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# Dynamics of Nitric Oxide Rebinding and Escape in Horseradish Peroxidase

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Studies of the kinetics of diatomic ligand recombination to heme proteins following photodissociation can be used effectively to probe protein dynamics[1] and help to reveal the diffusion pathway of the photodissociated ligand (NO, CO, and $O_2$) within the dynamically fluctuating protein matrix. For example, cavities within the protein matrix of Mb, known as xenon pockets (i.e., Xe1−4), have been shown to play a crucial role in the diatomic ligand migration process.[2] A variety of other kinetic studies involving ligand rebinding to myoglobin (Mb) and its mutants have been performed to help map out the ligand entrance and exit pathways of this prototypical heme system.[1−4]

In contrast to the diatomic ligand binding role of Mb, horseradish peroxidase (HRP) is a heme protein with peroxidase activity.[5] Although it utilizes the same histidine-ligated heme prosthetic group as Mb to form the functional active site, HRP has a significantly altered distal pocket architecture and plays a very different physiological role than Mb. As a result, it would not be surprising if ligand diffusion inside the protein matrix was quite different.

The rebinding of CO to HRP has been studied extensively,[6,7] but only a single picosecond kinetics study has been reported,[8] which found a relatively small CO geminate amplitude compared to the noise. On the other hand, we are aware of no prior study of the geminate recombination of nitric oxide to HRP. Since a large geminate amplitude ($I_g$) is observed for NO rebinding, a comparative study of NO rebinding in HRP and Mb reveals the effect of the distal pocket protein environment on the ligand rebinding and escape processes. In the case of NO rebinding to Mb, the kinetics are known to be nonexponential.[9] Similar behavior is found in heme proteins, such as leghemoglobin[10] and nitric oxide synthase.[11] Recent temperature-dependent studies of the NO rebinding to Mb have revealed that the slower (∼200 ps) kinetic phase involves transitions of the NO ligand back into the distal pocket from a more distant site.[12]

The competitive inhibitor benzohydroxamic acid (BHA) is useful as a probe of the distal pocket substrate binding site of HRP. BHA binds with high affinity, and X-ray crystal structures of the BHA complexes of resting state (ferric) HRP and cyanide-ligated HRP have revealed that it binds on the distal side of the heme plane.[13] BHA binding was observed to significantly change the CO rebinding kinetics,[8,14] so that $I_g$ increased from ∼20 to ∼90%, with a rate constant of $2 \times 10^9$ $s^{-1}$. In addition, it was recently shown that binding of BHA significantly impedes oxygen access to the heme pocket.[15]

Here we report the first kinetic studies of the NO rebinding kinetics to HRP and investigate the effects of BHA on the rebinding kinetics. The transient spectra of the photolyzed ferric HRPNO display the photoproduct absorption near 390 nm,[14] on the blue side of the ligated Soret peak, as is expected for a five-coordinated ferric heme species.[4] Unlike native Mb, which shows multiple geminate phases, the NO geminate rebinding in HRP in both the ferric and ferrous states is well fit using a single exponential decay

