. 2. Plot of the observed rate constants ($k_{1,obs}$) vs. [O_2] in the reaction of ferric α-hydroxyheme–HO-1 complex with O_2 at 25 °C, and pH 7.4 (■), 9.0 (●), and 6.5 (▲).

Effect of pH on the rate constants for ferric verdoheme formation

The k_1 value at pH 9.0 was 3.8×10^5 M^{-1} s^{-1}, which is close to that at pH 7.4 (3.9×10^5 M^{-1} s^{-1}), while it decreased to 2.8×10^5 M^{-1} s^{-1} at pH 6.5 (Fig. 2). Although the pH profile of the rate constants has not yet been adequately investigated, this observation suggests that the deprotonation of at least one group with a pK_a near 6.5 is involved in catalysis. It is reasonable to assign the α-hydroxy group to this deprotonating group, because the deprotonation of the α-hydroxy substituent is required for formation of the π-neutral radical. The effects of pH on k_1 should reflect the formation of this species.

Supporting evidence was provided by ESR spectra of the α-hydroxyheme–HO-1 complex measured at pHs 6.5 and 9.0 (Fig. 3). At pH 9.0 (Fig. 3A), a strongly rhombic high spin signal with g values of 6.05, 7.78, and 2.00 together with a radical signal ($g = 2.004$) indicates the coexistence of the ferric anion and ferrous radical forms; they became evident at lower microwave powers. Similar ESR signals were also observed at pH 7.4 [10]. At pH 6.5 (Fig. 3B), however, only the rhombic signal was observed and the radical signal was nominal even at lower powers. This can be attributed to a relatively smaller population of the radical form of α-hydroxyheme at pH 6.5, probably due to insufficient deprotonation of the α-hydroxy group. Even if the difference of temperatures between stopped-flow and ESR measurements is taken into account, the population of the π-neutral radical form must be small at acidic pH. Therefore, we conclude that the spectral changes observed in Fig. 1 represent the reaction of the radical form of α-hydroxyheme with O_2.

Conversion of ferrous α-hydroxyheme to ferrous verdoheme

Fig. 4 shows changes in absorption spectra during the reaction of the ferrous α-hydroxyheme–HO-1 complex with O_2. The reaction can be divided into two phases. In the first phase lasting up to 10 ms after mixing, the Soret band at 430 nm decreased and shifted to 415 nm with a concomitant increase and decrease of absorptions around 815 and 540 nm, respectively (Fig. 4A). A small amount of ferrous verdoheme was also formed, as indicated by the increase of broad absorption band at 685 nm. In the subsequent 90 ms, the broad Soret band at 415 nm shifted to 400 nm with a slight loss in intensity while a marked increase of the absorption at 685 nm occurred (Fig. 4B). These changes were associated with a decrease of the 815-nm band, suggesting that this species is an intermediate that transiently accumulates in the course of the reaction. The appearance of absorption at 535 nm together with clear increase of the 685-nm band indicates that the major product of the reaction is ferrous verdoheme.

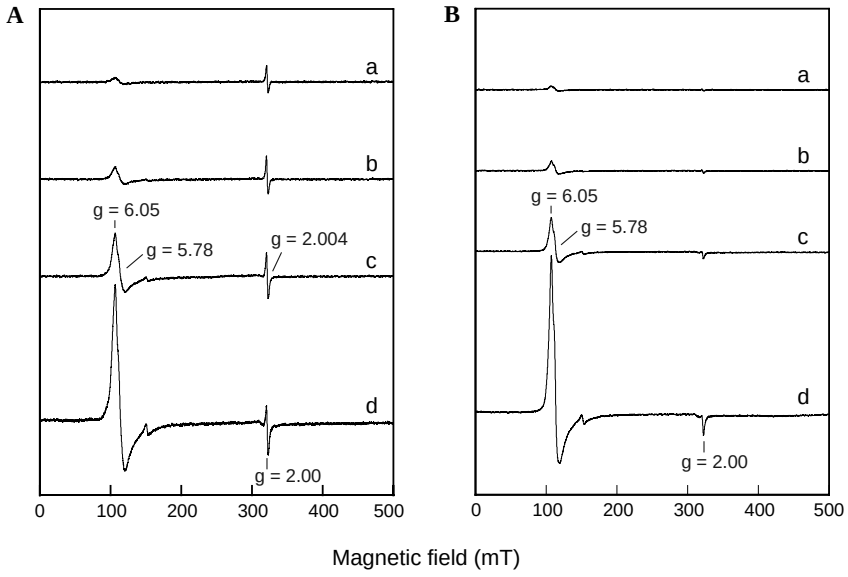


Fig. 3. ESR spectra of the ferric α-hydroxyheme–HO-1 complex at pH 9.0 (A) and 6.5 (B) recorded at different microwave powers. Traces (a–d) represent the spectra measured at 1, 10, 100, and 1000 μW, respectively. Other instrumental conditions are described under Materials and methods.

