Heme oxygenases have an increased binding affinity for O₂ relative to CO. Such discrimination is critical to the function of HO enzymes because one of the main products of heme catabolism is CO. Kinetic studies of mammalian and bacterial HO proteins reveal a significant decrease in the dissociation rate of O₂ relative to other heme proteins such as myoglobin. Here we report the kinetic rate constants for the binding of O₂ and CO by the heme oxygenases from *Neisseria meningitidis* (nmHO) and *Pseudomonas aeruginosa* (paHO). A combination of stopped-flow kinetic and laser flash photolysis experiments reveal that nmHO and paHO both maintain a similar degree of ligand discrimination as mammalian HO-1 and the HO from *Corynebacterium diphtheriae*. However, in addition to the observed decrease in dissociation rate for O₂ by both nmHO and paHO, kinetic analyses show an increase in dissociation rate for CO by these two enzymes. The crystal structures of nmHO and paHO both contain significant differences from the mammalian HO-1 and bacterial C. diphtheriae HO structures, which suggests a structural basis for ligand discrimination in nmHO and paHO.

Heme oxygenase (HO) catalyzes the oxidative degradation of heme to biliverdin, iron, and CO (Fig. 1). HO has been identified in a wide array of organisms, including mammals (1, 2), insects (3, 4), and photosynthetic organisms (5, 6). Of particular interest, HO is present in many pathogenic bacteria (7–10), including *Neisseria meningitidis* (11) and *Pseudomonas aeruginosa* (12). These pathogenic bacteria have developed sophisticated heme uptake systems that harness the iron from heme-containing proteins present in the host (13–15). The HO enzymes from *N. meningitidis* (nmHO) and *P. aeruginosa* (paHO) are both essential for the utilization of iron from imported heme, and the crystal structures of these two bacterial HO enzymes have now been solved (16, 17). Although most HOs hydroxylate exclusively the α-meso heme carbon (Fig. 1), paHO is unusual because the γ-meso carbon is the predominant site of hydroxylation (18) even though the structure paHO is very much the same as other HOs. The difference in hydroxylation patterns is due to an ~100° rotation of the heme in paHO relative to other HOs which places the γ-meso carbon at the same position in the active site as the α-meso carbon in other HOs (16).

The Fe(II) atom of the heme prosthetic group of heme proteins is an efficient binder of O₂, NO, and CO, and the binding by heme proteins of these diatomic molecules is of critical importance to physiological processes such as respiration, vasodilation, and neurotransmission (19–21). A common feature shared by all heme proteins is the need to not only bind its target ligand but also to discriminate against the binding of heme ligands of similar size and shape. Fe(II) adducts of CO are normally linear because backbonding is optimized by the overlap of Fe(II) d-orbitals with the empty π* orbitals of CO (22–24). In contrast, Fe(II)-NO and Fe(II)-O₂ are naturally bent in order to maximize overlap with the occupied π* electrons of the ligand with the dz² iron orbitals. Thus, the intrinsic binding geometries of these ligands can be utilized by heme proteins to increase ligand discrimination (25, 26).

It has been suggested that ligand discrimination by globins results from the preferred ligand geometries of bound O₂ and CO (27). The greater polarity of the bound O₂ ligand also plays a role in enhancing the affinity of O₂ relative to CO for both the globins and HO enzymes. The partition constant (M), defined as the ratio of the equilibrium association constants for CO and O₂ (K_{CO}/K_{O2}), is ~30,000 for free heme, whereas that for sperm whale myoglobin is only ~40 and is further reduced to ~4 for human heme oxygenase (28, 29). The O₂ affinities of mammalian HO-1 and HO-2 are 30–90-fold higher than those of mammalian myoglobins (28), whereas the O₂ affinity of the bacterial HO from *C. diphtheriae* is 20-fold higher than that of myoglobin (29). This increased O₂ affinity is largely due to a 100-fold slower rate of dissociation from HO relative to the globins. Thus, even though the inherent affinity of the ferrous heme for O₂ is lower than that for CO, myoglobin and heme oxygenase clearly alter the binding characteristics of the heme, dramatically increasing the O₂ affinity relative to CO. Here we report the kinetic rate constants for the binding of CO and O₂ by the bacterial heme oxygenases, nmHO and paHO, and provide a structural basis for ligand discrimination.

