# Kinetics of Nitric Oxide Dissociation from Five- and Six-Coordinate Nitrosyl Hemes and Heme Proteins, Including Soluble Guanylate Cyclase†

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ABSTRACT: Kinetics of NO dissociation were characterized for three five-coordinate systems, heme−NO, HSA−heme−NO (human serum albumin), GC−NO (soluble guanylate cyclase), and for the six-coordinate system, Im−heme−NO. Nitrosyl myoglobin was redetermined for comparison. Previously known, six-coordinate R and T state nitrosyl hemoglobins are also included in the comparison. The data indicate that NO dissociates more than 1000 times faster from five-coordinate model heme than it does from the six-coordinate analog. Such a negative *trans*-effect between NO and a proximal base is in sharp contrast to carboxy heme derivatives, in which ligand dissociation rates are greatly slowed in when a *trans* base is present. As a result of opposite *trans*-effects, six-coordinate carboxy and nitrosyl derivatives have comparable dissociation rates, even though the five-coordinate species are very different. In proteins, five- and six-coordinate forms do not show a large difference in dissociation rates. Part of the reason may be due to different probabilities for geminate recombination in the different proteins, but this cannot explain all the facts. There must also be influences of the protein structure on bond-breaking rate constants themselves. With the exception of hemoglobin in the T state, nitrosyl guanylate cyclase shows the highest NO dissociation rate constant, $k_{obs} = 6 \times 10^{-4}$ s$^{-1}$. This would yield a half-life of about 2 min at 37 °C for dissociation of NO from GC−NO, a number that has implications for the mechanism of regulation of the activity of this key heme enzyme.

For half a century, reactions of $O_2$, CO, and NO with easily obtained hemoglobins and myoglobins have been studied both for their intrinsic interest and with the hope that the lessons learned about structure−function relationships can be extended to other proteins (Antonini & Brunori, 1971). The three diatomic ligands behave very differently. In Hb,[1] for example, oxygen binding is readily reversible, while CO binds more strongly and is a well-known poison, and NO binds even more tightly. Furthermore, CO displays a positive *trans*-effect when bound opposite a histidine or imidazole, that is, the two bonds *trans* to each other are both strengthened and dissociation is slowed, while NO appears to have a negative *trans*-effect, with opposite results (Traylor & Sharma, 1992). In tetrameric Hb and in Mb, the liganded form of the prosthetic group is six-coordinate, except under extreme conditions, such as at low pH with NO as ligand (Duprat et al., 1996). Other heme proteins can be quite different in how they react with ligands. Soluble guanylate cyclase is a recently recognized enzyme that catalyzes the formation of cGMP and is implicated in a wide variety of physiological processes, including memory, signal transduction, vascular regulation, immune response, and so on. Dioxygen does not bind to GC, and the binding of CO is weaker by at least an order of magnitude than it is in Mb (Kharitonov et al., 1995a). Reaction with NO is of particular interest, since NO is a powerful activator of GC (Gerzer et al., 1981; Ignarro, 1989; Humbert et al., 1990; Kim & Burstin, 1994; Stone & Marletta, 1996). Heme in GC is different from heme in Hb in that it is five-coordinate in the active state. In a different arena, EPR signals characteristic of five-coordinate NO−heme have also been found in several tumors from various sources (Bastian et al., 1994). In comparison with CO, rather less is known about NO reactivity, and compared with six-coordinate heme proteins, five-coordinate heme proteins are less familiar. Consequently, we undertook a systematic characterization of reactions of NO with both classes of proteins and with related bare model hemes.

Since NO association with ferro heme proteins is diffusion controlled, association rate constants are unlikely to provide much useful information (Cassoly & Gibson, 1975). In contrast, rate constants for deligation depend on protein structure, and their variation accounts for most of the differences in NO affinity. Only for nitrosyl derivatives of Mb and Hb have rates of NO dissociation from ferro nitrosyl heme proteins been reported (Moore & Gibson, 1975; Sharma & Ranney, 1978). In this report, we compare the kinetics of NO dissociation from GC−NO, HSA−heme−NO, heme−NO, Im−heme−NO, Mb−NO, and Hb−NO.

