The Oxygen and Carbon Monoxide Reactions of Heme Oxygenase*

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The O₂ and CO reactions with the heme, α-hydroxymyoglobin, and verdoheme complexes of heme oxygenase have been studied. The heme complexes of heme oxygenase isoforms-1 and -2 have similar O₂ and CO binding properties. The O₂ affinities are very high, $K_{O2} = 30-80 \text{ M}^{-1}$, which is 30–90-fold greater than those of mammalian myoglobins. The O₂ association rate constants are similar to those for myoglobins ($k_{O2} = 7-20 \text{ M}^{-1} \text{s}^{-1}$), whereas the O₂ dissociation rates are remarkably slow ($k_{O2} = 0.25 \text{ s}^{-1}$), implying the presence of very favorable interactions between bound O₂ and protein residues in the heme pocket. The CO affinities estimated for both isoforms are only 1–6-fold higher than the corresponding O₂ affinities. Thus, heme oxygenase discriminates much more strongly against CO binding than either myoglobin or hemoglobin. The CO binding reactions with the ferrous α-hydroxymyoglobin complex are similar to those of the protoheme complex, and hydroxylation at the α-meso position does not appear to affect the reactivity of the iron atom. In contrast, the CO affinities of the verdoheme complexes are $>10$ times weaker than those of the heme complexes because of a 100-fold slower association rate constant ($k_{CO} = 0.004 \text{ M}^{-1} \text{s}^{-1}$) and a 300-fold greater dissociation rate constant ($k_{CO} = 3 \text{ s}^{-1}$) compared with the corresponding rate constants of the protoheme and α-hydroxymyoglobin complexes. The positive charge on the verdoporphyrin ring causes a large decrease in reactivity of the iron.

Heme oxygenase (HO) is an amphibathic microsomal protein that 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 as an electron donor (1–3). In the catalytic cycle the enzyme first binds 1 equivalent of hemin. This binding results in the formation of the heme-enzyme complex, which exhibits spectral properties similar to those of ferric myoglobins and hemoglobins (4, 5). The first electron donated from the reductase reduces the heme iron to the ferrous state, and then O₂ binds rapidly to form a metastable oxy complex (6). Electron donation to the oxy form initiates the three-step conversion of oxyheme to the ferric iron-biliverdin complex through α-hydroxymyoglobin and verdoheme intermediates (Scheme 1). The final step involves electron donation from the reductase to convert the ferric iron-biliverdin complex to ferrous iron and biliverdin (7). Thus, heme participates both as a prosthetic group and as a substrate, a property unique to heme oxygenase (5, 7).

HO has two isoforms, referred to as HO-1 and HO-2 (3). HO-1, an inducible form with a molecular mass of 33 kDa, is distributed mainly in reticuloendothelial cell-rich tissues such as spleen and liver. HO-2, with a molecular mass of 36 kDa, is expressed constitutively and is distributed mainly in the brain and testis. The amino acid sequence similarity between HO-1 and HO-2 is about 40%. However, both isoforms display the same enzymatic activity, active site structure, and heme iron coordination and electronic structures, hence the molecular mechanism of enzyme action is considered to be analogous between the two isoforms (8).

We and others have demonstrated that a neutral form of the imidazole group of histidine (His$^{25}$ in the HO isoform-1 sequence) is the axial heme ligand in the heme-enzyme complex of the water-soluble forms of recombinant HO (9–12). Although the distal residues have yet to be identified (13), an ionizable group with a $pK_a$ value of 7.6 is linked to the water molecule, which is coordinated to the ferric heme-HO-1 complex (9). The heme-HO complex appears to have an axial ligand coordination structure that is similar to that in myoglobins and hemoglobins. Resonance Raman studies on the oxy form of the heme-HO complex show that the bound oxygen molecule assumes a highly bent configuration because of strong interac-

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1 The abbreviation used is: HO, heme oxygenase.