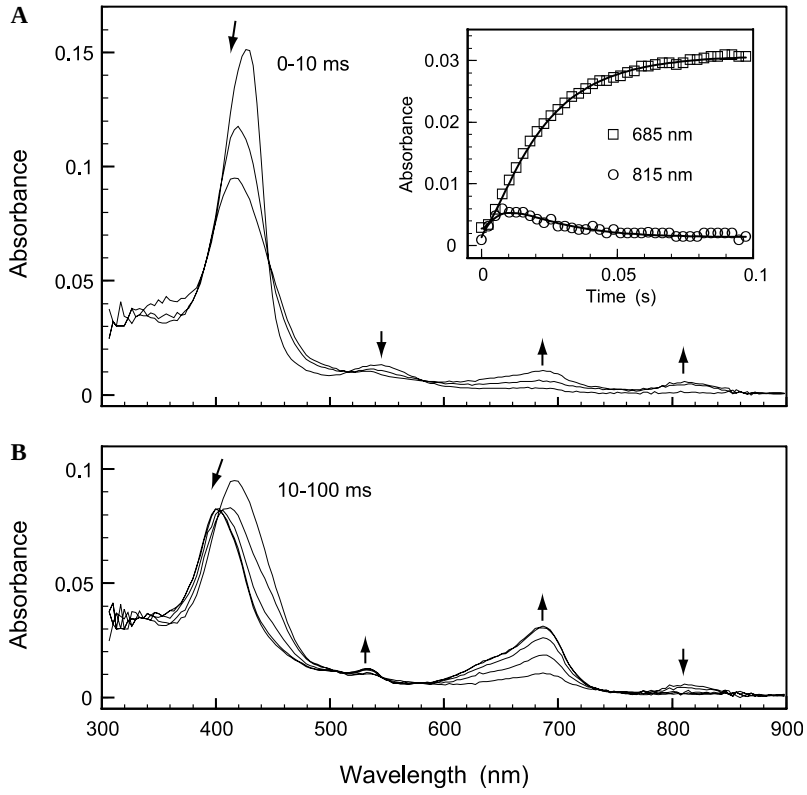


Fig. 4. Spectral changes of the ferrous α-hydroxyheme–HO-1 complex (15 μM) in the reaction with O_2 (76 μM) at 25 °C and pH 7.4. Rapid-scan absorption spectra were recorded at 0, 5, and 10 ms (A), and 10, 20, 40, 80, and 100 ms (B) after mixing. (A) Inset: single wavelength kinetic time traces of the reaction at 685 (□) and 815 nm (○). The solid lines with each trace represent the best fit to the successive single exponential functions.

The kinetic traces of the absorbance changes at 685 and 815 nm are shown in the inset to Fig. 4A. The absorbance at 685 nm showed a sigmoidal increase, whereas the absorbance at 815 nm showed a transient increase followed by a slow decay. This time course is typical of two consecutive first-order reactions with a transient intermediate, the 815-nm species, prior to formation of the ferrous verdoheme. The respective rate constants for the formation of the 815-nm species and ferrous verdoheme were obtained by fitting the data to the scheme shown in Scheme 1.

The observed rate constants, $k_{2,obs}$ and $k_{3,obs}$, were 53 and 137 s^{-1}, respectively, at an oxygen concentration of 76 μM.

$$\text{Fe}^{2+}\text{-}\alpha\text{-hydroxyheme} \xrightarrow{k_2} \text{815-nm species} \xrightarrow{k_3} \text{Fe}^{2+}\text{-verdoheme}$$

Scheme 1.

Plots of $k_{2,obs}$ and $k_{3,obs}$ as a function of oxygen concentration are shown in Fig. 5. As expected, the $k_{3,obs}$ was independent of $[O_2]$ and an average value of 145 s^{-1} was obtained for k_3. On the other hand, the plot of $k_{2,obs}$ showed an apparent hyperbolic increase, indicating the existence of rapid association/dissociation equilibrium between the ferrous α-hydroxyheme and O_2; this is contrast to the linear dependence of $k_{1,obs}$ upon oxygen concentration in the reaction of ferric α-hydroxyheme with O_2. The value of k_2 was estimated to be 68 s^{-1} by nonlinear least square fitting of the data to Eq. (1).

$$k_{2,obs} = k_2[O_2]/(K_{app} + [O_2]) \quad (1)$$

K_{app} represents the dissociation constant between the complex and O_2. In the present study, however, we could not obtain a reliable K_{app} value because of the lack of data at extremely low oxygen concentrations. It proved difficult to obtain pseudo first-order rate constants at such low oxygen concentration and no further effort was made to determine a precise K_{app}. Although the true nature of this intermediate has yet to be identified, it should be noted that a transient band at 820 nm appeared during the titration of ferric α-hydroxyheme–HO-1 complex with O_2 [10]. Furthermore, a similar absorption band was observed in the anaerobic reaction of the heme–HO complex with an equimolar amount of H_2O_2 that disappeared upon subsequent exposure to O_2 [7].

