Reaction Intermediates and Single Turnover Rate Constants for the Oxidation of Heme by Human Heme Oxygenase-1*

(Received for publication, September 18, 1999, and in revised form, November 19, 1999)

Yi Liu and Paul R. Ortiz de Montellano‡
From the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446

Heme oxygenase converts heme to biliverdin, iron, and CO in a reaction with two established intermediates, a-meso-hydroxyheme and verdoheme. Transient kinetic studies show that the conversion of Fe³⁺-heme to Fe³⁺-verdoheme is biphasic. Electron transfer to the heme (0.11 s⁻¹ at 4 °C and 0.49 s⁻¹ at 25 °C) followed by rapid O₂ binding yields the ferrous dioxy complex. Transfer of an electron (0.056 s⁻¹ at 4 °C and 0.21 s⁻¹ at 25 °C) to this complex triggers the formation of a-meso-hydroxyheme and Verdoheme. Transient kinetic studies show that the conversion of Fe³⁺-verdoheme to Fe³⁺-biliverdin is biphasic. Thus, reduction of Fe²⁺ to Fe²⁺-verdoheme (0.15 s⁻¹ at 4 °C and 0.55 s⁻¹ at 25 °C) followed by O₂ binding and an electron transfer produces Fe²⁺-biliverdin (0.025 s⁻¹ at 4 °C and 0.10 s⁻¹ at 25 °C). The conversion of Fe³⁺-biliverdin to free biliverdin is triphasic. Reduction of Fe³⁺-biliverdin to Fe²⁺-biliverdin (0.035 s⁻¹ at 4 °C and 0.15 s⁻¹ at 25 °C), followed by rapid release of Fe²⁺ (0.19 s⁻¹ at 4 °C and 0.39 s⁻¹ at 25 °C), yields the biliverdin-enzyme complex from which biliverdin slowly dissociates (0.007 s⁻¹ at 4 °C and 0.03 s⁻¹ at 25 °C). The rate of Fe²⁺ release agrees with the rate of Fe³⁺-biliverdin reduction. Fe²⁺ release clearly precedes biliverdin dissociation. In the absence of biliverdin reductase, biliverdin release is the rate-limiting step, but in its presence biliverdin release is accelerated and the overall rate of heme degradation is limited by the conversion of Fe²⁺-verdoheme to the Fe³⁺-biliverdin.

---

Heme oxygenase catalyzes the NADPH and P450 reductase-dependent oxidation of heme to biliverdin, iron, and CO (1) (Scheme 1). The enzyme, which employs heme as both the prosthetic group and substrate, regiospecifically oxidizes the heme at the a-meso position. This enzyme is physiologically important, in part because of the biological properties of its organic reaction products. Biliverdin is reduced by biliverdin reductase to bilirubin, which is then excreted as the glucuronic acid conjugate (2). The excretion of bilirubin is frequently impaired in newborn children as well as in individuals with genetic glucurontransferase deficiencies (3). High concentrations of unconjugated bilirubin are neurotoxic, and the prevention of its accumulation through phototherapy or inhibition of heme oxygenase is of clinical importance (4–6). CO, the other organic product of heme oxygenase, appears to play a role akin to nitric oxide as a signaling molecule (7–9). A role for CO in signaling pathways has received strong support from recent studies with heme oxygenase knockout mice (10, 11).

The existence of two heme oxygenase isoforms, HO-1 and HO-2, is well established (12–14), and a third isoform whose significance is unclear has been described (15). HO-1 is induced by chemical agents and a variety of stress conditions and is found in highest concentration in the spleen and liver. HO-2 is not induced by exogenous stimuli and is found in highest concentrations in the brain and testes. The heme oxygenases are membrane-bound proteins (16, 17), but water-soluble, catalytically active versions of rat and human HO-1 without the 23 carboxyl-terminal amino acid membrane anchor have been expressed in Escherichia coli (18–20).

His-25 has been identified as the proximal iron ligand in the heme-HO-1 complex by site-directed mutagenesis and resonance Raman spectroscopy (21–26). Replacement of the proximal histidine residue by an alanine produces a catalytically inactive protein that binds heme without providing a strong axial iron ligand (22). Recovery of both the iron-histidine coordination and full catalytic activity when imidazole binds to the heme-H25A mutant confirms the role of His-25 as the proximal iron ligand (23). The assignment of His-25 as the proximal iron ligand is confirmed by the human HO-1 crystal structure (27).

The HO-1-catalyzed oxidation of heme involves sequential a-meso-hydroxylation, oxygen-dependent fragmentation of the a-meso-hydroxymethylen to verdoheme, and oxidative cleavage of verdoheme to biliverdin (28). The formation of a-meso-hydroxyheme (29–31) and verdoheme (18, 32, 33) in the enzymatic reaction have been directly demonstrated. The intermediates and reaction steps involved in the formation of a-meso-hydroxyheme and its subsequent conversion to verdoheme have been the focus of several studies (18, 29, 33, 34). a-meso-Hydroxylation, the first step, proceeds via a P450 reductase-dependent reduction of the iron to the ferrous state, binding of oxygen to the reduced iron, and a second one-electron reduction of the ferrous-dioxy complex (28). The resulting ferric peroxide complex undergoes electrophilic addition of the distal oxygen to the porphyrin ring to yield a-meso-hydroxyheme (28). The a-meso-hydroxyheme exists as a resonance mixture of the Fe(III) phenolate, Fe(III) keto anion, and Fe(II) π neutral radical. Oxygen rapidly reacts with the Fe(II) π neutral radical structure, via as yet undetected intermediates, to give Fe(II)-verdoheme (29). Even less is known about the reaction intermediates involved in the conversion of verdoheme to biliverdin. Yoshida and Kikuchi (35) reported that the reconstituted heme-HO-1 complex was oxidized to the Fe⁵⁺-biliverdin complex instead of free biliverdin when ascorbate was used as a

