Nitric-oxide synthase (NOS) catalyzes conversion of L-arginine to nitric oxide, which subsequently stimulates a host of physiological processes. Prior work suggests that NOS is inhibited by NO, providing opportunities for autoregulation. This contribution reports that NO reacts rapidly (\(k_a = 2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\)) with neuronal NOS in both its ferric and ferrous oxidation states. Association kinetics are almost unaffected by L-arginine or the cofactor tetrahydrobiopterin. There is no evidence for the distinct two phases previously reported for association kinetics of CO. Small amounts of gaseous recombination of NO trapped in a protein pocket can be observed over nanoseconds, and a much larger amount is inferred to take place at picosecond time scales. Dissociation rates are also very fast from the ferric form, in the neighborhood of 50 s\(^{-1}\), when measured by extrapolating association rates to the zero NO concentration limit. Scavenging experiments give dissociation rate constants more than an order of magnitude slower; still quite fast. For the ferrous species, extrapolation is not distinguishable from zero, while scavenging experiments give a dissociation rate constant near \(10^{-4} \text{ s}^{-1}\). Implications of these results for interactions near the heme binding site are discussed.

Nitric-oxide synthases (NOS)\(^1\) constitute a family of heme proteins that catalyze conversion of L-arginine to citrulline and nitric oxide (1). The production of nitric oxide in specific cell types fulfills certain physiological roles for which each isoform is suited according to its structure and regulation (2). Neuronal NOS\(^\dagger\) (nNOS) (1) isoforms are localized in brain and in skeletal muscle, and induced by cytokines, produces NO for cytotoxic action. Endothelial NOS (eNOS) produces NO as a vasodilator. It was reported some years ago that NOS activity is inhibited by shear stress due to blood flow. The functional significance of eNOS association with plasma membrane is poorly understood. Perhaps blood flow modulates eNOS activity by, among other mechanisms, continuously depleting local NO concentrations. An optimum regulatory role for eNOS, or any isoform of NOS, would seem to require finely tuned affinity for NO.

In the much studied hemoglobins and myoglobins, there is a coordinate covalent bond between the heme iron and a proximal histidine; and the iron is exclusively Fe(II). In NOS, the bond is with the thiolate function of a cysteine residue; and the iron is exclusively Fe(III) state and seems to make significant effects. Proximal Cys favors the Fe(III) state and seems to make the ligation kinetics of ferric and ferrous species more similar, but heme proteins with either proximal base exhibit wide variation in rate constants. Tuning of the reactivity in heme proteins is achieved by varying the electrostatic and steric environment in the neighborhood of the iron, both at the proximal bond and on the opposite, or distal, side.

Association rate constants of NO with heme proteins range from \(-2 \times 10^7\) to \(-200 \text{ M}^{-1} \text{ s}^{-1}\), and the dissociation rate constants range from 100 to 0.03 s\(^{-1}\) for the ferric species and at least 1000-fold slower yet for the ferrous form (11, 12). The