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[1] Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; CTAB, cetyltrimethylammonium bromide; DTT, dithiothreitol; GC, soluble guanylate cyclase; GSH, reduced glutathione; Hb, human hemoglobin; Hb−NO$^{R\ or\ T}$, nitrosyl hemoglobin in the R quaternary state or in the T state; HSA, human serum albumin; IHP, inositol hexaphosphate; Mb, horse heart myoglobin; SDT, sodium dithionite; SNAP, *S*-nitroso-*N*-acetyl-*dl*-penicillamine; TEA, triethanolamine; TPP, α,*β*,*γ*,*δ*-tetraphenylporphyrin; 1-MeIm, 1-methylimidazole.

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The first three have five-coordinate nitrosyl heme, and the last three are six-coordinate.

Loss of NO is measured by rapid mixing with an efficient NO trap:

$$\text{Hm}-\text{NO} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Hm} + \text{NO} \quad (1)$$

$$\text{NO} + \text{trap} \xrightarrow{k_2} \text{products} \quad (2)$$

The trap must compete effectively against the back reaction $k_{-1}$, which is very fast ($>10^7$ $M^{-1}$ $s^{-1}$ for Hb and Mb). Consequently, large concentrations of the NO-reactive trap are required. This presents certain difficulties with nitrosyl heme proteins, which are not very stable, particularly over the long time required for kinetic experiments. The dithionite method commonly used for studying NO dissociation for Mb−NO and Hb−NO (Moore & Gibson, 1975) requires high concentrations of SDT, which may accelerate any instability. In fact, the applicability of the dithionite method to any five-coordinate nitrosyl heme derivative has not been tested. The other method used, introduced by Sharma and Ranney (1978), employs a large excess of Mb or Hb as a trap. For enzymes such as GC, limited supply restricts concentrations so that absorbance changes accompanying NO loss are small and difficult to measure in the presence of high absorption by the excess Hb or Mb trap. Fortunately, the two methods are complementary, and by comparing results from both, one expects that potential pitfalls can be discovered and avoided.

## MATERIALS AND METHODS

Protoheme, horse heart myoglobin, and human serum albumin (fatty acid free) were obtained from Sigma. Human hemoglobin was prepared from red blood cells hemolyzed by the toluene−water method. SDT and NO solutions were prepared as described previously (Kharitonov et al., 1995b). Solutions of protohemin were prepared in 0.01 M NaOH; hemin concentration was determined by the pyridine hemochromogen method (Antonini & Brunori, 1971a). Protohemin was reduced to protoheme with SDT. HSA−heme complex was prepared by adding 10-fold molar excess of HSA to hemin in 0.1 M phosphate buffer at pH 7.4. Under these conditions, hemin binds to the protein mostly at high-affinity sites (Beaven et al., 1974). The complex was reduced with SDT. HSA concentration was determined by absorbance, assuming that the absorbance of a 1 mg/mL solution at 280 nm is 0.55 (Beaven et al., 1974; Adams & Berman, 1980; Gattoni et al., 1996). Soluble GC was purified and characterized as described elsewhere (Humbert et al., 1990). The native enzyme and its carboxy and nitrosyl derivatives showed Soret absorption bands at 431, 424, and 398 nm, respectively. Enzyme activity increased 200-fold upon reaction with NO. All of these observations were consistent with earlier reports (Humbert et al., 1990; Gerzer et al., 1981; Stone & Marletta, 1996).

Unless otherwise noted, kinetic experiments were carried out at 20 °C in 0.1 M phosphate buffer, pH 7.4. Specifics for each system are listed with the results. Reactions were slow, and concentration changes could be monitored with an ordinary spectrophotometer; however, measurements were also made using stopped-flow spectrophotometry to ensure that no fast initial phase was missed. First-order rate constants ($k_{obs}$) were obtained from the slope of plots of ln($A$) *vs* $t$ or by computerized least-squares analyses of reaction time courses.