**EXPERIMENTAL PROCEDURES**

Preparation of Ligand-bound Heme Oxygenases—Expression, purification, and reconstitution of the recombinant nmHO and paHO with heme were carried out as described.
Diatomic Ligand Discrimination by Bacterial Heme Oxygenases

**FIGURE 1. Overall reaction catalyzed by heme oxygenase.** HO enzymes have an increased binding affinity for O$_2$ relative to CO. Such discrimination is critical to HO function because one of the main products of heme catabolism is CO. Oxidation of the heme substrate by nmHO occurs exclusively at the $\alpha$-meso position (shown at top left), whereas paHO oxidizes both the $\beta$- and $\delta$-meso heme carbons.

Association Rate Constants—Association rate constants were obtained from the slopes of the linear plots of the observed pseudo-first-order rate constants versus ligand concentration.

Dissociation Rate Constants—Dissociation rate constants for both O$_2$ and CO were measured directly by carrying out replacement reactions with an Applied Photophysics SX18MV-R. The ligands of a 10 $\mu$M solution of the O$_2$- and CO-bound forms of the HO enzymes were replaced by a high concentration of displacing ligand (500 $\mu$M CO and 900 $\mu$M NO, respectively). Solutions were measured at $\lambda = 410$ nm and $\lambda = 420$ nm in order to follow the absorbance changes due to the dissociation of O$_2$ and CO, respectively. The dissociation rate constants were then calculated from the expressions $k_{\text{O}_2} = k_{\text{obs}}(1 + k'_{\text{O}_2}[\text{O}_2]/k'_{\text{CO}}[\text{CO}])$ and $k_{\text{CO}} = k_{\text{obs}}(1 + k'_{\text{CO}}[\text{CO}]/k'_{\text{NO}}[\text{NO}])$. Because $k'_{\text{NO}} \gg k'_{\text{CO}}$ for all heme proteins, the observed replacement rate constant ($k_{\text{obs}}$) was directly equal to the CO dissociation rate constant ($k_{\text{CO}}$).

Molecular Dynamics—Molecular dynamics simulations were carried out with Amber 8.0. Heme parameters were provided by Dr. Dan Harris (Molecular Research Institute). Partial charges for the heme and CO were taken from the ferrous-CO complex provided by Dr. John Straub (Boston University). The crystal structures for CO complexes of rat HO-1 (1IX4) and nmHO (1P3V) were stripped of crystallographically defined water molecules except for those in the active site near the bound CO. The solvent-stripped structure was solvated with water molecules within a 30 Å radius of the CO molecule. The entire solvated protein was energy minimized as follows. First, water and H atoms were allowed to move in a short 10-ps low temperature (50 °C) MD simulation followed by 500 cycles of energy minimization for both protein and solvent. Second, both protein and solvent were allowed to move in a 10-ps 50 °C MD simulation followed by 500 cycles of energy minimization. Finally, a 2-ns MD simulation was carried out at 300 °C. Resi-
Ligand Binding and Dissociation Kinetics—The association and dissociation rate constants for the binding of CO and O2 by nmHO and paHO are compared with those of myoglobin, mammalian HO-1, and bacterial cdHO, and protoheme in Table 1. The CO and O2 equilibrium constants (\( K_{\text{CO}} \) and \( K_{\text{O2}} \)) and the ratios of equilibrium constants (\( K_{\text{CO}}/K_{\text{O2}} \)) for nmHO and paHO are also provided in Table 1.