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\(^{\dagger}\) The abbreviations used are: NOS, nitric-oxide synthase; BH\(_4\), tetrahydrobiopterin; Cyt c, horse heart cytochrome c; eNOS, endothelial NOS; iNOS, inducible NOS; Hb, hemoglobin(s); Mb, myoglobin(s); NOS, neuronal NOS; SDT, sodium dithionite (Na\(_2\)S\(_2\)O\(_4\)).
fastest association rates, which are similar for a large number of heme proteins, are limited by the rate of diffusion of the ligand through solvent to the protein and entry into the protein, after which bonding is very efficient. Apparently, entry of ligands into the protein and access to the iron is quite similar for many proteins. The lower association rate constants apply for proteins having substantial steric hindrance to bonding or when there is a need to displace water or some other ligand. The slow association in horse heart Cyt c is attributed to the need to displace a methionine sulfur (11). Like other heme proteins, NO has charged or polar amino acid side chains in the distal pocket, which might stabilize or destabilize coordination of a ligand, such as H2O (13). Ligand dissociation rate constants are also affected by factors that stabilize ligand bonding. For example, NO dissociates very slowly in many Mb and Hb, because it is stabilized by a particular distal His. In contrast, NO dissociates is much more rapidly from Mb(EL) (Asian elephant), which lacks the distal histidine (14). Effects of distal histidines have been examined recently in great detail using site-directed mutations in Mb (15, 16). The overall mechanisms proposed (10, 17, 18) for NOS-catalyzed conversion of L-Arg to citrulline specify both an interaction of L-Arg and N-hydroxylarginine bound to the enzyme in the distal heme pocket with a ligand (a reduced oxygen species, in this case) coordinated to the iron, as well as formation of hydroxymethemoglobin (NOS-•H2O) just before release of NO in the last step. Furthermore, it has been speculated that a coordinated water molecule in the hydroxy intermediate of NOS may be responsible for slowing down NO ligation long enough for the NO to diffuse away from the binding site and, consequently, minimizes self-inactivation of NOS.

For ferrous heme proteins, affinity of NO is usually extremely high, so that NO binds more strongly than other ligands, such as O2, and often acts as a “poison.” One significant exception is Cyt c, in which the affinity of the ferrous species for NO is quite low and only 20-fold greater than that of its ferric counterpart (11). This is due to an exceptionally small association rate, only 8.3 M⁻¹ s⁻¹ (11). In a different, recently described case, that of soluble guanylate cyclase in the presence of substrate GTP, low affinity is achieved in the opposite manner, i.e. primarily by unusually rapid dissociation (19). Since both guanylate cyclase and NOS are involved in the NO regulatory cycle, it is appropriate that they both be regulated by NO but not poisoned by very high affinity for NO. So, questions for NOS are as follows: Is affinity for NO reasonably low? Does affinity differ between ferric and ferrous oxidation states? Is low affinity achieved by slow association or by fast dissociation? Are the kinetics or the equilibria affected by cofactors or substrate? In the studies reported here, we answer those questions by determining the kinetics of NO ligation with both ferric and ferrous derivatives of nNOS holoenzyme and its heme domain (residues 1–714). We investigated the effects of tetrahydrobioprotein (Bhf) and L-arginine. From the bimolecular rate constants, we could derive an equilibrium constant for NO binding to nNOS.

MATERIALS AND METHODS

Nitrax oxide, high purity argon, and premixed NO diluted in argon were all obtained from Matheson. Nitrax oxide was further purified by passage through aqueous KOH solution. Buffers (20 mM Tris-HCl, pH 7.8, 100 μM EDTA, 100 mM NaCl) were prepared in a gas-tight syringe and deoxygenated by bubbling with argon for at least 40 min. When indicated, buffers were prepared with 250 μM BH4 and/or 15 mM Arg.

NOS was expressed in Escherichia coli and purified and reconstituted as described previously (20). The amino-terminal heme-binding domain (residues 1–714) was also rat nNOS expressed in E. coli and purified as reported previously (21). Assay for activity and other characterization and controls were as reported previously (9). Proteins were degassed very gently by blowing argon over the solution in a cuvette with a large surface-to-volume ratio. For studies of the ferrous oxidation state, protein solutions were reduced by adding small amounts of 100 μM sodium dithionate. Sample solutions were prepared by vigorously bubbling premixed NO diluted in argon through degassed buffer to obtain the desired [NO] and then adding small amounts of degassed concentrated protein solution. Measurements were made at 23 ± 0.5°C.