## RESULTS

*Five-Coordinate Nitrosyl Derivative of Bare Model Heme.* Spectroscopic characterization was carried out first with a sample prepared by blowing purified NO over a deoxygenated solution of hemin in a spectrophotometer cell sealed with a rubber septum to ensure that no species other than heme−NO could be present. The ferric heme−NO initially produced was rapidly converted to ferro heme−NO. Excess NO was removed by blowing nitrogen over the solution for 20 min. The near-UV absorption spectrum showed a broad absorption maximum at 375 nm. On addition of CTAB (0.01%−2%), the spectrum shifted to a sharp maximum at 398 nm, which is characteristic of five-coordinate nitrosyl heme (Rose & Hoffman, 1983). Since it seemed that heme−NO is polymeric in the absence of CTAB, experiments were performed with 2% CTAB. For kinetic experiments, nitrosyl heme was prepared by adding 1.2 equiv of DTT and 1.0 equiv of SNAP (which releases NO) to 1.0 equiv of hemin. This gave identical spectra.

Loss of NO was measured by mixing heme−NO with $HbO_2$. The trapping reaction (eq 3) is fast [$(3-5) \times 10^7$ $M^{-1}$ $s^{-1}$] and irreversible (Eich et al., 1996; Doyle & Hoekstra, 1981):

$$HbO_2 + NO \rightarrow Hb^+ + NO_3^- \quad (3)$$

Neither $HbO_2$ nor any reaction product can act as a ligand and bind at the free site present in five-coordinate heme−NO. Oxyhemoglobin concentration was varied over the range 3.7−88 $\mu$M, and absorbance changes were monitored at 575 nm. The reaction was studied at two heme−NO concentrations of 3.4 and 8 $\mu$M. The kinetic data yielded $k_{obs} = (2.1 \pm 0.8) \times 10^{-5}$ $s^{-1}$. This corresponds to $t_{1/2} \approx 9$ h and explains why one might be concerned about "down regulation" or shutting off activity in GC or any other enzyme having five-coordinate heme activated by NO.

*Six-Coordinate Nitrosyl Model Heme (Im−Heme−NO).* Large concentrations of imidazole were added to the preparations of heme−NO described above in an effort to make six-coordinate model heme. Depending on equilibrium constants, there might be enough of the six-coordinate form to observe spectroscopically or only a small, steady-state concentration. In either case, if there is a strong negative *trans*-effect, there could be a much larger dissociation rate than that described above. One might caricature the situation as imidazole "forcing" NO off the other side.

Kinetics were studied by adding a small volume of nitrosyl heme solution to a solution of SDT in CO-saturated buffer with or without various amounts of imidazole and observing absorbance changes at 420 nm. In one set of experiments, SDT concentration (in CO-saturated buffer) was kept constant at 28 mM and imidazole concentration was varied over the range 0−2 M. The expectation is that when imidazole binds, NO will dissociate quickly. In the presence of high imidazole and CO concentrations, any Im−heme produced by NO dissociation will be converted to Im−heme−CO, while the NO reacts with dithionite. A detailed mechanism is proposed below, which explains that the apparent rate of

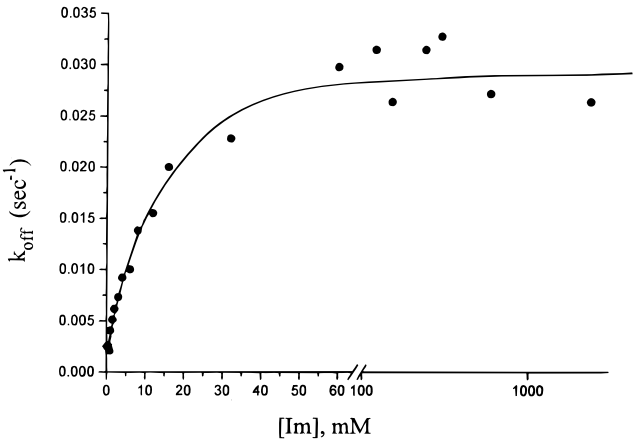


FIGURE 1: Rate constant for NO dissociation from heme−NO in the presence of CO, SDT, and 1-MeIm, plotted against imidazole concentration, $T = 20$ °C, 0.1 M sodium phosphate, pH 7.4, [CO] = 945 $\mu$M; [SDT] = 28 mM.

