Kinetics of CO Ligation with Nitric-oxide Synthase by Flash Photolysis and Stopped-flow Spectrophotometry*

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Interaction of CO with heme proteins has physiological importance. This is especially true for nitric-oxide synthases (NOS), heme/flavoenzymes that produce NO and citrulline from L-arginine (Arg) and are inhibited by CO in vitro. The kinetics of CO ligation with both neuronal NOS and its heme domain module were determined in the presence and absence of tetrahydrobiopterin and Arg to allow comparison with other heme proteins. Geminate recombination in the nanosecond time domain is followed by bimolecular association in the millisecond time domain. Complex association kinetics imply considerable heterogeneity but can be approximated with two forms, one fast (2–3 × 10^4 M^-1 s^-1) and another slow (2–4 × 10^3 M^-1 s^-1). The relative proportions of the two forms vary with conditions. For the heme domain, fast forms dominate except in the presence of both tetrahydrobiopterin and Arg. In the holoenzyme, slow forms dominate except when both reagents are absent. Geminate recombination is substantially, ~50%, only when fast forms predominate. Stopped-flow mixing found dissociation constants near 0.3 s^-1. These data imply an equilibrium constant such that very little CO should bind at physiological conditions unless large CO concentrations are present locally.

Nitric-oxide synthase is a heme protein that catalyzes conversion of the amino acid L-arginine to citrulline and nitric oxide (1, 2). The NO produced activates another heme enzyme, guanylate cyclase (see Reaction 1), that catalyzes formation of cyclic guanosine monophosphate, a second messenger that mediates numerous biochemical events including vascular smooth muscle relaxation, platelet disaggregation, photoreceptor cell differentiation, ion transport in gastrointestinal cells, and myeloid cell differentiation.

At least three isoforms of NOS have been purified and characterized. In each, a heme prosthetic group, iron protoporphyrin IX, is attached to the protein by a proximal cysteine. Visible absorption spectroscopy and other physicochemical measurements reveal similarities to cytochromes P450 (3–8). Like that family of proteins, reduced (ferro) NOS forms carbonyl derivatives with an absorption maximum at ~446 nm (4). Marletta (9) and Stuehr et al. (10) have proposed mechanisms for the oxidation of Arg catalyzed by NOS, based largely upon the similarity of NOS and P450, and Masters (11) has proposed a scheme involving redox interactions with the flavoprotein module of NOS. A key observation was that enzyme activity is inhibited by CO (4, 6, 9) as well as by NO (12), the physiological significance of which remains uncertain. Enzyme inhibition in vitro required large concentrations of either CO or NO. If those reagents play a role in vivo, the mechanism must involve locally enhanced concentrations. For NO, fast turnover and slow diffusion away from the site of production in unstirred cytoplasm might possibly lead to large concentrations locally and, consequently, to autoregulation of NO production. Recent evidence suggests a role for CO produced by heme oxygenase as a messenger molecule in the same cell types as nNOS (13–15).

Could the activity of heme oxygenase produce high concentrations? The CO concentration needed depends upon the equilibrium constant for CO binding. Unfortunately, equilibrium constants for binding CO and NO are not known for NOS. Matsuoka et al. (16) reported CO combination rate constants for neuronal NOS but not CO dissociation data, which could have been combined with the combination rate constant to calculate an equilibrium constant. In heme proteins, CO dissociation rate constants vary from 6.5 s^-1 in P450 to 7.2 × 10^-5 s^-1 in horseradish peroxidase. Therefore, knowledge of CO association and dissociation is important for determining NOS reactivity relative to myoglobin, guanylate cyclase, and other heme proteins, which potentially could compete with NOS for reactive ligands.

In addition to the interest generated by its physiology, this sensitivity of CO dissociation rate constants to protein structure makes it an excellent parameter for providing qualitative information about the local heme environment. A review of CO dissociation constants reveals two trends. 1) CO off rates are markedly affected by the nature of any base proximal to the heme–CO bond, whereas for a given base, any weakening of the base–heme bond, or its total rupture, dramatically increases the CO dissociation rate. 2) Steric hindrance on the distal side reduces both association and dissociation rates for CO in all cases known (17).