Kinetic measurements were carried out as described previously (9). For the laser flash photolysis, 3 mJ in 4 ns at 550 nm was used for excitation over an area of 0.1 cm² collinear with the probe light. Protein concentrations were 10 μM for the heme domain and 5 μM for holoenzyme. Protein dissociations were 10 μM for the heme domain and 5 μM for holoenzyme. Digitization was by a LeCroy 9361 oscilloscope. Typically, 200–1000 laser “shots” were averaged for each run. Kinetics were always characterized at two different wavelengths, as a check that they gave similar results, one near 445 nm corresponding to bleaching of the original solution and the other near 405 nm corresponding to maximum transient absorption. Preliminary measurements were made from 390 nm to 450 nm to search for any anomalous behavior, but none was found. The isosbestic region near 420 nm was examined to confirm that only two states were involved, at least within available resolution. Steady state absorption was monitored before and after laser irradiation in order to verify that there was very little photodegradation. Statistical noise was reduced to ΔA(Δt = 0) = 0.00002, typically. This was necessary because the low quantum yield for escape of dissociated NO from the protein resulted in small transient absorbance changes (at best, ΔA(Δt = 0) = 0.01). A more extensive discussion of signal-to-noise issues has been given elsewhere (9).

Kinetics of NO dissociation from ferric nNOS-NO were determined using a Durrum stopped-flow instrument. NO dissociation was monitored at 412 nm during reaction with oxymyoglobin, which is converted to metmyoglobin by reaction with NO, a method we used recently for another protein (22). Trapping of free NO by MbO2 is fast and irreversible. Four different concentrations of scavenger were used in order to establish that the absorption changes were independent of scavenger concentration and, therefore, rate-limited by NO dissociation. Dissociation from ferrous nNOS-NO is so slow that it could be measured in an ordinary spectrophotometer, utilizing sodium dithionate (SDT) as a scavenger, in the presence of CO, and monitoring absorbance change at 420 nm (23). Concentrations of some components are slightly different for the dissociation measurements than for photolysis; they are listed in figure captions.

RESULTS

Definition of the Rate Constants—Rate constants for bimolecular association are defined in Reaction 1.

\[
\begin{align*}
\text{NOS} + \text{Fe} + \text{NO} \rightleftharpoons \text{NOS} + \text{Fe} + \text{NO} \\
\end{align*}
\]

\[
\text{Reaction 1}
\]

Photolysis perturbs the equilibrium and creates a temporary excess population on the right. Flooding the sample with excess NO ensures pseudo-first-order conditions under which relaxation back to equilibrium is described by Equation 1.

\[
\text{h}_{\text{obs}} = k_{\text{obs}}[\text{NO}] + k_{\text{d}}
\]

Equation 1

When \( k_{\text{obs}} \) is plotted versus [NO], the slope and intercept determine \( k_{\text{d}} \) and \( k_{\text{a}} \). We used 100% NO (2000 μM) and certified mixtures of 10%, 0.5%, and 0.1% NO in Ar in order to ensure known [NO]. In all cases, there was good linear behavior extrapolating close to the origin. Since association was quite fast, we needed 0.5% and 0.1% NO mixtures to establish the intercept. Repeated measurements verified that the y intercept was measurable only above the origin for ferric nNOS-NO. For ferrous nNOS-NO the intercept was indistinguishable from the origin and could be measured only by a mixing experiment. The overall bimolecular rate constants \( k_{\text{a}} \) and \( k_{\text{d}} \) for a variety of conditions are collected in Tables I and II.

An observation that is only semiquantitative, but nonetheless important, is that the net photodissociation yield for ligands to leave the protein was quite low, roughly 1%. Even this
TABLE I
Rate constants for binding NO to ferric nNOS heme domain and holoenzyme

| System | $k_a$ | $k_t$, flash | $k_t$, mixing |
|--------|-------|--------------|--------------|
| Dom    | 2.1   | 0            | 0            |
| Dom + Arg | 16   | 50           | 5            |
| Dom + BH₄ | 19   | 50           | 5            |
| Dom + Arg + BH₄ | 2.1  | 0            | 0            |
| Holo   | 6.0   | 70           | 2            |
| Holo + Arg | 5.9  | 120          | 2            |
| Holo + BH₄ | 6.1  | 60           | 2            |
| Holo + Arg + BH₄ | 6.2  | 120          | 3            |
| Cytø   | 0.00072 | 0.044       | 14           |
| Mb (SW) | 0.053 | 14           | 40           |
| Mb (EL) | 2.2   | 2            | 0            |

$^a$ Dom, heme domain; Holo, holoenzyme.
$^b$ Data from Ref. 11.
$^c$ Data from Ref. 33. SW, sperm whale.
$^d$ Data from Ref. 14. EL, Asian elephant.