* This work was supported by National Institutes of Health Grant DK30297. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
‡ To whom correspondence should be addressed: School of Pharmacy, University of California, San Francisco, CA 94143-0446. Tel.: 415-476-2903; Fax: 415-502-4728 or 476-0688; E-mail: ortizr@gel.ucsf.edu.
† The abbreviations used are: heme, iron protoporphyrin IX regardless of oxidation and ligation state; HO-1, heme oxygenase isoform 1; hHO-1, truncated human HO-1; EPR, electron paramagnetic resonance; P450 reductase, NADPH-cytochrome P450 reductase; MOPS, 4-morpholinepropanesulfonic acid.

This paper is available on line at http://www.jbc.org

5297
Deionized, doubly distilled water was used for all experiments. 

**Scheme 1. Reaction intermediates in the HO-1-catalyzed oxidation of heme to biliverdin.** The substituents on the porphyrin are vinyl (V) and propionate (Pr).

surrogate-reducing agent. The Fe$^{3+}$-biliverdin thus obtained was readily converted to free biliverdin when NADPH and P450 reductase were added (35). However, these studies did not establish that the ferric complex is a true precursor of biliverdin in the P450 reductase-dependent oxidation of heme by heme oxygenase.

Through the use of stopped-flow spectroscopy, we provide here kinetic and spectroscopic evidence on the reaction steps involved in the conversion of Fe$^{3+}$-verdoheme to biliverdin. Our findings indicate that Fe$^{3+}$-verdoheme is first reduced to Fe$^{2+}$-verdoheme, which upon binding of oxygen and transfer of a second electron is converted to the Fe$^{3+}$-biliverdin complex. One-electron reduction of the Fe$^{3+}$-biliverdin complex by P450 reductase is followed by release of the ferrous iron atom. In the final step of the catalytic cycle, the metal-free biliverdin dissociates from the enzyme. Determination of the rate constants for formation of verdoheme, Fe$^{3+}$-biliverdin, and the subsequent steps of the reaction establish that biliverdin dissociation is the rate-limiting step in the heme degradation pathway in the absence of biliverdin reductase. In the presence of biliverdin reductase, the overall rate of heme degradation appears to be limited by the conversion of Fe$^{2+}$-biliverdin to Fe$^{3+}$-biliverdin.

**EXPERIMENTAL PROCEDURES**

**Materials**—H$_2$O$_2$ (30%), NADPH, heme, protoporphyrin IX, bovine serum albumin, sodium dithionite, sodium ascorbate, and ferrozine were obtained from Aldrich or Sigma. High purity argon (99.998%), CO (99.95%), and O$_2$ (99.9%) were obtained from Matheson and Aldrich, respectively. All chemicals were used without further purification.

**General Methods**—The hHO-1 construct that was used encoded the human hHO-1 complex without the 23 amino-terminal amino acids (19). The plasmid used was pET22b+ (F' era Dlac-proABirpL, R300 lacZDM15 hsdR17) and overexpressed human hHO-1 using the recombinant biliverdin reductase was 3470 nmol mg$^{-1}$ h$^{-1}$ at 37°C. This activity is comparable to that obtained with the biliverdin reductase from rat liver cytOS (18).

**Stopped-flow Absorption Spectroscopy**—Single turnover reactions of the recombinant heme-hHO-1 complex were monitored by stopped-flow using an Applied Photophysics instrument with a diode array detector. Spectra were collected over the range of 320–740 nm at given time intervals during the course of the heme-hHO-1 reaction. The turnover of the heme-hHO-1 complex can be considered to consist of three different stages as follows: (a) the conversion of heme to Fe$^{3+}$-verdoheme; (b) the conversion of Fe$^{3+}$-verdoheme to Fe$^{2+}$-biliverdin; and (c) the conversion of Fe$^{2+}$-biliverdin to free biliverdin. The Fe$^{2+}$-biliverdin-hHO-1 complex was prepared by aerobically adding 1 eq of H$_2$O$_2$ to the heme-hHO-1 complex. The Fe$^{2+}$-biliverdin-hHO-1 complex was prepared by adding 200–300-fold sodium ascorbate to the heme-hHO-1 complex until no further changes were observed in the UV-visible spectrum (approximately 40–50 min). The excess sodium ascorbate was then removed by passing the protein through a desalting PD-10 column (Amersham Pharmacia Biotech) before it was loaded into the stopped-flow syringe.

In general, syringe A of the stopped-flow instrument contained 15 μM heme, Fe$^{3+}$-verdoheme, or the Fe$^{3+}$-biliverdin-hHO-1 complex as well as 0.1 or 1 eq of human P450 reductase in 100 mM NaMOPS, pH 7.4. Syringe B of the stopped-flow instrument contained 15 or 30 μM NADPH (1 or 2 eq, respectively, relative to hHO-1) in the same buffer. After the solutions in syringes A and B were mixed, 400–800 spectra over the range of 320–740 nm were recorded during the reaction time course. When biliverdin reductase was used, 1 eq (15 μM) relative to hHO-1 of this protein was added to syringe A. When a ferrous iron-chelator was employed, an excess amount (1 mM) of ferrozine was added to syringe B. The O$_2$ concentration in the reaction was taken to be 250 μM (calculated for an air-saturated solution). All experiments were performed at both 4 and 25°C. The kinetic time traces for individual wavelengths between 320 and 740 nm were also recorded with the diode array detector.