These same issues arise for carboxy guanylate cyclase activation. In vitro activity is significantly affected by 1 atm of CO, but equilibrium constants indicate that in vivo, little, if any, guanylate cyclase activation by CO should be expected. Nevertheless, several biological phenomena seem to be affected by small amounts of CO (15). We have no explanation, but the example points out the difficulty in extrapolating from in vitro to in vivo conditions. Nevertheless, a knowledge of equilibrium and rate parameters is the essential starting...
By accumulating 80–200 recordings, the root mean square uncertainty allowed us to estimate the equilibrium binding constant of CO in the case of the heme domain. In addition to bimolecular ability in (25 °C). The amino-terminal heme-binding domain (residues 1–714) L-[3H]citrulline assay (23) and found to be 420 nmol min

NaCl) in a gas-tight syringe and deoxygenated by bubbling with argon purified by passage through a column of fresh KOH. Buffers were used to determine association and dissociation rate constants. We investigated the effects of tetrahydrobiopterin (BH_4) and L-arginine (Arg). For the latter, advantage was taken of the ability in *E. coli* to express nitric-oxide synthases in the absence of BH_4 and with diminished Arg concentrations (18, 20) in the case of the heme domain. In addition to bimolecular association, we also investigated geminate recombination of CO-iron pairs confined within the protein. These studies allowed us to estimate the equilibrium binding constant of CO with NOS and provided insights into the nature of the heme pocket in the enzyme.

**MATERIALS AND METHODS**

Carbon monoxide (99.8%), nitric oxide (99.9%), high purity argon, and premixed CO in argon were all from Matheson. NO was further purified by passage through a column of fresh KOH. Buffers were prepared (20 mM Tris-HCl, usually pH 7.8, 100 µM EDTA, 100 mM NaCl) in a gas-tight syringe and deoxygenated by bubbling with argon for >40 min. When indicated, buffers also contained 250 µM BH_4 and/or 0.5 or 10 mM Arg. Microperoxidase solutions were prepared as described elsewhere (21).

Rat nNOS holoenzyme was expressed in two different systems. Unless otherwise indicated, it was in *E. coli*, purified and reconstituted as described previously (18). Nitric oxide formation, measured in *E. coli*-expressed nNOS after Arg and BH_4 replication using a hemoglobin capture assay described previously (22), was found to be 250 nmol min

m

1 (25 °C) or 540 nmol min

2

m

1 (25 °C). Expression of rat nNOS in human embryonic kidney cell was performed as reported previously (4); nitric oxide formation was measured using an l-[3H]citrulline assay (23) and found to be 420 nmol min

m

1 (25 °C). The amino-terminal heme-binding domain (residues 1–714) of rat nNOS was expressed in *E. coli* and purified as described previously (19). All proteins were >90% homogenous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein solutions were degassed and then reduced using degassed 1% sodium dithionite solution at 25 °C is 930

m

1 atm (105 pascals) total pressure. We used CO in argon at molar ratios of 5, 10, 20, and 50% as well as 100% CO. Molar concentrations were calculated assuming that the solubility of CO in aqueous solution at 25 °C is 930 µmol atm

1.

**RESULTS**

The kinetics of the reaction of CO with both the complete nNOS holoenzyme and the heme domain fraction of nNOS were characterized, and the rates in the presence and absence of Arg and BH_4 were measured. Thus, there are eight cases to distinguish. The effect of Ca^2+~/calmodulin on rates of CO binding has been previously shown to be negligible (16).

**Association—Flash photolysis measurements were made at 23 ± 0.5 °C.** They were carried out at both pH 7.0 and pH 7.8, with identical results. There was also no discernible difference between nNOS from kidney cells and that from *E. coli*. There was no irreversible photochemistry. Transient recovery was >99.5% after each flash, and spectra did not change during a series of flashes. Association rates were determined by monitoring recombination after photodissociation in the presence of excess CO. Kinetics were quite complex. In general, we observed three phases on different time scales. There was a very fast process with a half-life on the order of 100 ns that was unaffected by changing CO concentration and was assigned as geminate recombination of protein-caged heme-ligand pairs. All slower processes varied systematically with [CO]. Solutions were equilibrated with premixed gas mixtures at 1 atm (10^5 pascals) total pressure. We used CO in argon at molar ratios of 5, 10, 20, and 50% as well as 100% CO. Molar concentrations were calculated assuming that the solubility of CO in aqueous solution at 25 °C is 930 µmol atm

1.