**RESULTS**

The association and dissociation rate constants for $O_2$ and CO binding to ferrous forms of the heme-HO-1 and HO-2 complexes are listed in Table I. The kinetic traces for bimolecular recombination of $O_2$ to the heme-HO complex after flash photolysis are monophasic, and the observed rates depend linearly on $O_2$ concentration (Fig. 1). The association rate constants, $k_{O_2}$, are similar to those of mammalian myoglobin. However, the $O_2$ dissociation rate constants, $k_{O_2}$, of the heme-HO complexes are $0.25$ $s^{-1}$; about 100 times slower than the dissociation rate constants of myoglobin (15 $s^{-1}$) (22). The $O_2$ equilibrium constants, $K_{O_2}$, are 28 and 77 $M^{-1}$ for the HO-1 and HO-2 complexes, respectively, 30–90 times greater than the equilibrium constant of myoglobin. The $O_2$ affinity of the heme-HO complexes is equal to or higher than that of leghemoglobin, which has one of the highest known $O_2$ affinities.

The association reactions of both the HO-1 and HO-2 heme complexes are biphasic with the faster phase showing a bimolecular rate that is about 4–5 times larger than that of the slower phase. The amplitudes of the two phases are independent of CO concentrations (between 0.1 and 1 mM). The association rate constants, $k_{CO}$, for the slower phases are between 0.3 and 0.6 $M^{-1} s^{-1}$, which are similar to those of myoglobin (0.5–0.8 $M^{-1} s^{-1}$) (22). The kinetic traces for CO dissociation from the heme-HO complexes are monophasic. The dissociation
rate constants, $k_{CO}(0.007–0.009 \text{ s}^{-1})$ are slightly smaller than those of myoglobin ($0.02 \text{ s}^{-1}$) (22). Because of the biphasic nature of the CO association reactions, the overall CO equilibrium association constants, $K_{CO}$, can only be estimated as between 34 and 150 $\mu\text{M}^{-1}$ and 89 and 420 $\mu\text{M}^{-1}$ for the HO-1 and HO-2 complexes, respectively. It should be noted that the ratios of CO and O$_2$ affinities, $K_{CO}/K_{O2}$, are between 1.2 and 5.6 for the heme-HO complex. These ratios are much smaller than those for myoglobin (40) and hemoglobin (200) (23). Thus, O$_2$ binding to the ferrous heme iron in HO is inhibited to a much lesser extent by CO than it is in myoglobin and hemoglobin.

The reaction of CO with the ferrous $\alpha$-hydroxyheme-HO-1 complex is also biphasic (Table II), and the association rate constants for the fast and slow phases are similar to those of the heme-HO-1 complex. Again, CO dissociation is monophasic, and the dissociation rate constant is essentially the same as that of the heme-HO complex. Thus, the hydroxyl group at the $\alpha$-meso position does not affect the reactivity of the ferrous iron toward CO. The oxy form of the $\alpha$-hydroxyheme-HO complex is unstable (17) and cannot be prepared for O$_2$ binding and dissociation rate measurements.

The reaction of CO with verdoheme-HO complexes is different from that of the heme- and $\alpha$-hydroxyheme-HO complexes. The rebinding reaction is monophasic after flash photolysis. Plots of the dependence of the observed pseudo-first order rate constant, $k_{obs}$, on CO concentration are shown in Fig. 2A for the verdoheme-HO-1 complex. The slope and the $y$ axis intercept of this plot yield association and dissociation rate constants equal to $0.0054 \mu\text{M}^{-1} \text{s}^{-1}$ and $2.9 \text{ s}^{-1}$, respectively, and a CO equilibrium association constant equal to $0.0018 \mu\text{M}^{-1}$. Thus, the CO affinity of the verdoheme-HO-1 is more than 10,000 times weaker than that of the protoheme complex (Table II). Low CO affinity is confirmed by the CO equilibrium curve generated by plotting the absorption changes induced by the flash photolysis as a function of the CO concentration (Fig. 2B). Because the quantum yield of the CO form of the verdoheme-HO complex is almost 1 (15), the absorption changes after photolysis are proportional to the equilibrium fraction degree of saturation with this ligand. The experimental points are compared with a CO equilibrium curve calculated from the association and dissociation rate constants. The agreement between the calculated and observed results is excellent. The verdoheme HO-2 complex has the CO binding parameters similar to those of the corresponding HO-1 complex. As in the case of $\alpha$-hydroxyheme-