**Kinetic Data Analysis**—Except where noted, experimental conditions were used that resulted in first order or pseudo-first order reaction kinetics. In many cases, the reaction consisted of several steps, result-
ing in more than one summed exponential kinetic phase in the data. Data were fit using a global analysis algorithm kinetic program "Pro/Kineticist" (Pro/K) provided by Applied Photophysics. After a kinetic model with single or multiple phases was selected, the reciprocal relaxation times (rate constants) of the phases were determined using the program by fitting all the absorption changes at each individual wavelength over the collected wavelength range. With the determined rate constants, the absorption spectrum of each intermediate in the kinetic model over the wavelength range examined can be generated. For single wavelength kinetic time courses, the reciprocal relaxation times and phase amplitudes of the phases were determined by nonlinear regression fitting using the program KFIT developed by Dr. Neil C. Millar, Kings College, London, UK.

RESULTS

Kinetic Time Course and Rate Constants for the Single Turnover of Heme hHO-1 to Fe$^{3+}$-Verdoheme—The major intermediates in the first stages of the heme oxygenase reaction have been clearly shown to be α-meso-hydroxyheme and verdoheme. In this study, we have focused on determining the rate constants for the reactions that produce these intermediates. A stoichiometric concentration of P450 reductase was used in these studies to more closely mimic the in vivo situation. Time course studies of the single turnover reaction of Fe$^{3+}$-heme to Fe$^{3+}$-verdoheme in the presence of P450 reductase and 1–3 eq of NADPH (relative to heme:hHO-1) show that, under these conditions, maximal Fe$^{3+}$-verdoheme formation requires approximately 2 eq of NADPH (data not shown). The reaction proceeds beyond Fe$^{3+}$-verdoheme with 3 eq of NADPH. Only two electrons are required to convert the heme-hHO-1 complex to Fe$^{3+}$-verdoheme, but stopped-flow experiments show that approximately 2 eq of NADPH (4 electron equivalents) are required to maximize Fe$^{3+}$-verdoheme formation. The requirement for NADPH in excess of that formally required to turn over the enzyme is due to the fact that the electrons are provided by P450 reductase. The two flavin groups in P450 reductase can store up to four electrons, and studies of the role of P450 reductase in other systems have shown that electron transfer to acceptor hemoproteins usually leaves the flavoprotein in a partially reduced state (38, 39). One equivalent of NADPH is therefore presumably required to prime the P450 reductase by putting it into the appropriate redox state.

The changes in the absorption spectrum between 360 and 740 nm were monitored by stopped-flow spectroscopy when the heme-hHO-1 complex was mixed at 4 °C, pH 7.4, with 0.1 eq of heme and 0.1 eq of NADPH. The single wavelength time traces at 410 nm (Fe$^{2+}$-dioxy intermediate), 425 nm, 535 nm (β-band), 575 nm (α-band), and 680 nm (Fe$^{3+}$-verdoheme) are shown in Fig. 1, C and D. The reaction appears to go through two phases as follows: (a) in the first phase, the Fe$^{2+}$-dioxy intermediate is formed, as indicated by a shift of the Soret band from 405 to 410 nm, and (b) in the second phase, the Fe$^{2+}$-dioxy intermediate is transformed into Fe$^{3+}$-verdoheme, as signaled by an increase at 680 nm with a concomitant disappearance of the Fe$^{3+}$-dioxy species at 410 nm. The data in Fig. 1, A and B, were fit to a two-step reaction model using a global analysis algorithm Pro/K kinetic fitting program. A good fit of the data in Fig. 1, A and B, at both at 4 and 25 °C was thus obtained (Table I; see Reaction 1).

\[
A \rightarrow B \rightarrow C
\]

\[
k_1 \quad k_2
\]

REACTION 1

In addition, the single kinetic traces at the multiple wavelengths shown in Fig. 1, C and D, were fit with two exponential phases using the KFIT program as described under "Experimental Procedures." A good fit was obtained for each kinetic trace at 4 and 25 °C, respectively, with the rate constants shown in Table II.

The rate constants obtained from the single wavelength traces (Table II) agree well with those obtained with the Pro/K fitting program (Table I). The UV-visible spectra and the reaction concentration profiles for each intermediate in the single turnover of Fe$^{3+}$-heme to Fe$^{3+}$-verdoheme generated from the rate constants in Table I are shown in Fig. 2, A and B. The intermediates A, B, and C correspond to the Fe$^{3+}$-heme-hHO-1, Fe$^{3+}$-heme-dioxy, and Fe$^{3+}$-verdoheme complexes, respectively. The distinct features of the spectrum of each intermediate are consistent with those experimentally observed earlier, confirming that the curve fitting procedure was reliable.

Assignment of the Reaction Rate Constants to the Reaction Steps from Heme-hHO-1 to Fe$^{3+}$-Verdoheme—The reaction steps that convert the Fe$^{3+}$-heme-hHO-1 complex to Fe$^{3+}$-verdoheme involve the following: (a) reduction of the ferric heme, (b) binding of oxygen to give the Fe$^{2+}$-dioxy complex, (c) reduc-
Kinetic rate constants obtained from the fit of the single kinetic traces at the multiple wavelengths in the conversion of ferric heme to ferric verdoheme at 4 and 25 °C, pH 7.4

The data were fit using the KFIT kinetic program as described under Experimental Procedures.

| Wavelength | Temperature | $k_1$ | $k_2$ |
|------------|-------------|-------|-------|
| nm         | °C          |       |       |
| 410        | 4           | 0.124 | 0.054 |
| 425        | 25          | 0.46  | 0.26  |
| 538        | 4           | 0.091 | 0.057 |
| 575        | 4           | 0.38  | 0.26  |
| 680        | 4           | 0.18  | 0.055 |

Fig. 2. The UV-visible spectra and reaction concentration profiles for the intermediates in the single turnover conversion of Fe$^{3+}$-heme to Fe$^{3+}$-verdoheme. A. Absorption spectra of the reaction intermediates Fe$^{3+}$-heme (--), Fe$^{2+}$-heme-oxy (→), and Fe$^{3+}$-verdoheme (→→) generated from the best fit of the rate constants $k_1$ and $k_2$ at 4 °C. B. Concentration profiles for each of the reaction intermediates during the single turnover reaction at 4 °C.