TABLE II
Rate constants for binding NO to ferrous nNOS heme domain and holoenzyme

| System | $k_a$ | $k_t$, flash | $k_t$, mixing |
|--------|-------|--------------|--------------|
| Dom    | 11    | 0            | 0            |
| Dom + Arg | 10   | 0            | 0            |
| Dom + BH₄ | 9.8  | 0            | 0            |
| Holo + Arg | 9.0  | 0            | 0            |
| Holo + BH₄ | 9.0  | 0            | 0            |
| Cytø   | 8.3 x 10⁻⁶ | 2.9 x 10⁻⁵   | 0            |
| Mb (SW) | 17    | ~10⁻⁴        | ~10⁻⁴        |

$^a$ Dom, heme domain; Holo, holoenzyme.
$^b$ Solutions without L-arginine were unstable.
$^c$ Data from Ref. 11.
$^d$ Data from Ref. 23. SW, sperm whale.

FIG. 1. Base-10 logarithms of absorbance changes over seven half-lives following flash photolysis of solutions of nNOS-NO holoenzyme in the presence of l-Arg and BH₄. Top panel; ferric form; bottom panel; ferrous; both have the same time scale. Dots are data points; lines are best fits; fits to a double exponential are indistinguishable from the data, except where visible in the tails; fits to single exponentials intersect the data curve at two points, but fail in the tails and also at early times, although the semilogarithmic plot obscures how bad they are in that region. Still, the two rates used differ only by a factor of just over 2 in the lower panel and under 2 in the upper panel.

protein conformations that the simple oxygen carriers do not, and this leads to a narrow distribution of $k_a$; however, at this point in time, it is difficult to rule out absolutely the possibility that the more complex isolation and purification procedures required for the enzymes might lead to slightly damaged protein that is avoided in the much simpler procedures used for the globins. The main point is that with precision capable of detecting such inconsequential heterogeneity even in the very difficult case of NO photolysis, with its low photodissociation yield, we can be very confident that no major feature is obscured by inadequate sensitivity.

Variation of $k_{on}$—All the association rates for both ferrous and ferric nNOS are quite fast, in the neighborhood of 10 μM⁻¹ s⁻¹. In ferric nNOS⁺⁺, the NO association rate for the heme domain alone is consistently 2–3 times faster than that for the holoenzyme. Ferrous nNOS does not show this variation, and its $k_a$ have an intermediate value. For ferric nNOS⁺⁺, we were able to prepare protein with and without substrate, l-Arg, and cofactor, BH₄. Any differences are very small. All of the holoenzyme cases lie within statistical scatter. For the heme domain, the difference with and without l-Arg is probably real, but just barely measurable. For ferrous nNOS-NO, solutions without arginine are unstable, a point of considerable significance in itself, and could not be characterized. Instability in nNOS has been discussed by others at some length (25), although that study put more emphasis on the role of BH₄⁺⁺.

Once BH₄⁺⁺ is present, it is difficult to remove completely, so it is important that we started with protein prepared (21) so that BH₄⁺⁺ was never present, by employing recombination in E. coli that itself does not make BH₄⁺⁺. It is also difficult to exclude arginine completely. The cases listed as lacking it may have trace amounts.

For comparison, we note that the common Hb and Mb proteins have $k_a$ that differ substantially between ferrous and ferric forms. Ferrous globins usually have $k_a$ roughly similar to what we find for nNOS; but the ferric globins have much smaller association rate constants.