NO loss will increase with imidazole concentration up to some limiting value. At 30 mM imidazole, the reaction rates became independent of imidazole concentration at $k_{obs} = (2.9 \pm 0.5) \times 10^{-2}$ s$^{-1}$, which is more than 1000-fold faster than dissociation from five-coordinate heme−NO. The data are shown in Figure 1. Solutions of heme−NO in the presence of excess imidazole, even up to 2 M, still show $\lambda_{max}$ near 400 nm, which is characteristic of five-coordinate heme−NO. This suggests that, despite the large $k_{obs}$, only a fractional, steady-state population of six-coordinate heme was present and that the $k_{obs}$ measured is only a lower limit for six-coordinate nitrosyl heme.

In a second set of experiments, imidazole was kept constant at 32 mM and SDT concentration (in CO-saturated buffer) was varied in the range 0.1−56 mM. In that case, the reaction rates became independent of SDT concentration at 20 mM SDT with $k_{obs} = (2.9 \pm 0.5) \times 10^{-2}$ s$^{-1}$, which is the same as the result from the first set of experiments.

*HSA−Heme−NO.* This solution shows $\lambda_{max}$ at 400 nm, characteristic of five-coordinate nitrosyl heme (Kharitonov et al., 1995a; Rose & Hoffman, 1983). Kinetics were measured using both methods: In the dithionite method, NO dissociation was studied by reacting 5 $\mu$M HSA−heme−NO with 0.1%−2% SDT, in CO-saturated buffer. Absorbance changes were followed at 418 nm. These studies yielded $k_{obs} = (8 \pm 2) \times 10^{-5}$ s$^{-1}$. In the $MbO_2$ method, $MbO_2$ concentration was varied from 3 to 43 $\mu$M and absorbance was monitored at 581 nm. This method gave $k_{obs} = (1.7 \pm 0.5) \times 10^{-5}$ s$^{-1}$. The discrepancy between methods in this case is the greatest observed, about twice the combined experimental variations, but is not significant for the inferences drawn below.

*GC−NO.* These solutions show $\lambda_{max}$ at 398 nm, again characteristic of five-coordinate nitrosyl heme. They were prepared in 25 mM TEA, 0.5 mM EDTA, 1 mM GSH at pH 7.5. A 0.9 $\mu$M solution of GC in a sealed spectrophotometer cell with total volume of 0.15 mL was deoxygenated with high-purity argon. To this was added NO solution to yield a final concentration of NO equal to 8 $\mu$M. Excess NO was removed by flushing with CO for 5 min. In the dithionite method, the reaction was studied at 423 nm and SDT concentration was varied from 0.8 to 26 mM. Figure 2 shows a reaction time course with fit. The result was $k_{obs} = (8.2 \pm 2) \times 10^{-4}$ s$^{-1}$.

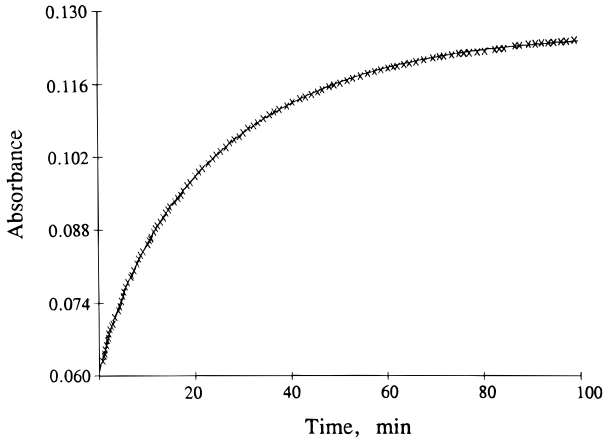


FIGURE 2: Reaction time course for NO dissociation from nitrosyl guanylate cyclase, $T = 20$ °C, pH 7.5, [TEA] = 25 mM, [EDTA] = 0.5 M, [GSH] = 1 mM, [GC] = 0.9 $\mu$M, [CO] = 945 $\mu$M, $\lambda$ = 423 nm; symbols are measurements, and the continuous line is the fit to a single exponential with $k_{obs} = 8.2 \times 10^{-4}$ s$^{-1}$.

For the $HbO_2$ method, GC−NO solutions were prepared as above except that after removing excess NO, solutions were equilibrated with air just prior to adding $HbO_2$. Reaction of NO with free $O_2$ is very slow compared to reaction with $HbO_2$ and air equilibration causes no difficulties (Kharitonov et al., 1994). $HbO_2$ concentrations were varied from 1.7 to 13 $\mu$M. The reaction was studied at both 433 and 576 nm. These experiments yielded $k_{obs} = (5.5 \pm 1) \times 10^{-4}$ s$^{-1}$.