The changes in the absorption spectrum after the Fe$^{3+}$-verdoheme-hHO-1 complex was mixed at 4 °C, pH 7.4, with 0.1 eq of P450 reductase, and 2 eq of NADPH were monitored by stopped-flow spectroscopy (Fig. 3, A and B). The reaction appears to be biphasic. In the first phase, Fe$^{2+}$-verdoheme is formed, as indicated by a shift of the Soret band from 405 nm to 400 nm, and an increase in the absorption at 700 nm. In the second phase, Fe$^{3+}$-biliverdin is formed, as indicated by both the appearance of a broad band at 380 nm with an almost featureless visible region (Fig. 3, A and B). The reaction proceeded all the way to free biliverdin (data not shown). Approximately 1 eq of NADPH were required to convert Fe$^{3+}$-verdoheme to Fe$^{3+}$-biliverdin in these single turnover experiments, in agreement with the theoretical requirement of four electrons. This is the first direct evidence that Fe$^{3+}$-biliverdin is a true intermediate in the reaction sequence to free biliverdin.
The single kinetic traces at 400, 535, and 700 nm (Fig. 3, C and D) were also fit to a two-exponential phase equation using the KFIT program (see "Experimental Procedures"). A good fit was obtained for the kinetic traces at both 4 and 25 °C, respectively, with the rate constants in Table III.

The rate constants obtained from the single wavelength traces (Table III) agree well with those obtained with the Pro/K fitting program (Table I). The UV-visible spectra and the reaction concentration profiles of each intermediate in the single turnover reaction of Fe3⁺-verdoheme to Fe3⁺-biliverdin generated using the rate constants from the best fit in Table I are shown in Fig. 4, A and B. The intermediates C, D, and E represent the Fe3⁺-verdoheme, Fe2⁺-verdoheme, and Fe3⁺-biliverdin complexes, respectively. The distinct features of the spectrum of each intermediate are consistent with those observed experimentally, indicating that the fitting procedure was reliable.

Assignment of the Reaction Rate Constants to the Reaction Steps from Fe3⁺-Verdoheme to Fe3⁺-Biliverdin—The reaction from Fe3⁺-verdoheme to Fe3⁺-biliverdin is triggered by the reduction of Fe3⁺- to Fe2⁺-verdoheme. Oxygen binding and a second electron transfer then led to the formation of Fe2⁺-biliverdin. The constant \( k_3 \) was assigned to the rate of formation of Fe2⁺-verdoheme. The second order rate constant for the binding of oxygen to Fe2⁺-verdoheme is not known. The rate constant \( k_4 \) could, therefore, be assigned to any steps involved in the conversion of Fe2⁺-verdoheme to Fe3⁺-biliverdin, which includes oxygen binding to Fe2⁺-verdoheme followed by the second electron transfer step to the Fe2⁺-verdoheme-dioxy complex. The rate constant for oxygen binding can, in principle, be determined by directly measuring the rate of formation of the verdoheme-dioxy complex from Fe2⁺-verdoheme. However, the verdoheme-dioxy complex has yet to be spectroscopically identified.

When the single turnover reaction of Fe3⁺-verdoheme to Fe3⁺-biliverdin was carried out in the presence of a stoichiometric amount (1 eq) of P450 reductase at 4 °C, the value of the rate constant \( k_3 \) increased to 1.27 s⁻¹, but the value of \( k_4 \) only increased slightly to 0.07 s⁻¹ when the data were fitted to the
same two-step reaction model. The fact that an 8-fold increase in $k_3$ was observed with a 10-fold increase in the reductase concentration confirms the assignment of this rate constant to the reduction of Fe$^{3+}$-verdoheme. The rate constant $k_4$ could be attributed to the coordination of O$_2$ to Fe$^{2+}$-verdoheme, the reduction of the resulting oxygen complex, or some other chemical reaction involved in cleavage of the verdoheme ring. The finding that only a slight increase in $k_4$ is associated with a 10-fold increase in the reductase concentration suggests that $k_4$ is not directly dependent on the electron transfer step.

Formation of Fe$^{3+}$-Biliverdin and Its Conversion to Metal-free Biliverdin by Release of Ferrous Iron—Fe$^{3+}$-biliverdin was formed using a coupled oxidation system by adding a 250–300-μM ferrioxamine, to the putative Fe$^{3+}$-biliverdin complex by the single turnover reaction of 20 μM heme-hHO-1 in the presence of a 50-fold excess of ferrozone (---).

To examine this possibility, the Fe$^{3+}$-biliverdin complex was made anaerobic and was then treated with 1 eq of dithionite. The iron-biliverdin was readily converted to free biliverdin, as shown by the appearance of the expected 680 nm band (Fig. 5). This indicates that the conversion of Fe$^{3+}$-biliverdin to metal-free biliverdin requires reduction of the iron but does not require oxygen. The EPR spectrum of the Fe$^{3+}$-biliverdin complex exhibits a rhombic EPR signal indicative of a high spin ferric iron that differs from those of the ferric heme (high spin axial signal) or Fe$^{3+}$-verdoheme (low spin rhombic signal) complexes.