Bimolecular rebinding of O2 by nmHO and paHO after flash photolysis is monophasic, and the observed rates show a first-order dependence on the O2 concentration. The association rate constants for O2 binding by nmHO and paHO are 5.0 and 4.5 \( \mu \text{M}^{-1} \text{s}^{-1} \), respectively. These values are 4-fold smaller than that of myoglobin but very similar to both the mammalian and bacterial HO enzymes. The CO association reactions of both the nmHO and paHO heme complexes are also monophasic, with the observed rates showing a linear dependence on the CO concentration. Although the time courses for the binding of CO by HO-1 and cdHO were moderately biphasic and could be fit to a two-exponential expression (29), the time courses for CO binding by nmHO and paHO were observed to be monophasic and were fit to a single exponential expression at several different CO concentrations. The association rate constants for CO binding by nmHO and paHO are 1.10 and 0.90 \( \mu \text{M}^{-1} \text{s}^{-1} \), respectively. These values are similar to that of myoglobin, as well as those of HO-1 and cdHO. Sample traces for the CO and O2 association reactions are provided in Fig. 2, A and C, respectively. The second-order association rate constants for the binding of CO and O2 by
nmHO and paHO were obtained from the slopes of the linear plots of the observed rate constants \(k_{\text{obs}}\) versus ligand concentration (Fig. 2, E and F). The \(\text{O}_2\) dissociation rate constants for nmHO and paHO are 1.5 and 1.7 s\(^{-1}\), respectively. These data indicate that the dissociation rate constants for \(\text{O}_2\) binding by nmHO and paHO are \(\sim 14\)-fold slower than that of myoglobin but are \(\sim 7\)-fold faster than those of mammalian HO-1 and bacterial cdHO. The CO dissociation rate constants for nmHO and paHO are 0.16 and 0.12 s\(^{-1}\), respectively. The dissociation rate constants for CO binding by nmHO and paHO are \(\sim 6\)-fold faster than that of myoglobin but are \(\sim 22\)-fold faster than those of HO-1 and cdHO. Sample traces for the CO and \(\text{O}_2\) dissociation reactions are provided in Fig. 2, B and D, respectively.

The oxygen equilibrium constants \(K_{\text{O}_2}\) for nmHO and paHO are 3.3 and 2.6 M\(^{-1}\), respectively, which are \(\sim 3\)-fold greater than that of myoglobin but \(\sim 8\)-fold smaller than those of HO-1 and cdHO. The CO equilibrium constants \(K_{\text{CO}}\) for nmHO and paHO are 6.9 and 7.5 M\(^{-1}\), respectively, which are \(\sim 5\)-fold smaller than that of myoglobin but are \(\sim 17\)-fold smaller than those of HO-1 and cdHO. It should be noted that although the CO and \(\text{O}_2\) equilibrium constants \(K_{\text{CO}}\) and \(K_{\text{O}_2}\) for nmHO and paHO are smaller than those of HO-1 and cdHO, the ratios of equilibrium constants \(K_{\text{CO}}/K_{\text{O}_2}\) for nmHO and paHO are 2 and 3, respectively, which are similar to those of HO-1 and cdHO. The smaller \(\text{CO}\) and \(\text{O}_2\) affinities by nmHO and paHO are largely due to the much faster dissociation rate constants for these enzymes compared with HO-1 and cdHO.

**Structural Basis for Ligand Discrimination**—The problem in understanding ligand discrimination is illustrated in Table 1. Protoheme binds CO \(\sim 30,000\)-fold more tightly than \(\text{O}_2\) but only 40-fold more tightly in myoglobin. Steric factors were long thought to be the predominant structural underpinning of such ligand discrimination in heme proteins. Early on, Collman et al. (32) proposed that a strategically placed distal histidine in myoglobin could accommodate a bent \(\text{O}_2\) ligand but would inhibit the binding of a normally linear CO ligand. However, in recent years structural, spectroscopic, and theoretical studies have revealed the importance of electrostatic interactions for diatomic ligand discrimination (25, 33, 34). In particular, it has been shown that the H-bond between the distal His in myoglobin and \(\text{O}_2\) is quite important in controlling \(\text{O}_2\) affinity (35).

The HO crystal structures show that steric crowding is an important factor in HO although electrostatics also is quite important. Dioxygen-bound cdHO has two strong hydrogen bonds that can preferentially stabilize the highly polar Fe-\(\text{O}_2\) complex (29). One interaction is with the amide NH of Gly-139, and the other is with the distal pocket water molecule. Mutagenesis studies have also demonstrated that increasing the strength and number of hydrogen bonds donated from the distal pocket amino acids to the iron-bound \(\text{O}_2\) decreases the
rate of O₂ dissociation from myoglobin (36–38). Therefore, the more extensive H-bonding in HO-1 compared with myoglobin explains why \( k_{\text{off}} \) of O₂ is slower in HO-1 and thus is a major contributing factor in ligand discrimination.