### Table II

Parameters for CO binding to the $\alpha$-hydroxyheme- and verdoheme-HO complexes in 0.1 M phosphate, pH 7, 20 °C

| Heme-protein complexes | $k_{CO}$ | $k_{DO}$ | $K_{CO}$ |
|------------------------|---------|---------|---------|
| $\alpha$-Hydroxyheme-HO1 | 0.17 (33 ± 10%) | 0.011 | 100 |
| Verdoheme-HO-1 | 0.0054 | 2.9 | 0.0018 |
| Verdoheme-HO-2 | 0.0026 | 2.3 | 0.0011 |
| Verdoheme-myoglobin | 0.024 | 3 | 0.008 |

Fig. 1. The observed rate ($k_{obs}$) dependence of the O$_2$ concentration for the O$_2$ reaction with the ferrous heme-HO complex after the flash photolysis measured in 0.1 M phosphate buffer, pH 7, measured at 20 °C.

Fig. 2. Panel A, observed rate ($k_{obs}$) dependence of the CO concentration for the CO reaction with the verdoheme-HO complex after the flash photolysis measured in 0.1 M phosphate buffer, pH 7. Panel B, absorbance changes at 636 nm induced by the flash photolysis of the CO-bound verdoheme-HO complexes. The squares are experimental data points, and the curve is calculated from the equilibrium association constant derived from the kinetic parameters (Table II). The measurements were carried out at 20 °C.

### DISCUSSION

The oxygen equilibrium constants, 30–80 $\mu\text{M}^{-1}$, of the heme-HO complex are similar to those of leghemoglobin (23...
μm⁻¹) (24). However, the mechanism responsible for the extraordinarily high O₂ affinity for the heme-HO complex is different from that for leghemoglobin. The high O₂ affinity of leghemoglobin is caused by a very large association rate constant that is determined by enhanced reactivity of the heme iron toward O₂ (24). In the heme-HO complex, high O₂ affinity is caused by a very slow O₂ dissociation rate constant. Decreases in O₂ dissociation rate constants for myoglobins and related heme proteins are associated with increases in hydrogen bonding or other favorable electrostatic interactions between bound oxygen and surrounding amino acids. For example, replacing the distal valine [E11] with an asparagine residue in sperm whale myoglobin decreases k₉₀ from 15 s⁻¹ to 0.5 s⁻¹ presumably by creating a second hydrogen bond with the bound ligand (22). The presence of strong interactions between distal residues in heme oxygenase and bound O₂ has already been proposed based on resonance Raman studies (14) and on cobalt EPR work (25).

The CO association reactions with the heme- and α-hydroxymyoglobin-HO complexes are biphasic. One of the possible origins of the biphasic reaction is heme orientational disorder around the porphyrin α-γ axis, which has been reported for the heme-HO complex (26). However, if this were the case, the O₂ association reaction would also be expected to be biphasic, but this is not the case. In addition, the ligand binding properties of myoglobin are independent of the heme orientation disorder (27). Thus, the structural origin of the biphasic CO association reactions with the heme- and α-hydroxymyoglobin-HO complex is not clear.

There are subtle differences in the kinetic parameters between the HO-1 and HO-2 complexes. The neutral imidazole form of histidine is the proximal ligand in both isoforms (8–10). In the hemin-enzyme complex, the pK₆ value of the acid-base transition of the HO-1 complex (pK₆ 7.6) is 0.9 unit lower than that in the HO-2 complex (pK₆ 8.5) (8, 9). Thus, the polarities of the distal pockets appear to be different between the isoforms, which might explain the small difference in kinetic parameters.