Reduction of Fe$^{3+}$-biliverdin produces biliverdin, as indicated by the increase in the 680 nm absorption, and presumably also an iron atom. To examine the iron release directly, we employed ferrozone, a chelating agent that specifically binds ferrous (as opposed to ferric) iron to give a purple complex with an absorption maximum at 564 nm. Control reactions were performed to determine whether the Fenton reaction of Fe$^{2+}$ with H$_2$O$_2$ formed by uncoupled turnover of hHO-1 interferes with quantitation of the iron. Formation of the Fe$^{2+}$-ferrozene complex in ferrous iron solutions in the absence and presence of increasing amounts of added H$_2$O$_2$ showed that the Fenton reaction becomes competitive with ferrozene coordination at higher levels of H$_2$O$_2$, i.e. when the H$_2$O$_2$:ferrous iron ratio was more than 2-fold. Formation of the Fe$^{2+}$-ferrozene complex was also perturbed in phosphate buffer, presumably because phosphate coordinates to the ferrous iron in competition with ferrozone. Therefore, prior to these studies, the phosphate buffer of the normal heme-hHO-1 preparation was exchanged for 100 mM NaMOPS, pH 7.4, using a PD-10 desalting column. When single turnover reactions of the heme-hHO-1 complex were carried out in the presence of a 50-fold molar excess of ferrozone, the Fe$^{3+}$-ferrozene complex was formed (Fig. 5). The amount of Fe$^{3+}$ released during the heme-hHO-1 single turnover reaction, calculated with an extinction coefficient at 564 nm for the complex of 26.9 mM$^{-1}$ cm$^{-1}$ (100 mM NaMOPS, pH 7.4), agrees well with that expected from stoichiometric ferrous iron release. Thus, essentially all the iron released from the heme-hHO-1 single turnover reaction was trapped as the verdoheme complex.

Together, the above studies establish that (a) the reaction of ascorbate with the heme-hHO-1 complex produces Fe$^{3+}$-biliverdin, (b) the conversion of Fe$^{3+}$-biliverdin to free biliverdin requires one-electron reduction of the iron, (c) reduction of Fe$^{3+}$-biliverdin cannot be mediated by ascorbate, and (d) iron is released in the ferrous state. Thus, the ascorbate-supported reaction ends at Fe$^{3+}$-biliverdin because the redox potential of ascorbate is not negative enough to reduce Fe$^{3+}$-biliverdin. The Fe$^{3+}$-biliverdin complex obtained with ascorbate is not stable, however, and over a period of hours loses the iron atom, as indicated by gradual appearance of the 680 nm absorbance of free biliverdin.

Kinetic Time Course and Rate Constant Measurement for Biliverdin and Ferrous Iron Formation from Fe$^{3+}$-Biliverdin—The changes in the absorption spectrum between 480 and 740 nm when the pre-formed (with ascorbate) Fe$^{3+}$-biliverdin-hHO-1 complex was mixed with 0.1 eq of P450 reductase and 1 eq of NADPH at 4 °C, pH 7.4, are shown in Fig. 6. The single wavelength kinetic time traces at 508, 580, and 680 nm of the same single turnover reaction are shown in Fig. 6D. The reaction time course appears to have three phases (best seen in the 508 nm time trace), suggesting that there are at least three kinetically distinguishable steps in the overall reaction. The data were fit as described under "Experimental

---

2 Yi Liu and Paul R. Ortiz de Montellano, unpublished results.
Heme Oxygenase Kinetic Rate Constants

Fig. 6. Kinetic time course for the conversion of Fe\(^{3+}\)-biliverdin to free biliverdin under single turnover conditions as observed by stopped-flow spectroscopy at 4 °C, pH 7.4. Changes in the absorption spectrum between 480 and 740 nm during the reaction time 0–36 (A), 36–54 (B), and 54–326 s (C). The single wavelength kinetic time traces of the reaction at 508, 580, and 680 nm (D) are also shown.

Procedures” using the Pro/K kinetic program provided by Applied Photophysics. The analysis showed that the overall single turnover reaction was well fit using the following three-step model, Reaction 3.

\[
E \rightarrow F \rightarrow G \rightarrow H \quad k_5, \quad k_6, \quad k_7
\]

**REACTION 3**

The rate constants for the three steps generated from the best fit of the data are \(k_5 = 0.035, k_6 = 0.19,\) and \(k_7 = 0.007 \text{ s}^{-1}\) at 4 °C, and \(k_5 = 0.15, k_6 = 0.39,\) and \(k_7 = 0.03 \text{ s}^{-1}\) at 25 °C, pH 7.4 (Table I). The kinetic rate constants vary slightly from one batch of enzyme to another.

The reaction steps in the kinetic model can be reasonably proposed to be the one-electron reduction of Fe\(^{3+}\)-biliverdin (intermediate E) to Fe\(^{2+}\)-biliverdin (intermediate F), release of Fe\(^{2+}\) to produce the biliverdin-enzyme complex (intermediate G), and release of biliverdin (H) from the enzyme. Assignment of the three rate constants to the indicated reaction steps is difficult because a relatively good fit of the kinetic data can also be obtained if the three rate constants are interchanged. That is, all six possible different combinations of rate constants give an acceptable fit. However, the rate constant of 0.19 s\(^{-1}\) at 4 °C cannot be \(k_7\) because the UV-visible spectra of intermediates B and C generated from this model are not reasonable. Thus, four different sets of rate constants remain to be considered. In the absence of authentic spectra for intermediates B and C, alternative methods have been pursued to make possible the assignment of the rate constants.