We must distinguish a kinetic “phase,” as the term was used above, from an exponential component of a fit. The two words are frequently used as synonyms. Here, however, a phase may be amplitudes, which sum to unity, and \( k_i \) is the rate constant. The nanosecond geminate phase involved only a single exponential, characterized by \( a_\text{gem} \) and \( k_\text{gem} \). The bimolecular association required as many as four additional components, but they could be grouped into fast and slow phases characterized by \( k_f \) or \( k_s \), each defined as a sum over one, two, or, occasionally, three exponentials as follows.

\[
\Delta A(t) = s \sum \{a e^{-t/k_i}\}
\]

where \( s \) is an overall scale factor, \( a_i \) are amplitudes, which sum to unity, and \( k_i \) is the rate constant. The nanosecond geminate phase involved only a single exponential, characterized by \( a_\text{gem} \) and \( k_\text{gem} \). The bimolecular association required as many as four additional components, but they could be grouped into fast and slow phases characterized by \( k_f \) or \( k_s \), each defined as a sum over one, two, or, occasionally, three exponentials as follows.
for a fast phase (exponential fit of the major slow phase plus a small single exponential difficult to see in the semilog plot, also at short times. Several half-lives, but it clearly deviates at long times and, although due to very small, time-dependent shifts in the transient spectrum related to conformational relaxation of the holoprotein.

Principal data for all measurements are collected in Table I, but the following sections add information about certain details, mainly the multiple exponentials included in certain phases.

Heme Domain with neither l-Arginine nor BH₄—The fast phase could be fit fairly well using two exponentials of equal amplitude with rates differing by a factor of 3, but at very high S/N it became clear that two exponentials were not completely adequate. The steady-state absorption spectrum showed a small amount of extra absorption near 420 nm, consistent with that reported and explained by Wang et al. (28). We also noticed additional complexity in the transient spectra, particularly around the usual isosbestic point. This could be pursued, if one were interested in the so-called P420 form of NOS, but for our present purpose we believe that by choosing wavelengths characteristic of the P450-like spectrum, we can include this case in the comparison study, with minimal contamination by the P420 form.

Heme Domain with l-Arginine but Not BH₄—The fast phase required at least two exponentials with rates differing by about a factor of 3.

Heme Domain with BH₄ but Not l-Arginine—The fast phase required two exponentials differing by a factor of 4–5, slightly more than in the cases above.

Heme Domain with both l-Arginine and BH₄—Adding both reagents had a striking effect, not observed with either alone. Geminmate recombination was greatly reduced so that the slow phase dominated bimolecular association. The slow phase could be well fit using two exponentials differing in rate by a factor of 3, but instead of the amplitudes being approximately equal, the slower contributed 90% to the phase.

Holoenzyme with neither BH₄ nor l-Arginine—The bimolecular combination could be described very well using three exponentials, of which two could be grouped into a fast phase. The slow phase dominated bimolecular association. The slow phase could be well fit using two exponentials differing in rate by a factor of 3, but instead of the amplitudes being approximately equal, the slower contributed 90% to the phase.

Holoenzyme with BH₄ but Not l-Arginine—The slow phase was well fit using two exponentials differing by a factor of only 2, with the slower carrying the majority of the amplitude.

Holoenzyme with BH₄ but Not l-Arginine—The slow phase
required two exponentials differing in rate by a factor of about 3, with the slower carrying most (about 70%) of the weight.

**Holoenzyme with l-Arginine and BH₄**—Protein with 0.25 mM BH₄ and either 0.5 or 10 mM Arg showed no detectable geminate recombination (<3%), and the bimolecular association was almost exclusively (>95%) a slow phase. That phase, however, was not at all a single exponential (see Fig. 1). It required at least two and often three exponentials, distributed in rate over a range of 5–10. The small, fast phase was at least as fast as the fast phase observed in the absence of Arg. Although a small fast phase at the beginning of a decay can be quite apparent, it is difficult to characterize since it is affected by the random noise in the much larger slow phase.

**Dissociation**—Plots of association rates versus [CO] were linear and could be extrapolated to the y intercept, but the plots were not very useful for estimating dissociation constants. Only upper limits could be assigned, typically <20 s⁻¹ for the fast phase and <2 s⁻¹ for the slow phase. Consequently, we resorted to stopped-flow methods carried out at 20 °C. With one exception, all stopped-flow measurements were well fit by single exponentials over the time scale that could be monitored. A typical trace is shown in Fig. 2. The absorbance change of 0.07 is what was expected for the change from reactants to products, so that any additional component cannot be very large. Results are included in Table I. The holoenzyme lacking Arg showed about 15–20% faster phase with rate constant 15 ± 2. Perhaps surprisingly, the heme domain did not show any fast process under any conditions.