As shown in Scheme 2, α-hydroxymyoglobin undergoes a redox-linked transition between ferric oxophlorin and ferrous α-hydroxymyoglobin (17). In the ferrous form, the prosthetic group assumes a porphyrin macrocycle structure with a hydroxyl group at the α-meso position (structure 2 of Scheme 2). The α-hydroxyl group should be electron-donating, and electron withdrawal or electron donation from heme side chains has been shown to affect the reactivity of the heme iron (23, 28). However, the CO binding properties of the α-hydroxymyoglobin-HO complex are similar to those of the heme complex, indicating that the α-meso hydroxyl group does not affect either heme iron reactivity or iron-CO bond strength. The porphyrin π-electron spin density at the α-meso carbon could be so low that the electron donation at this position does not affect the iron reactivity appreciably. The Raman results show that the Fe-CO and C-O stretching frequencies of the CO-bound α-hydroxymyoglobin-HO complex are similar to those of the heme-HO complex (15). This implies that the electronic states of the Fe-CO unit and the Fe-C bond strengths are similar in these complexes, which is consistent with our kinetic results.

In the verdoheme-HO complex the iron atom is less reactive, and the iron-CO bond is much weaker than that in the heme and α-hydroxymyoglobin-HO complexes. Two resonance structures have been suggested for verdoheme and are shown in Scheme 2 (29). Structure 4 possesses a positive charge on the ring oxygen atom, whereas the charge is on the iron atom in structure 3. The resonance Raman studies show that a significant contribution from structure 3 is present in ferrous verdoheme-HO (15). Because CO binds only to ferrous iron, the presence of a significant amount of a ferric character will reduce the reactivity of verdoheme-HO toward CO significantly.

The Fe-CO stretching frequency of the CO verdoheme-HO complex is 474 cm⁻¹, whereas those for the heme- and α-hydroxymyoglobin-HO complexes are 503 and 508 cm⁻¹, respectively (10, 15). Clearly, the Fe-CO bond order in verdoheme-HO is lower than that in the heme- and α-hydroxymyoglobin-HO complexes. Because the rate of CO dissociation is determined by the rate of the thermal Fe-CO bond breakage (30), the lower Fe-CO bond order is probably the cause of the large k₉₀ value shown by verdoheme-HO.

The conversion of α-hydroxymyoglobin to verdoheme by HO generates CO stoichiometrically (16). Yoshida and co-workers (16, 18) have shown that CO can inhibit the mono-oxygenation reactions of the HO catalytic cycle by binding to the ferrous heme- or verdoheme-HO complex. Hence, CO could potentially act as a product inhibitor. When catalysis is carried out in air-saturated buffer, hemin is stoichiometrically converted to biliverdin, and the product inhibition by CO does not appear to occur. The results in Tables I and II explain why the CO produced does not severely inhibit heme oxygenase activity. The first oxygenation cycle is not inhibited because the ferrous iron atom in protoheme-HO has an extremely high affinity for O₂ which is roughly equal to that for CO. In air, the concentration of O₂ will always be much greater than that of CO, preventing product inhibition. When α-hydroxymyoglobin is converted to verdoheme in HO, the CO molecule generated by oxygenation of the heme group could also bind to the verdoheme product. In this case, CO binding to the verdoheme-HO complex is extremely weak, and the CO dissociation rate constant is very large, preventing product inhibition until the CO concentration is greater than 0.5 atmospheres or 500 μM. Previous experimental work has also shown that CO does not inhibit the conversion of α-hydroxymyoglobin to verdoheme (17, 18). Thus, the active site of HO has evolved to discriminate strongly.

![Scheme 2](image)
against CO binding and in favor of the binding of \( \text{O}_2 \), which is the oxidizing substrate for the overall reaction.

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