**Assignment of the Reaction Rate Constants in the Reaction Steps from Fe\(^{3+}\)-Biliverdin to Biliverdin—**Efforts to generate the Fe\(^{2+}\)-biliverdin:hHO-1 complex by reduction of the Fe\(^{3+}\)-biliverdin complex in the presence of CO and/or ferrous iron were not successful. It appears that reduction of Fe\(^{3+}\)-biliverdin is followed by rapid dissociation of the iron without the accumulation of Fe\(^{2+}\)-biliverdin. Our failure to observe the Fe\(^{2+}\)-biliverdin complex or to trap it with CO suggests that \(k_5\) is much larger than \(k_6,\) if \(k_5\) is the reduction rate constant. An alternative approach to determination of the rate of reduction of the Fe\(^{3+}\)-biliverdin complex is to measure the NADPH consumption rate. A parallel experiment was therefore carried out with 1 eq of NADPH in which the absorption decrease at 340 nm due to NADPH oxidation was monitored. The changes in the absorption at 340 nm at 4 and 25 °C are shown in Fig. 7. The NADPH consumption rate constants obtained with the rate constants 0.04 s\(^{-1}\) at 4 °C and 0.17 s\(^{-1}\) at 25 °C, respectively, at pH 7.4.

If P450 reductase is rapidly reduced by NADPH and the reductase has a high affinity for heme oxygenase, as usually observed in the cytochrome P450 systems, then the NADPH consumption rate represents the rate of reduction of Fe\(^{3+}\)- to Fe\(^{2+}\)-biliverdin with a limiting amount of reductase. In the presence of a substoichiometric amount (0.05 eq) of P450 reductase and 1 eq of NADPH, the electron transfer rate from the reduced reductase to the Fe\(^{3+}\)-biliverdin-hHO-1 complex will be essentially equal to the NADPH consumption rate because NADPH oxidation will be limited by the electron transfer step. The finding that the measured NADPH consumption rate agrees well at both temperatures with rate constant \(k_5\) (Table I) indicates both that the assumptions in this method are valid and that \(k_5\) is the rate constant for the reduction of Fe\(^{3+}\)-biliverdin.

The constant \(k_5\) (Table I) can be reasonably assigned to the biliverdin-hHO-1 formation step for the following reasons. First, if the \(k_7\) rate constants 0.007 s\(^{-1}\) at 4 °C and 0.03 s\(^{-1}\) at 25 °C represented the biliverdin-hHO-1 formation rate, the half-lives at the two temperatures for formation of the biliverdin-hHO-1 complex would be 99 and 23 s, respectively. As it usually requires 5–6 half-lives to complete a reaction, the finding that the 600–700 nm absorbance band (biliverdin product) reaches a maximum after 40–50 s at 4 °C and 15–20 s at 25 °C suggests that \(k_5\) in Table I is too slow to be that for formation of the biliverdin-hHO-1 product. Furthermore, the earlier observation that the Fe\(^{2+}\)-biliverdin intermediate does not accumulate suggests that \(k_6\) should be much larger than \(k_5.\)

It is therefore likely that \(k_6\) (0.19 s\(^{-1}\) at 4 °C and 0.39 s\(^{-1}\) at 25 °C) is the rate of formation of the biliverdin-hHO-1 complex and possibly also the rate of Fe\(^{2+}\) release. To determine if this is the case, we have directly measured the rate of ferrous iron release.

Parallel experiments were performed in the presence of a
Heme Oxygenase Kinetic Rate Constants

50-fold excess of ferrozene. Fig. 8 shows the kinetic time traces for formation of the Fe$^{2+}$-ferrozene complex (564 nm) during the conversion of Fe$^{3+}$-biliverdin to biliverdin at both 4 and 25 °C. The time traces were fit using one exponential phase. The data show that formation of the ferrous-ferrozene complex occurs with a single rate constant of 0.021 s$^{-1}$ at 4 °C and 0.126 s$^{-1}$ at 25 °C. The rate constants for formation of the Fe$^{2+}$-ferrozene complex at both temperatures are consistent with the rate constants $k_5$ for the reduction of Fe$^{3+}$-biliverdin (see above). This result indicates that the conversion of intermediate F to G involves the release of Fe$^{2+}$ and the formation of the biliverdin-enzyme complex. Fe$^{2+}$ release occurs rapidly after reduction of the iron and is limited by the rate of reduction of Fe$^{3+}$-biliverdin. In turn, this finding confirms that the rate constant for the reduction step is $k_5$, with values of 0.035 s$^{-1}$ at 4 °C and 0.15 s$^{-1}$ at 25 °C.

The last, and slowest, step in the sequence can therefore be reasonably attributed to the dissociation of biliverdin. The rate constant for this step is thus $k_7$, although this assignment is based on inference rather than direct evidence. Biliverdin release is the rate-limiting step in the reaction from Fe$^{3+}$-biliverdin to free biliverdin in single turnover experiments. With the three rate constants assigned, it is possible to reconstruct the UV-visible spectra of the intermediates and the reaction concentration profiles for each intermediate in the single turnover reaction of Fe$^{3+}$-biliverdin (Fig. 9, A and B, respectively).

When the single turnover reaction of Fe$^{3+}$-biliverdin to biliverdin was carried out in the presence of a stoichiometric amount (1 eq) of P450 reductase at 4 °C, the value of the rate constant $k_5$ increased to 0.195 s$^{-1}$, whereas the values of $k_6$ and $k_7$ only changed slightly to 0.34 s$^{-1}$ and 0.011 s$^{-1}$, respectively, when the data were fitted to the same three-step reaction model. The fact that a 5-6-fold increase of $k_5$ was observed with a 10-fold increase in the reductase concentration confirms the assignment of this rate constant to the reduction of Fe$^{3+}$-biliverdin. Based on the above assignment, $k_6$ and $k_7$ are not expected, as observed, to depend on the reductase concentration.