Variation of $k_{off}$—For ferric nNOS, the values derived from the intercept of $k_{obs}$ in the flash photolysis experiments are
A small photodissociation yield, as NOS-NO has, entails very efficient geminate recombination. The corollary is that almost every ligand that enters the protein will bond to the iron, so that $k_d$ is controlled by the time needed to reach the protein and pass through whatever "gate" exists at entry. This means that steric and electronic effects exercised at the heme iron binding site are not very effective in modulating bimolecular association $k_a$. In contrast, the net ligand dissociation rate $k_d$ remains directly proportional to the rate of breaking the Fe-NO bond, albeit reduced by a constant factor dependent on the geminate recombination probability. Protein control of ligand affinity in the case of NO can be exerted effectively through the dissociation constant, utilizing steric and electronic variation on either proximal or distal sides of the iron. The situation is different for CO and any other situation with large photodissociation yield, in which variation near the iron immediately affects both $k_a$ and $k_d$, and, consequently, $K_a$.

**DISCUSSION**

The single exponential with parameters as in Table I.

near 50 s$^{-1}$, with a significantly higher rate constant only for the holoenzyme in the presence of L-Arg. There was scatter in measurements made during several repetitions on different days, as much as a factor of 2, but no observations anywhere near 10 s$^{-1}$, which would have been easy to detect. This presented a conundrum, because such a fast flow mixing experiments were then undertaken. They gave no way to guess whether the intercept in flash photolysis might actually be a factor of 10 higher than the case of NO can be exerted effectively through the dissociation constant, utilizing steric and electronic variation on either proximal or distal sides of the iron. The situation is different for

For ferrous nNOS the flash data extrapolated close to the origin, implying slower $k_d$ and larger $K_a$. The equilibrium association constant $K_a$ was, in fact, very large, beyond our ability to measure by titration, since we worry that NO is sufficiently reactive to cast some doubt on efforts to prepare very low [NO] with accuracy. We determined $k_d$ by trapping spontaneously released NO and found the small $k_d$ reported in the table. We have no way to guess whether the intercept in flash photolysis might actually be a factor of 10 higher than the $k_d$ obtained by scavenging, as there is no way to measure by extrapolation such a small departure from the origin.

**FIG. 2.** Reaction time course for NO dissociation from ferric nitrosyl heme domain of nNOS, measured using MbO$_2$ as scavenger for NO. $\lambda = 409$ nm; T, 20 °C; pH, 7.9; Tris, 20 mM; EDTA, 100 $\mu$M; NaCl, 100 mM; BH$_4$, 250 $\mu$M; protein, 0.5 $\mu$M; MbO$_2$, 6 $\mu$M; NO, 2.5 $\mu$M. Symbols are measured absorbances; continuous line is a best fit to a single exponential with parameters as in Table I.

**FIG. 3.** Reaction time course for NO dissociation from ferrous nitrosyl heme domain of nNOS, measured using sodium dithionite as scavenger for NO in the presence of CO. $\lambda = 420$ nm; T, 20 °C; pH, 7.8; Tris, 20 mM; EDTA, 100 $\mu$M; NaCl, 100 mM; L-Arg, 10 mM; protein, 2 $\mu$M; SDT, 30 mM; NO, 20 $\mu$M; CO, 945 $\mu$M. Symbols are measured absorbances; continuous line is a best fit to a single exponential with parameters as in Table II.
NO Ligation with Nitric-oxide Synthase

It could also explain the different results we obtained for two different methods of measuring $k_d$.

Rate constants in Table I for $k_d$ of the heme domain are consistently higher than for the holoenzyme by a factor of 3–4, which demonstrates that the active site is influenced in some manner by more distant parts of the protein.