Whether or not this view holds with all HOs will require additional structures of oxy complexes, but unfortunately obtaining such structures is very difficult. However, NO is a good mimic of O₂ because, like O₂, NO prefers a bent geometry. Moreover, the structure of the rat HO-1/NO (39) complex exhibits a very similar H-bonding pattern as the cdHO/O₂ complex (Fig. 3D). Thus, a comparison between various CO and NO complexes provides some insights on O₂/CO discrimination.

Currently, the only two heme oxygenases that have crystal structures available for both the CO- and NO-bound forms are those of nmHO and rat HO-1 (39, 40). As shown in Fig. 3D, the NO has three H-bonding partners, the peptide NH of Gly-143 and a water molecule which, as already noted, is very similar to the cdHO-oxy complex (29). The situation with nmHO is slightly different. In this case the NO forms only one H-bond, with the active site water. The peptide NH group of Gly-120 (Fig. 3B) is too far and geometry not optimal for good H-bonding. If, as we expect, the oxy complex is similar, then the missing H-bond in nmHO compared with HO-1 helps to explain why \( k_{\text{off}} \) for O₂ is faster in nmHO than in HO-1 or cdHO.

The more significant difference between nmHO and HO-1 or cdHO is that \( k_{\text{off}} \) for CO is \(~18\) times faster, indicating that the nmHO-CO complex is less stable than in HO-1. More specifically, in HO-1/CO the active site water is 2.9 Å from the CO whereas this distance is 3.1 Å in nmHO (Fig. 3, A and C). In addition, the CO is very close, 2.7 Å, to the carbonyl oxygen of Ser-117 whereas the corresponding distance is 2.9 Å in HO-1 (Fig. 3, A and C). A 0.2 Å difference is small and probably within the margin of error in comparing two structures. Thus, it is difficult to conclude that differences in steric crowding are the structural basis for the differences in CO \( k_{\text{off}} \). Here is where the molecular dynamics simulations provided some insights that point toward electrostatics as being an important factor in controlling the stability of the CO complex. The average MD structure (average of 100 structures) taken over the last 1 ns of the simulation superimposed on the crystal structures is shown in Fig. 4. Also shown in panels B and D are the average MD structures showing key distances indicated by the dashed lines.

**FIGURE 4.** Panels A and C are the average MD structures (green) taken over the last 1 ns of the simulation (100 structures) superimposed on the crystal structures (cyan). The key water molecule interacting with CO is indicated by green (MD) and red (crystal) spheres. Panels B and D are the average MD structures showing key distances indicated by the dashed lines.
ing greater electrostatic destabilization of the CO O atom than in HO-1. Second, the water near the CO in HO-1 is closer to the CO than in nmHO, thus providing greater electrostatic stabilization of the CO O atom. Over the 2-ns simulation the water-CO distance is 4.61 ± 0.97 Å in HO-1 and 5.83 ± 1.29 Å in nmHO-1. There is a fair amount of fluctuation owing to large movement of solvent as expected, but it is clear that the CO is better solvated in HO-1. Thus, less electrostatic stabilization of the CO in nmHO can help to explain the faster \( k_{off} \) for CO.

One problem with this argument is that CO is normally considered to be the least polar of heme diatomic ligands and, as such, electrostatics should not play that important a role in CO binding. However, electrostatic control of CO binding is supported by model heme studies. One comprehensive study on CO- and \( \text{O}_2 \)-bound twin-coronet porphyrins (TCPs) established that CO/\( \text{O}_2 \) ligand discrimination may be controlled by the polar effects exerted by overhanging hydroxyl groups (41). Specifically, the TCP-\( \text{O}_2 \) complex was stabilized by hydrogen bonding with two overhanging hydroxyl groups, whereas the TCP-CO complex was destabilized because of suppression of \( \pi \) backbonding from the iron atom to bound CO. The decrease in backbonding was caused by strong negative electrostatic interactions of bound CO with the lone pairs of the hydroxyl groups in the distal pocket, which resulted in an increase in the dissociation rate of CO from TCP compared with chelated protoporphyrin (41). These model heme studies thus support the view that the reason nmHO exhibits a slower CO \( k_{off} \) than HO-1 is due to less favorable electrostatic stabilization of the CO complex compared with HO-1.

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