**Rate-limiting Step in the Presence of Biliverdin Reductase**—To determine if biliverdin reductase alters the kinetics of product release, and therefore heme oxygenase turnover, we cloned biliverdin reductase into a pET23 vector, expressed the vector in E. coli BL-21 pLys cells, purified the protein by affinity chromatography, and examined its effect on heme oxygenase kinetics. In the purification procedure the cell supernatant was loaded onto the Ni$^{2+}$ affinity column, and the column was washed with buffer containing 60 mM imidazole to remove contaminating bacterial proteins. The biliverdin reductase with a six-histidine tag on the carboxyl terminus was eluted from the column with 250 mM imidazole. SDS-polyacrylamide gel electrophoresis analysis showed that the protein was highly purified and had the expected molecular mass of ~37
kDa. The protein was active, as indicated by disappearance of the biliverdin absorption at 680 nm and appearance of the bilirubin maximum at 468 nm when biliverdin reductase was added to biliverdin and NADPH. As expected, the solution color changed from green to yellow during the reaction.

Under physiological conditions, biliverdin binds to biliverdin reductase and is reduced to bilirubin. We therefore investigated whether the rate of biliverdin release is altered in the presence of biliverdin reductase by monitoring the rate of bilirubin formation (468 nm) from Fe$^{3+}$-biliverdin supported by P450 reductase and NADPH in the presence of biliverdin reductase (Fig. 10). The rate constants generated from single exponential fits of the data are 0.035 s$^{-1}$ at 4 °C and 0.14 s$^{-1}$ at 25 °C. These rate constants are essentially the same as the $k_{5}$ values at the corresponding temperatures (Table I). The rate constant for bilirubin formation in the single turnover conversion of Fe$^{3+}$-biliverdin thus agrees well with that for the reduction of Fe$^{3+}$-biliverdin. This requires that the rate of release of biliverdin ($k_{7}$) be greatly increased when biliverdin reductase is present.

The reaction steps and the rate constants for single turnover of heme to biliverdin by hHO-1 are summarized in Scheme 2. The reaction of ferric heme to Fe$^{3+}$-verdoheme follows two phases, and the corresponding rate constants $k_{1}$ and $k_{2}$ have been assigned, respectively, to the reduction of ferric heme and electron transfer to the ferrous-dioxy intermediate. The reaction of Fe$^{3+}$-verdoheme to Fe$^{3+}$-biliverdin also follows two phases. The rate constants $k_{3}$ and $k_{4}$ determined for these two phases were assigned, respectively, to the reduction of Fe$^{3+}$-verdoheme and either the pseudo-first order rate for coordination of oxygen to Fe$^{3+}$-verdoheme or reduction of the resulting verdoheme-dioxy species. The single turnover reaction of Fe$^{3+}$-biliverdin to biliverdin has three detectable steps as follows: (a) one-electron reduction of Fe$^{3+}$- to Fe$^{2+}$-biliverdin, (b) release of ferrous iron with formation of the biliverdin-hHO-1 complex, and (c) release of biliverdin. The final step, release of biliverdin, is rate-limiting in the single turnover experiments. However, in the presence of biliverdin reductase, biliverdin release is greatly enhanced and is no longer rate-limiting. In the presence of biliverdin reductase and multiple turnover conditions, the conversion of Fe$^{2+}$-verdoheme to Fe$^{3+}$-biliverdin appears to become the rate-limiting step.
Heme Oxygenase Kinetic Rate Constants

is associated with increased oxidative damage at the same time that the fraction of iron in hemoglobin decreases (42). The first report of a human with a heme oxygenase deficiency suggests that a similar derangement of iron homeostasis is present (43). It is possible that in the absence of heme oxygenase heme is degraded in inappropriate compartments or by non-physiological, peroxidative pathways that release ferric iron in a form unsuitable for efficient physiological utilization.

The rate-limiting step in the single turnover of heme oxygenase is dissociation of biliverdin from the protein. The rate constant $k_7 = 0.007 \text{ s}^{-1}$ at 4 °C for biliverdin dissociation is one-third as large as $k_4 = 0.025 \text{ s}^{-1}$, the next smallest rate constant. A similar difference is observed at 25 °C. However, at least two other factors may alter the rate-limiting step. First, the rate of binding of heme to HO-1, if slow, could limit catalytic turnover. The physiological rate of heme binding is difficult to ascertain because it is difficult to define the concentration of heme and whether it is free or bound to proteins. Second, under physiological conditions, heme oxygenase turnover occurs in the presence of biliverdin reductase, and interactions with this second protein could alter the enzyme kinetics. Indeed, we show here that biliverdin reductase accelerates biliverdin release sufficiently that the rate-limiting step becomes the conversion of ferrous verdoheme to ferric biliverdin ($k_4$). The increase in the biliverdin dissociation rate caused by interaction of heme oxygenase with biliverdin reductase presumably involves an allosteric weakening of the binding of biliverdin to HO-1 or even a direct transfer of biliverdin from HO-1 to biliverdin reductase. The crystal structure of human HO-1 indicates that the heme is bound in a cleft with an edge exposed to the solvent (27). The electrostatic potential of the amino acids surrounding the cleft and exposed heme edge is positive. This led to the proposal that P450 reductase, which is negatively charged (44), binds directly over the exposed heme edge. It is possible that biliverdin reductase binds in a similar manner and helps to extract the biliverdin product. However, efforts to demonstrate that heme oxygenase and biliverdin reductase form a stable complex have been unsuccessful, indicating that any such protein-protein interaction is transient.