*Mb−NO.* Near neutral pH, Mb−NO is six-coordinate according to its absorption spectrum, as confirmed by many other techniques. In the dithionite method, SDT concentration was varied in the range 5.8−58 mM and the reaction was studied at 437 nm. Dissociation was also measured using $MbO_2$ as a trap. In those experiments 2.4 $\mu$M Mb−NO was reacted with 3−28 $\mu$M $MbO_2$ and absorbance changes were followed at 581 nm. Both methods yielded very similar values for $k_{obs}$: $(9 \pm 1) \times 10^{-5}$ s$^{-1}$. Kinetics of NO dissociation from Mb−NO had been studied earlier by both methods. The measurements were repeated here for comparison. A previously reported value of $k_{obs}$ is $1.2 \times 10^{-4}$ s$^{-1}$ at 20 °C in 0.05 M phosphate buffer of pH 7.0, close to our value (Moore & Gibson, 1975).

## DISCUSSION

*Bare Model Hemes.* The data presented above and collected in Table 1 show that bare nitrosyl heme in the presence of excess imidazole loses NO at least a thousand times faster than five-coordinate nitrosyl heme. Since added imidazole has little or no effect on the spectrum, we believe that the best explanation is that a small steady-state fraction of the six-coordinate species forms and undergoes facile dissociation. The true dissociation rate from six-coordinate heme is then at least an order of magnitude faster than 0.029 s$^{-1}$. This is all very plausible in view of the X-ray crystallographic studies of Picciulo et al. (1974) and Scheidt and Frisse (1975), who characterized five- and six-coordinate forms of nitrosyl tetraphenylporphyrin. They found that in five-coordinate TPP−NO the iron is displaced from the plane of the four porphinato nitrogen atoms by 14 pm toward the coordinated NO with an Fe−NNO distance of 1717 pm. Addition of a sixth ligand increased the Fe−NNO distance to 1743 pm and decreased the out-of-plane displacement of

Table 1: Rate Constants $k_{obs}$ for Dissociation of NO from Five- and Six-Coordinate Nitrosyl Heme Derivatives in 0.1 M Phosphate Buffer, pH 7.4, at 20 °C

| | method | $k_{obs}$ ($s^{-1}$) | coordination |
|---|---|---|---|
| models | | | |
| heme−NO | $HbO_2$ | $(2.1 \pm 0.8) \times 10^{-5}$ | 5 |
| heme−NO | (SDT + CO)[a] + Im | $>0.029 \pm 0.005$ | 6 |
| heme−NO | SDT + (CO + Im)[a] | $>0.029 \pm 0.005$ | 6 |
| proteins | | | |
| HSA−heme−NO | $MbO_2$ | $(1.7 \pm 0.5) \times 10^{-5}$ | 5 |
| HSA−heme−NO | SDT + (CO)[a] | $(8 \pm 2) \times 10^{-5}$ | 5 |
| GC−NO | SD + (CO)[a] | $(8.2 \pm 2.0) \times 10^{-4}$ | 5 |
| GC−NO | $HbO_2$ | $(5.5 \pm 1.0) \times 10^{-4}$ (576 nm) | 5 |
| | | $(7 \pm 3) \times 10^{-4}$ (433 nm) | 5 |
| Mb−NO | SDT + (CO)[a] | $(8.0 \pm 0.2) \times 10^{-5}$ | 6 |
| Mb−NO | $MbO_2$ | $(1.1 \pm 0.1) \times 10^{-4}$ | 6 |
| $Hb-NO^{R}$ [b] | SDT | $1.8 \times 10^{-5}$ | 6 |
| $Hb-NO^{T}$ [c] | Mb, SDT | $1.0 \times 10^{-3}$ | 6 |

[a] Reagents in parentheses were kept at a constant concentration: [CO] = 945 $\mu$M, [Im] = 32 mM, [SDT] = 28 mM. [b] Moore and Gibson (1975). [c] Sharma and Ranney (1978).