There is a striking qualitative difference between association of NOS with CO and with NO, aside from any quantitative differences. Two independent studies (9, 29) demonstrated that CO association exhibits two distinct phases separated in rate by a factor of about 100. Reaction with NO either has none of the slower phase or so little that we have not been able to resolve it. The major effect of cofactor, BH$_4$, or substrate, l-Arg, on CO reactivity is to shift the relative contributions of the two phases. Those two agents have very little effect on NO reactivity, which is reasonable since there is only one phase present.

Several considerations: the proposed overall reaction mechanism, the recently determined (26) crystal structure for part of iNOS (residues 66–498 of the oxygenase domain), as well as ENDOR studies of intact nNOS (30), all entail interaction between activated oxygen coordinated to heme and the guanidino group of l-Arg or N-hydroxy-Arg. The NO association rates do not reflect such interactions. The lack of sensitivity of association rates to l-Arg and BH$_4$ has consequences given our finding that the rate-limiting step for $k_d$ is diffusion to and entry into the protein. Diffusion should not be affected, and protein entry apparently is not affected by l-Arg or BH$_4$.

Ligand dissociation rate constants are also unaffected by l-Arg or BH$_4$. The $k_d$ for ferric nNOS$^-$, as determined by extrapolation of flash photolysis transient kinetics, all lie within a factor of 3 of each other and are very fast, suggesting that NO coordination has little stabilization by hydrogen bonding with distal residues. This was discussed above in the context of $k_d$ for different species and mutations of Mb. It has also been observed previously for catalase (11) and cytochrome c peroxidase (11), as confirmed in our own studies (12). However, $k_d$ for the ferric species are lower by a factor of 10 or more when determined by extrapolation by stopped-flow mixing with a scavenger. The latter values are closer to the value for ferric Hb$_{a^+}$-NO, in which coordinated NO is stabilized somewhat by hydrogen bonding with distal histidine. A plausible explanation of the difference between flash photolysis and scavenging is the same one invoked above for another purpose: that following bimolecular addition of NO there is a structural change on a slow time scale that stabilizes NO binding. The hydrophilic residue nearest to the heme is Glu$_{592}$. The analogous residue in iNOS, Glu$_{771}$, although a little removed from the ligand binding site, is known to interact with l-Arg in the heme pocket (26). It has been found that l-Arg and the mechanism-based inactivator aminoguanidine bind in the distal pocket adjacent to the heme and interact directly with Glu$_{592}$. We speculate that either substrate arginine or, in its absence, Glu$_{592}$ may be stabilizing NO binding via a hydrogen-bonded water molecule drawn into the pocket after initial NO ligation.

In the case of ferrous nNOS, extrapolation of observed association rates to zero nitric oxide concentration did not yield an intercept significantly different from zero, but that only means <10 s$^{-1}$. The $k_d$ measured by scavenging NO from ferrous nNOS-NO is near 10$^{-4}$ s$^{-1}$, and comparable to that in Mb-NO. It too is minimally affected by Arg or BH$_4$. This suggests that there is no serious steric interaction between coordinated NO and arginine. The fact that electron transfer occurs between these centers does not present a contradiction. Tuning donors and acceptors to facilitate efficient electron transfer is not just a matter of having them as close as possible; rather, they are optimized carefully to control both forward and reverse transfers. This was discussed above in the context of $k_d$ for the ferric state of NOS (8).

The affinity of NO for ferric nNOS is modest, due exclusively to rapid dissociation able to offset rapid association, which is actually quite fast. This keeps nNOS from being poisoned, but also allows rapid kinetic changes. The remaining question is how the enzyme escapes being inactivated by irreversible binding of NO to ferrous nNOS. The explanation probably lies in the observation that the ferrous nNOS-N0 complex is stable only in the presence of l-Arg. In a sense, the protein shifts to its ferric form to offload NO. Due to the anionic nature of the proximal ligand (Cys$^{415}$), the higher oxidation state is favored in NOS. Although the presence of the positively charged guanidino group would tend to stabilize the ferrous oxidation state, if the rate of heme oxidation is faster than the second order combination of NO with ferrous N0, then only very small amounts of ferrous NO will be formed during the enzyme turnover.