**DISCUSSION**

**Heterogeneous Kinetics**—Bimolecular association required three or four exponentials to fit, but the exponentials tended to group into two distinct time regimes. Fig. 1 displays a large amplitude slow phase requiring three exponentials varying in rate by only a factor of 4 between the extreme values together, with a minority fast phase that is 150 times faster. We attribute the different rates to different protein conformations, each reacting with its own rate constant. This is based on two considerations. 1) Protein expression should produce proteins with a common sequence identity. 2) Lack of spectral evidence for intermediates appears to rule out sequential mechanisms. (Additional spectral features can be detected under certain circumstances but not the conditions discussed here.) We postulate a quasi-continuous distribution of conformations, which is bimodal, clustering about the two most common forms. The effect of cofactors, substrates, and other reagents is to bias the probability distribution in ways that can be described approximately (but not completely) as shifting the protein between fast reacting forms and slow reacting forms. The one previous report of combination kinetics in NOS distinguished the two major phases, but did not recognize additional complexity (16).

Tentatively assuming that kinetic heterogeneity is due to
BH₄ and Arg. All other cases lie in between. The holoenzyme lacking both BH₄ and Arg is similar to the heme domain, but adding either reagent is sufficient to convert it largely to the slow reacting form even though both are needed for the full effect. For the heme domain, either reagent alone has a small effect, but both together have a dramatic effect. The limiting cases are reproducible. For the intermediate cases, different preparations vary slightly in the percentage of each phase, again suggesting heterogeneity. Thus, it is not surprising that there is some slight difference between our numbers and those reported previously, which we believe pertain to two intermediate permutations (16).

It is known that both the BH₄ and the Arg binding sites are located in the heme domain (19, 34). Our results confirm that model. It has also been reported that addition of Arg to the native enzyme is accompanied by changes in the UV-visible spectrum that are consistent with conversion of a low-spin, six-coordinate iron to a high-spin, five-coordinate iron (35). This implies that the Arg does not bind directly to the iron but perturbs or displaces some as yet unidentified ligand (or protein side chain) coordinated to the iron (35, 36). Steric interference by Arg of the ligand binding site was suggested (37). It is not surprising, therefore, that in the presence of both BH₄ and Arg the CO association rate is reduced by a factor of 100 below the fast phase. Lack of any profound effect by Arg alone in the heme domain suggests that Arg can be incorporated into the heme domain, but its spatial orientation is different and directed away from the ligand binding site. In the holoenzyme, Arg alone is only slightly different from Arg + BH₄, showing that the options for spatial orientation are severely limited. These conformational constraints may also correlate with quaternary changes, that is, the formation of homodimer, which is facilitated by BH₄ and may be more favored in the holoenzyme.

The dissociation rate for CO from either NOS-CO itself or the heme domain subunit is close to 0.3 s⁻¹ in the presence of Arg and 0.16 s⁻¹ in its absence. Either number is high compared with the oxygen-binding hemoproteins but substantially less than in P450. Consider: five-coordinate carboxyhemec (a protein-free model compound) in cetyltrimethylammonium bromide detergent has a CO dissociation rate constant of 400 s⁻¹. Coordination of proximal imidazole trans to CO in either a protein or a model system reduces the CO dissociation constant to between 0.1 and 0.01 s⁻¹ (38). In six-coordinate bacterial cytochrome P450cam, in the absence of substrate, the CO dissociation rate constant is 6.3 s⁻¹ (38) upon extrapolation to room temperature, which represents a much smaller trans effect of only 64. In the presence of substrate, CO dissociation from carboxy-P450 decreases to 1.4 s⁻¹, and it is not surprising that there is some slight difference between our numbers and those reported previously, which we believe pertain to two intermediate permutations (16).

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We noted above the evidence for some steric crowding at the ligand binding site. As mentioned in the introduction, such crowding seems always to decrease rate constants for both association and dissociation of CO. Consequently, we can infer from kinetic measurements that the pocket in NOS is probably more constrained than in P450cam. The is no significant sequence homology between NOS and P450 and, according to computer modeling, no structural similarity. Consequently, differences in their heme pockets should be expected.