Fe to 7 pm. Perutz et al. (1976) had pointed out earlier that such a reduction in displacement from 14 to 7 pm will bring the NO nitrogen to within less than normal van der Waals distance from the porphyrin nitrogens (270 *vs* 300 pm). Such steric hindrance could produce a strong negative *trans*-effect when imidazole is bound opposite NO. The affinity is so low that only a small steady-state concentration of the six-coordinate intermediate, Im−heme−NO, is formed even at imidazole concentration as high as 2 M. The complete sequence of reactions we postulate is as follows:

$$\text{heme}-\text{NO} + \text{Im} \underset{K_A}{\overset{\text{SDT, CO}}{\rightleftharpoons}}$$

$$\text{Im}\cdots\text{heme}-\text{NO} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{Im}-\text{heme} + \text{NO} \underset{K_c}{\rightleftharpoons}$$

$$\text{Im}-\text{heme}-\text{Im} \rightleftarrows \text{Im}-\text{heme}-\text{CO} \quad (4)$$

Assuming $K_A$ is small, the reaction scheme predicts a limiting value for dissociation, $k_{obs}$, of

$$k_{obs} = K_A k_4 \quad (5)$$

This limiting value, the plateau in Figure 1, is obtained when the third and fourth steps are fast enough to compete with the back reaction $k_{-4}$. The bisimidazole species is the first product formed; but it is soon replaced by the final Im−heme−CO adduct, which has greater stability.

Finally, we note the interesting coincidence that NO and CO dissociation rate constants for six-coordinate heme are similar, near 0.1−0.01 $s^{-1}$, because of the opposite *trans*-effects, even though their dissociation rates from five-coordinate hemes are markedly different (White et al., 1979).

*Heme Proteins.* A key question in elaborating protein mechanisms is to decide whether reactivity of a prosthetic group, heme in the present instance, remains unchanged in its protein environment or has its reactivity perturbed by the protein. Are the rates of five- and six-coordinate heme−NO dissociation the same in models and in proteins? In particular, do we find the same 1000-fold difference between six- and five-coordinate species that is apparent in the models? According to the data in Table 1, we do not. The rate constants $k_{obs}$ for all the proteins in the table are restricted to the range from $1.7 \times 10^{-5}$ to $1.3 \times 10^{-3}$, and the two varieties are interleaved in the following order: $Hb-NO^{T}$ > GC−NO > Mb−NO > HSA−heme−NO ≈ $Hb-NO^{R}$, which is 6 > 5 > 6 > 5 ≈ 6 coordination. The dissociation rate for GC−NO is particularly striking. One might have expected it to be 1000-fold slower than that in Mb; in fact, it is 10-fold faster. This suggests distinctly different influences of protein structures in the two proteins, perhaps due to evolutionary selection. In GC, NO serves a regulatory function; enzyme activity is enhanced 200-fold when NO is bound. To be a regulator, NO must dissociate and not be permanently bound. Even the rate listed seems barely adequate. (Physiological studies have not yet defined the rate of down regulation in GC−NO, that is, the speed at which it turns off.) Assuming that the $k_{obs}$ approximately doubles for each 10 °C rise in temperature, $k_{obs}$ should be about $(4-6) \times 10^{-3}$ $s^{-1}$ at 37 °C, which implies a half-life not less than 2 min for NO dissociation from GC−NO *in vivo*. Deactivation of NO in less than 2 min would require additional mechanisms, perhaps an agent that can depress GC activity with NO bound, or an agent that can affect protein structure to further enhance dissociation *in vivo*. Even to achieve the 2-min response time, it is necessary to have a high concentration of a fast NO scavenger to prevent reactivation, and it is far from obvious what that could be. Reactions of NO with $O_2$, thiols, amines, and most other reactive groups are slow (Kharitonov et al., 1995b). Only $O_2^-$ and five-coordinate iron in ferro heme derivatives can compete with the back reaction of NO with GC (eq 1) (Padmaja & Huie, 1993; Cassoly & Gibson, 1975). The intracellular concentrations of $O_2^-$ in healthy tissue are very low. Furthermore, if modest concentrations of $O_2^-$ could scavenge NO dissociated from GC−NO, might they not also prevent GC activation by NO in the first place?