The affinity of NO for ferric nNOS$^-$ is sufficiently high that in unstirred solutions high local concentrations of NO may cause self-inactivation, but not so high as to render the enzyme unresponsive to changes in physiologically plausible local concentrations of nitric oxide. Since the ferrous form of the enzyme is present only in very small amounts, and NO is produced while the enzyme is in the ferric state, one may appreciate the observation by Griscavage et al. that NO inactivates by binding to the ferric state of NOS (8).

Kinetics of NO (and CO) binding to iNOS were reported very recently by Abu-Seoud et al. (31), after the experiments described here were complete. In some ways, their study was similar to ours: both compared the Fe(II) and Fe(III) species, and both investigated the effects of l-Arg and BH$_4$. In other ways, the two studies were complementary: we studied nNOS, they studied iNOS; we compare the heme domain with the holoenzyme, they studied only the heme (oxygenase) domain; we worked at 23 °C, they at 10 °C; we used flash photolysis, they used stopped-flow mixing; we used both extrapolation and scavenging to measure dissociation, they report only association measurements; we began a characterization of geminate recombination, their mixing experiments will never address geminate recombination; we merely mention protein instability for certain conditions, while they pursue that issue at considerable length. The differences in methods and conditions suggest that a number of general conclusions reached in both studies are robust and probably apply to all NOS isoforms; our association rate constants are generally about an order of magnitude faster, but after a plausible correction for different temperatures, this implies that the association rate constants are fairly similar in nNOS and iNOS. Both studies agree that NO association is similar for Fe(II) and Fe(III) and that the effects...
of L-Arg and BH₄ are minimal. Both studies agree that the dissociation rate for Fe(III) as measured by extrapolating association kinetics is very high. The $k_d$ are numerically very close in the two studies, but this means either that they will diverge slightly after a temperature correction is applied (which is entirely possible since nNOS and iNOS are, after all, different), or that the activation energy for dissociation is surprisingly small. Abu-Soud et al. (31) were able to extrapolate association data for ferrous iNOS-NO and determine intercepts that were as fast for Fe(II) as for Fe(III). Our best estimates place our intercepts lower and close to zero, and our scavenging experiments determined a very small $k_d$ for ferrous nNOS-NO.

The general similarity between our nNOS and their iNOS is particularly intriguing in light of a study by Raman spectroscopy (32), whose authors found similarities between iNOS and eNOS but differences between the two constitutive isoforms eNOS and nNOS, which might have been expected to be the more similar pair. They explain the difference by postulating a more open pocket in nNOS. That should have implications for ligand binding kinetics, although they might be subtle. Since $k_a$ is rate-limited by protein entry, one might expect an “open pocket” to affect $k_a$, but it does not. The situation with $k_d$ is unclear because of the lack of scavenging measurements on iNOS, but the only difference that seems like it may exist (faster $k_d$ in iNOS) is in the wrong direction; it should be nNOS that is faster, if it has the open pocket.

To summarize, we have shown that fast ligand association in nNOS combines with fast dissociation to keep affinities reasonable in the ferric form, but an additional mechanism (cy