Relevance to heme degradation in vivo by HO

As noted in the Introduction, whether or not α-hydroxyheme requires an electron before reacting with O_2 to yield

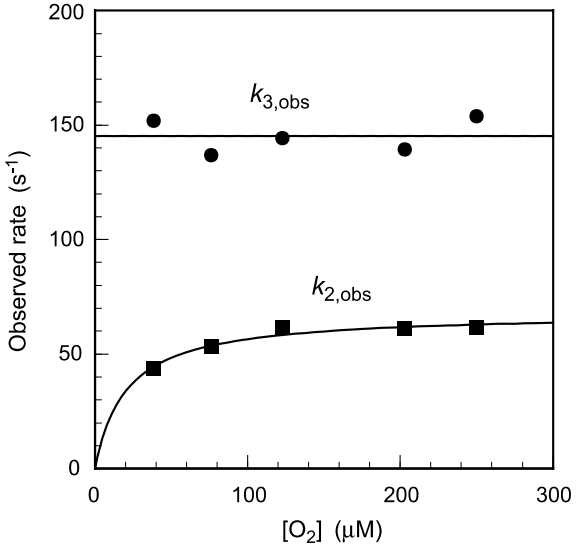
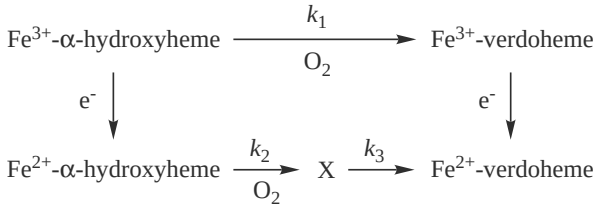


Fig. 5. Dependencies on $[O_2]$ of the observed rates, $k_{2,obs}$ (■) and $k_{3,obs}$ (●), in the reaction of ferrous α-hydroxyheme–HO-1 complex with O_2 at 25 °C and pH 7.4.



Scheme 2. Reaction pathways of conversion of α-hydroxyheme to verdoheme. X represents the 815-nm species.

verdoheme remains unsettled. Put another way, "Does verdoheme formation start from ferric or from ferrous α-hydroxyheme?" The present study documents that both can take place in vitro and delineates the kinetic properties of the two pathways.

Scheme 2 schematizes the two pathways. Simply comparing the rate constants at an oxygen concentration of 160 μM, which is almost equal to that of arterial blood or pulmonary alveoli, the ferric α-hydroxyheme to ferric verdoheme pathway ($k_{1,obs}$ = 63 s^{-1}) seems to be preferable to the two-step conversion of ferrous α-hydroxyheme to ferrous verdoheme; $k_{2,obs}$ of 60 s^{-1} and k_3 of 145 s^{-1} give a net rate constant of 42 s^{-1} for the overall reaction. On the other hand, at an oxygen concentration of 32 μM, which is comparable to the oxygen level in tissue, the net rate constant for the ferrous α-hydroxyheme to ferrous verdoheme conversion (32 s^{-1}) exceeds that of the ferric α-hydroxyheme to ferric verdoheme pathway (13 s^{-1}). From these considerations, the ferrous α-hydroxyheme to ferrous verdoheme pathway seems to be preferred in the peripheral tissues with a relatively low oxygen concentration. However, we also have to take the rate of reduction of the coordinated iron of α-hydroxyheme into account. Liu and Ortiz de Montellano [8] reported the rate constants for single turnover of degradation from heme to biliverdin by HO. Under their conditions employing substoichiometric amount of NADPH-cytochrome P450 reductase, which mimics the in vivo situation, the reduction rates of ferric heme and ferric verdoheme to the respective ferrous forms were estimated to be 0.21 and 0.55 s^{-1}, respectively, at 25 °C. These values are much smaller than the rate constants for the conversion of α-hydroxyheme to verdoheme obtained in this study. Although the reduction rate of ferric α-hydroxyheme is unknown, it is to be probably less than 1 s^{-1}. Thus, it seems unlikely that the reduction of the coordinated iron of ferric α-hydroxyheme should precede the reaction of ferric α-hydroxyheme with O_2 in vivo. Therefore, in the physiological HO reaction, it is plausible that the ferric α-hydroxyheme to ferric verdoheme pathway is dominant and that reduction of the verdoheme iron is subsequent.

In conclusion, we demonstrate that either the ferric or ferrous form of α-hydroxyheme can be converted to verdoheme in an O_2-depending manner in vitro. Ferric α-hydroxyheme is converted to ferric verdoheme by direct attack of O_2 on the π-neutral radical form and ferrous α-hydroxyheme is converted to ferrous verdoheme via the

815-nm species. In view of the ambient oxygen concentration and efficiency of NADPH-cytochrome P450 reductase system in tissues, we consider that the ferric α-hydroxyheme to ferric verdoheme pathway prevails in vivo.

Acknowledgments

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