In sum, the rate constants for seven steps in the heme oxygenase catalytic sequence have been determined in single turnover studies by stopped-flow spectroscopy. The final steps have been shown to involve mandatory reduction of the heme oxygenase ferric biliverdin complex, release of ferrous iron, and release of iron-free biliverdin. The last step of this sequence is the slowest, but it is accelerated in the presence of biliverdin reductase so that reduction of verdoheme becomes the rate-limiting step. This finding suggests that biliverdin reductase exerts an allosteric or direct effect on biliverdin release via some form of protein-protein interaction. Furthermore, release of ferrous rather than ferric iron may be an important feature of heme oxygenase catalysis in terms of the iron homeostasis of the organism.

Acknowledgments—We thank Mahin Maines (University of Rochester) and Clark Lagarias (University of California, Davis) for the rat biliverdin reductase cDNA.

REFERENCES

1. Tenhunen, R., Marver, H. S., and Schmid, R. (1969) J. Biol. Chem. 244, 6388–6394
2. Schmid, R., and McDonagh, A. F. (1979) in The Porphyins (Dolphin, D., ed. Vol. 6, pp. 257–292, Academic Press, New York
3. Maines, M. D. (1988) in Heme Oxygenase: Clinical Applications and Functions, pp. 253–260, CRC Press, Inc., Boca Raton, FL
4. Maines, M. D., and Trakshel, G. M. (1992) Biochim. Biophys. Acta 1131, 166–174
5. Kappas, A., Demas, G. E., and Clark Lagarias (University of California, Davis) for the rat biliverdin reductase cDNA.

SCHEME 2. Summary of the rate constants for the overall heme degradation sequence in the absence of biliverdin reductase. The rate constant $k_i$ is taken from the literature (40).

4°C, $k_i=0.11$ $k_2=0.056$ $k_3=0.15$ $k_5=0.25$ $k_6=0.35$ $k_7=0.19$ $k_8=0.007 \text{ s}^{-1}$
25°C, $k_i=0.49$ $k_2=0.21$ $k_3=0.55$ $k_4=0.10$ $k_5=0.15$ $k_6=0.39$ $k_7=0.03 \text{ s}^{-1}$
22. Sun, J., Loehr, T. M., Wilks, A., and Ortiz de Montellano, P. R. (1994) Biochemistry 33, 13734–13740
23. Wilks, A., Sun, J., Loehr, T. M., and Ortiz de Montellano, P. R. (1995) J. Am. Chem. Soc. 117, 2925–2926
24. Takahashi, S., Wang, J., Rousseau, D. L., Ishikawa, K., Yoshida, T., Host, J. R., and Ikeda-Saito, M. (1994) J. Biol. Chem. 269, 1010–1014
25. Takahashi, S., Wang, J., Rousseau, D. L., Ishikawa, K., Yoshida, T., Takeuchi, N., and Ikeda-Saito, M. (1994) Biochemistry 33, 5531–5538
26. Ito-Maki, M., Ishikawa, K., Matera, K. M., Sato, M., Ikeda-Saito, M., and Yoshida, T. (1995) Arch. Biochem. Biophys. 317, 253–258
27. Schuller, D., Wilks, A., Ortiz de Montellano, P. R., and Poulos, T. (1999) Nat. Struct. Biol. 6, 860–867
28. Ortiz de Montellano, P. R. (1998) Acc. Chem. Res. 31, 543–549
29. Liu, Y., Moenne-Loccoz, P., Loehr, T., and Ortiz de Montellano, P. R. (1997) J. Biol. Chem. 272, 6969–6974
30. Yoshida, T., Noguchi, M., Kikuchi, G., and Sano, S. (1981) J. Biochem. (Tokyo) 90, 125–131
31. Yoshinaga, T., Sudo, Y., and Sano, S. (1990) Biochem. J. 267, 659–664
32. Yoshinaga, T., Sudo, Y., and Sano, S. (1990) Biochem. J. 270, 659–664
33. 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
34. Wilks, A., Torpey, J., and Ortiz de Montellano, P. R. (1994) J. Biol. Chem. 269, 29553–29556
35. Yoshida, T., and Kikuchi, G. (1978) J. Biol. Chem. 253, 4230–4236
36. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
37. Dierks, E. A., Davis, S. C., and Ortiz de Montellano, P. R. (1998) Biochemistry 37, 1839–1847
38. Masters, B. S. S., and Okita, R. T. (1986) Pharmacol. Ther. 9, 227–244
39. Vermilion, J. L., Ballou, D. P., Massey, V., and Coon, M. J. (1981) J. Biol. Chem. 256, 266–277
40. Migitu, C. T., Matera, K. M., Ikeda-Saito, M., Olson, J. S., Fujii, H., Yoshimura, T., Zhou, H., and Yoshida, T. (1998) J. Biol. Chem. 273, 945–949
41. Richardson, D. R., and Punka, P. (1997) Biochim. Biophys. Acta 1331, 1–40
42. Poss, K. D., and Tonegawa, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10919–10924
43. Yachie, A., Niida, Y., Wada, T., Igarashi, N., Kaneda, H., Toma, T., Ohta, K., Kasahara, Y., and Koiwami, S. (1999) J. Clin. Invest. 103, 129–135
44. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. S., and Kim, J.-J. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8411–8416
Reaction Intermediates and Single Turnover Rate Constants for the Oxidation of Heme by Human Heme Oxygenase-1
Yi Liu and Paul R. Ortiz de Montellano

J. Biol. Chem. 2000, 275:5297-5307.
doi: 10.1074/jbc.275.8.5297

Access the most updated version of this article at http://www.jbc.org/content/275/8/5297

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 19 of which can be accessed free at http://www.jbc.org/content/275/8/5297.full.html#ref-list-1