REFERENCES

1. Knowles, R. G., Palacios, M., Palmer, R. M., and Moncada, S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5159–5162
2. Masters, B. S., McMillan, K., Sheta, E. A., Nishimura, J. S., Roman, L. J., and Martasek, P. (1996) FEBS Lett. 389, 1255–1260
3. Kato, T., Schmidt, K., and Mayer, B. (1992) Biochem. J. 288, 15–17
4. Stuehr, D. J., and Ikeda-Saito, M. (1992) J. Biol. Chem. 267, 20547–20550
5. White, K. A., and Marletta, M. A. (1992) Biochemistry 31, 6627–6631
6. Griscavage, J. M., Rogers, N. E., Sherman, M. P., and Ignarro, L. J. (1993) J. Immunol. 151, 6239–6237
7. Buga, G. M., Griscavage, J. M., Rogers, N. E., and Ignarro, L. J. (1993) Circ. Res. 73, 808–812
8. Griscavage, J. M., Fukuto, J. M., Komori, Y., and Ignarro, L. J. (1994) J. Biol. Chem. 269, 21644–21649
9. Scheele, J. S., Karbitzov, V. G., Martasek, P., Roman, L. J., Sharma, V. S., Masters, B. S. S., and Magde, D. (1997) J. Biol. Chem. 272, 12523–12526
10. Marletta, M. A. (1995) J. Biol. Chem. 268, 12251–12254
11. Hoshimo, M., Ozawa, K., Seki, H., and Ford, P. C. (1993) J. Am. Chem. Soc. 115, 9568–9575
12. Karbitzov, V. G., Bonaventura, J., and Sharma, V. S. (1996) in Method in Nitric Oxide Research (Feiisch, M., and Stamler, J. S., eds) 1st Ed., pp. 39–45, John Wiley & Sons, Chichester
13. Tsai, A-L. (1994) FEBS Lett. 341, 141–145
14. Sharma, V. S., Taylor, T. G., and Gardiner, R. (1987) Biochemistry 26, 3837–3843
15. Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. R., Aitken, J. F., Mathews, A. J., Johnson, K. A., Smith, R. D., Phillips, G. N., Jr., and Olson, J. S. (1996) Biochemistry 35, 6976–6983
16. Eich, R. F. (1997) Reactions of Nitric Oxide with Myoglobin. Ph.D. dissertation, Rice University
17. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., and Wiseman, J. (1991) J. Biol. Chem. 266, 6259–6263
18. Masters, B. S. S. (1994) Annu. Rev. Nutr. 14, 131–145
19. Karbitzov, V. G., Oates, M., Magde, D., Sharma, V. S., and Koesling, D. (1994) Biochem. Biophys. Res. Commun. 220, 8428–8432
20. Roman, L. J., Sheta, E. A., Martasek, P., Gross, S. S., Liu, Q., and Masters, B. S. S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9845–9849
21. McMillan, K., and Masters, B. S. S. (1996) Biochemistry 34, 3686–3693
22. Karbitzov, V. G., Sharma, V. S., Magde, D., and Koesling, D. (1997) Biochemistry 36, 6814–6818
23. Morey, C. G., and Gibson, Q. H. (1976) J. Biol. Chem. 251, 2788–2794
24. Jongeward, K. A., Magde, D., Taube, D. J., Marsters, J. C., Taylor, T. G., and Sharma, V. S. (1988) J. Am. Chem. Soc. 110, 380–387
25. Wang, J., Stuehr, D. J., and Rousseau, D. L. (1985) Biochemistry 24, 7089–7095
26. Crane, R. B., Arvai, A. S., Gachhui, R., Wu, C., Ghosh, D. K., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1997) Science 278, 425–431
27. Roman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S. S., and Poulos, T. L. (1998) Cell 95, 937–950
28. Hurshman, A. R., and Marletta, M. A. (1995) Biochemistry 34, 5627–5630
29. Matsusaka, A., Stuehr, D. J., Olson, J. S., Clark, P., and Ikeda-Saito, M. (1994) J. Biol. Chem. 269, 20335–20339
30. Tierney, D. L., Martasek, P., Doan, P. E., Masters, B. S. S., and Hoffman, B. M. (1998) J. Am. Chem. Soc. 120, 2983–2984
31. Abu-Soud, H. M., Wu, C., Ghosh, D. K., and Stuehr, D. J. (1998) Biochemistry 37, 3777–3786
32. Fan, B., Wang, J., Stuehr, D. J., and Rousseau, D. L. (1997) Biochemistry 36, 12660–12665
33. Sharma, V. S., Isaacs, R. A., John, M. E., Waterman, M. R., and Chevion, M. (1983) Biochemistry 22, 3897–3902