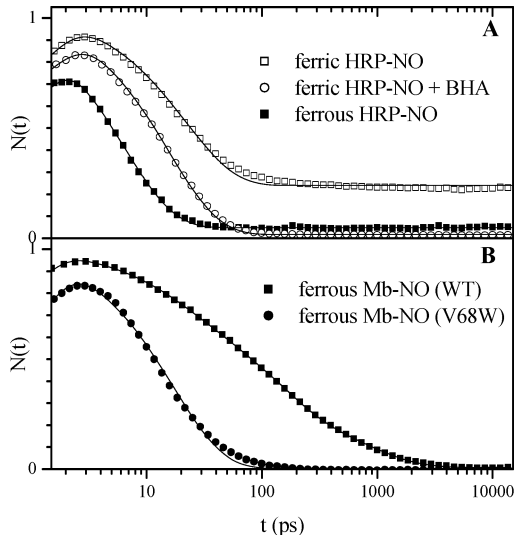


***Figure 1.*** (A) Kinetics of NO rebinding to ferric HRP (pumped at 403 nm and probed at 420 nm) and ferrous HRP (pumped at 403 nm and probed at 440 nm) using a dual synchronized picosecond−femtosecond laser system. (B) Kinetics of NO rebinding to ferrous native and mutant V68W Mb (pumped at 403 nm and probed at 440 nm). The data are fit by a single exponential decay convolved with a Gaussian response function (fwhm = 3 ± 0.5 ps), except native Mb, which is fit with three exponential decays where the slower exponentials mimic the time-dependent barrier[12] involved in the transition from the X- to B-state. The kinetics are normalized to unity at time zero.

(Figure 1). This demonstrates that nonexponential NO kinetics, as observed in Mb, do not arise from an inherent property of the heme relaxation.[9a] Additional measurements on ferric HRP also detect a small bimolecular rebinding phase with a 0.6 ms time constant.[14] The kinetics can be described by a standard three-state scheme

$$\mathrm{HRPNO} \underset{k_{BA}}{\longleftarrow} \mathrm{HRP{:}NO} \underset{k_{in}}{\overset{k_{out}}{\rightleftharpoons}} \mathrm{HRP} + \mathrm{NO}$$

The kinetic fitting parameters,[14] $I_g = k_{BA}/k_g$ and $k_g = k_{out} + k_{BA}$, along with the fundamental rates $k_{BA}$ and $k_{out}$, are presented in Table 1. For ferric HRP, the addition of BHA significantly reduces $k_{out}$ and increases the geminate amplitude to near unity.

These kinetic results suggest a very different process for internal ligand diffusion in HRP in comparison to Mb. The multiple exponential geminate rebinding in native MbNO reflects the dynamical process of ligand transitions between cavities.[12,16] On the other hand, the single exponential geminate phase of HRPNO is similar to that of the V68W mutant of Mb,[12] where the Xe4 cavity is blocked, indicating that there is no additional docking site or protein cavity in HRP that competes for the ligand following photolysis. The impact of BHA binding on the HRP kinetics further supports the existence of a single direct pathway for ligand escape from the distal heme cavity into the solvent. Blockage of this pathway by


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***Table 1.*** Kinetics of NO Rebinding to Horseradish Peroxidase ($T = 293$ K)

| NO bound sample | $\lambda_{pump}$ (nm) | $\lambda_{probe}$ (nm) | $k_{BA}$ ($10^{10}$ $s^{-1}$) | $k_{out}$ ($10^{10}$ $s^{-1}$) | $k_g$ ($10^{10}$ $s^{-1}$) | $l_g$ (%) |
|---|---|---|---|---|---|---|
| ferric HRP | 403 | 420 | $3.3 \pm 0.1$ | $1.0 \pm 0.06$ | $4.3 \pm 0.1$ | $76 \pm 1$ |
| ferric HRP+BHA | 403 | 420 | $6.0 \pm 0.1$ | $0.09 \pm 0.02$ | $6.1 \pm 0.1$ | $98.5 \pm 0.3$ |
| ferric HRP | 580 | 420 | $3.3 \pm 0.4$ | $0.9 \pm 0.2$ | $4.2 \pm 0.4$ | $78 \pm 3$ |
| ferric HRP+BHA | 580 | 420 | $4.0 \pm 0.2$ | $0.04 \pm 0.04$ | $4.0 \pm 0.1$ | $99 \pm 1$ |
| ferrous HRP[a] | 403 | 440 | $15 \pm 0.3$ | $0.8 \pm 0.2$ | $16 \pm 0.2$ | $95 \pm 1$ |
| ferrous Mb (V68W) | 403 | 440 | $5.8 \pm 0.2$ | $<0.05$ | $5.8 \pm 0.2$ | $100 \pm 0.8$ |
| ferrous Mb (WT)[b] | 403 | 440 | $5.6 \pm 0.2$ | $<0.05$[c] | $5.6 \pm 0.2$ | $26 \pm 1$ |
| | | | | | $0.77 \pm 0.03$ | $47 \pm 1$ |
| | | | | | $0.12 \pm 0.004$ | $26 \pm 1$ |
| | | | | | N/A | $0.9 \pm 0.1$[d] |