*Bond Breaking and Cage Effects.* The lack of systematic differences between five- and six-coordinate nitrosyl proteins, evident in Table 1, is due to five-coordinate proteins releasing NO "too fast," compared with models, while the six-coordinate species are "too slow." In order to understand how protein structure is modulating the intrinsic heme reactivity, one must realize that cage effects are prominent in NO reactivity for both model hemes and heme proteins. Following NO dissociation, there is a significant likelihood that NO will undergo geminate recombination with iron(II) before it escapes to the surrounding solvent. We have

discussed such geminate recombination previously, with citations to much other work (Jongeward et al., 1988). The simplest model is undoubtedly oversimplified (Walda et al. 1994), but it is adequate for the present purpose:

$$\mathrm{Mb{-}NO} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} [\mathrm{Mb\cdots NO}] \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \mathrm{Mb + NO} \qquad (6)$$

$$k_{\mathrm{obs}} = Yk_5 = \frac{k_6}{k_{-5} + k_6} k_5 \qquad (7)$$

The geminate pair intermediate, [Mb···NO], is formed by bond-breaking, $k_5$. The observed ligand dissociation rate constants, $k_{\mathrm{obs}}$, are reduced below $k_5$ by the fractional efficiency for escape from the cage to surrounding solvent, $Y$. In the simple model, $Y = k_6/(k_{-5} + k_6)$; but the relation between $k_{\mathrm{obs}}$ and $k_5$ remains valid even if $Y$ is related in some more complicated manner to underlying kinetic processes. Even the bare nitrosyl hemes have a significant probability for geminate recombination (Rose & Hoffman, 1983; Traylor et al., 1992), with $Y \approx 0.1-0.2$ in low-viscosity solvents and even less in high-viscosity solvents. In proteins $Y$ can be much smaller. In Mb−NO and in $Hb^R$−NO, cage escape, $Y$, is as low as $10^{-3}$, as might be inferred from the low quantum yield for photodissociation (Antonini & Brunori, 1971b), and is confirmed by picosecond kinetic studies (Walda et al., 1994). Together with $k_{\mathrm{obs}} = 10^{-4}$ s$^{-1}$ this implies that the NO bond-breaking rate constant $k_5$ in Mb is approximately 0.1 s$^{-1}$, which is a little slower than dissociation from six-coordinate heme−NO after the value of $k_{\mathrm{obs}} = 0.029$ s$^{-1}$ is corrected for the steady-state concentration of six-coordinate species and for the cage effect in the model. In $Hb^R$ $k_5$ is 5-fold smaller than in Mb. The fastest NO dissociation rate occurs in $Hb^T$, where it is 50-fold faster than in $Hb^R$. Part of the factor of 50 may be due to increased $k_5$, but it is likely that a portion is due to increased $Y$, which has not been measured. The fact that many six-coordinate proteins have $k_{\mathrm{obs}}$ reduced below that of the bare hemes is largely explained by reductions in $Y$, that is, increased cage recombination and decreased escape to solvent. Any additional factor can be attributed to slight changes in steric strain due to the bond to the proximal histidine.

Five-coordinate nitrosyl heme proteins dissociate surprisingly fast, if one expects them to be 1000 times slower than Mb. Understanding eq 6 reduces that surprise. We can only speculate at this time about the exact mechanism in five-coordinate species because $Y$ is not known for any of them. The two possibilities, however, are clear. Either $Y$ is very large, comparable to or even larger than in bare hemes, or $k_5$ itself must be enhanced. We are initiating efforts to measure $Y$ in order to distinguish between the possibilities. If the explanation does not lie entirely with large $Y$ values, then two possibilities exist to enhance bond breaking, $k_5$. Even a small steady-state population of the six-coordinate species, too small to noticeably affect the spectrum, would substantially enhance $k_{\mathrm{obs}}$. This would be a change from present models for GC−NO, which assume that the active form is exclusively five-coordinate. The other possibility involves a reduction in the transition state barrier that determines $k_5$ due to distal side steric and/or polar effects determined by details of the heme pocket. The fact that there is no proximal bond in the five-coordinate species may allow the heme to move and interact more strongly with distal residues. Crystal structures, when they become available, may help refine these hypotheses.

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