From the rate constants, one may calculate association equilibrium constants for the fast and slow phases of −1⁰⁸ and −1⁰⁵. (Conditions that generate some fraction of faster dissociation will reduce those numbers even further.) This affinity is 300 times less than in human Mb. It is unlikely that CO binding will be significant in vivo, where low CO concentrations are expected, unless there is much local enhancement and an absence of nearby traps, such as Hb or Mb. Ingi et al. (15) predicted, on the basis of different evidence, that a 1 mM concentration of CO would be needed to inhibit NOS activity. The equilibrium constant reported here suggests that their value is too high, but we concur with the conclusion that CO inhibition is unlikely. In fact, a recent study suggests just the opposite; far from being an inhibitor, small amounts of CO, 1000 ppm, may increase NO production (41). If that observation is confirmed, it will have important implications for the mechanism of NOS action. One possibility is that small amounts of CO may stabilize the reduced, ferro intermediate of iron in NOS in the reaction scheme proposed by Marletta (9).

Geminate Recombination—After bond dissociation by flash photolysis, CO may remain in the heme pocket for some time. The simplest model for such a geminate pair temporarily trapped in a protein pocket is as follows.

\[ k_1 \frac{[Fe\cdot CO]}{[Fe]} = \frac{k_{gem}}{k_{gem} + k_2} \]

In this model, admittedly oversimplified (24), the rate of disappearance of the caged pair intermediate is \( k_{gem} = k_2 + k_3 \), whereas the geminate yield (the fraction recombining directly from the geminate pair) is \( Y = k_{gem} / (k_2 + k_3) \). For NOS, Table I shows that \( k_{gem} \) is about 1.5 × 10⁵ s⁻¹ and varies by less than a factor of 2 among our eight conditions, while \( Y \) changes by a factor of 10, from <5% to almost 50%. This implies that \( k_3 \) remains approximately constant, while \( k_2 \), the rate of bond formation proper, changes significantly.

Changes in \( k_2 \) may be due to changes either in the distal environment or in strain on the proximal ligand, both of which are influenced by protein conformation. Hence, geminate recombination provides direct evidence that in addition to other consequences, when the two domains are combined or BH₄ or Arg is added, one effect is a substantial perturbation at the heme. Resonance Raman spectroscopy also probes the heme and its environment. Binding Arg causes a frequency shift of the Fe–NO stretch (37). Furthermore, that mode is broad, suggesting multiple conformations (7). All this echoes what is inferred from bimolecular combination rates.

The rates \( k_{gem} \) are similar in all hemoproteins. In P450, two components were detected, one 2–3 times faster than we observe in NOS, the other 2–3 times slower (42). For three different hemoglobins, \( k_{gem} \) varied from approximately equal to about half the rate measured in NOS (43). Part of that variation is due to the fact that \( k_{gem} = k_2 + k_3 \) is faster when \( k_2 \) is greater. Escape from the protein \( k_3 \) is surprisingly constant for different hemoproteins.

The geminate yield \( Y \) varies greatly among hemoproteins. For CO, \( Y \) is no more than about 5% in Mb, 20–50% in various HbR, depending on pH, and somewhat less in Hb under conditions that favor T-like structures (42). In P450cam, \( Y \) is reported to be as large as 90% in the absence of substrate, reduced to only 2% with camphor bound (41). NOS is like P450 in having very small \( Y \) with substrate bound. In the absence of substrate, P450 is like NOS without BH₄ or like the heme domain and unlike the competent holoenzyme lacking only substrate. This echoes the conclusion from measurements by magnetic circular...
dichroism that "the active sites of NOS and P450 may share some common structural features, but significant distinctions exist" (44).

Geminate yield is related to bimolecular association. According to equation 3,

$$k_a = k_i Y$$  \hspace{1cm} (Eq. 4)

where $k_a$ is a bimolecular rate constant that describes entry into the protein and involves both diffusion to the periphery of the protein and a steric factor describing the fraction of encounters in which the ligand penetrates the protein. After entry, only a fraction $Y$ of the ligands bind to the iron; the rest escape and must try again. From Table I, a correlation is evident between $Y$ and the fraction of the bimolecular fast phase. The fast binding forms have substantial, ~50%, geminate yield, whereas slow binding conformations show very little geminate recombination. The 100-fold ratio in association rates between fast and slow phases can be accounted for largely by their different $Y$.

Geminate recombination strongly suggests that conformational effects on ligand binding are mediated not by changes in ligand entry into the protein but rather by interactions related to binding at the iron, involving strain from the proximal cysteine or polar or steric effects from the distal side.

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