[a] Ferrous HRP−NO + BHA data are not available because the sample could not be stabilized. [b] Standard three-state model is not applicable.[12] [c] If NO escapes from the B-state, the $k_{out}$ in WT is assumed to be the same as that of V68W mutant. If NO escapes to the solvent from the X-state in WT and 0.9% is taken as the escape yield (i.e., bimolecular amplitude), the $k_{out}$ is less than $10^8$ $s^{-1}$. [d] Amplitude for ligand escape.

BHA significantly reduces the ligand escape rate, $k_{out}$, while only slightly increasing the rebinding rate, $k_{BA}$. The increase of $k_{BA}$ is probably due to the reduced accessible volume available to the dissociated ligand, which reduces the entropic part of the rebinding barrier.[16,17] BHA also affects the ligand migration from the solvent into the protein, as seen in the phosphorescent quenching studies, which find that BHA binding drastically impedes oxygen access to the heme pocket.[15]

In Mb, photolysis partitions the dissociated ligand (with pump wavelength dependence) between two sites: the distal pocket ("B-state") and the nearby xenon (Xe4) cavity or some other docking site in the vicinity ("X-state").[12] While the ligands in the B-state rebind with no barrier, the ligands projected into the X-state must first return to the B-state, as the protein relaxes,[12] before rebinding to the heme. Thus, the simplified (pump wavelength independent) NO rebinding kinetics of HRP indicate the absence of internal cavities and docking sites that compete for the initial partitioning of the ligand following the photolysis reaction. The clear differences between the B-state IR spectra of MbCO[18] and HRPCO[6] photolyzed at cryogenic temperatures also indicate a simpler environment for the photolyzed ligand in HRP. Photolyzed HRPCO shows one broad temperature-independent band, while the B-state IR spectrum of photolyzed MbCO is a doublet with a strong temperature dependence.

It is noteworthy that, in the absence of BHA, the time for NO escape from HRP is ~100 ps for both the ferric and ferrous states, much faster than the escape from Mb. The X-ray structure of Mb shows no channel for ligand exit or entry, and ligand escape in Mb is thought to be gated by the (pH-dependent) "opening" motion of the distal histidine.[1,19,20] Assuming similar NO escape for the V68W mutant and native Mb, $k_{out}$ for NO in HRP is at least 20 times larger than $k_{out}$ for Mb (Table 1). Moreover, CO escape from HRP[8,14] is similar to the NO escape from HRP and is $\sim 10^3$ times faster than CO escape from Mb ($k_{out} \sim 8 \times 10^7$ $s^{-1}$). The significantly faster $k_{out}$ in HRP is consistent with HRP having a distal pocket that is fully connected to the solvent[21] in contrast to the open and closed states of Mb.[1,19,20]

In summary, diatomic probe molecules have been used to deduce the presence of very efficient entry and escape channels in HRP compared to Mb. This result is likely a reflection of the need for substrate access to the distal pocket in an enzymatically active heme protein, such as HRP. In contrast, Mb and Hb probably evolved to trap diatomic molecules, and therefore, they have developed slower and much more circuitous escape pathways. The HRP kinetics also show that heme relaxation dynamics[9a] are not the source of the nonexponential NO rebinding to Mb.

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**Note Added after ASAP Publication:** In the version published on the Internet January 12, 2006, a footnote was omitted from Table 1. This has been corrected in the version published January 25, 2006, and in the print version.

**Supporting Information Available:** Experimental details. Transient absorption spectra and complete rebinding kinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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