Water and ligand entry in myoglobin: Assessing the speed and extent of heme pocket hydration after CO photodissociation

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A previously undescribed spectrokinetic assay for the entry of water into the distal heme pocket of wild-type and mutant myoglobins is presented. Nanosecond photolysis difference spectra were measured in the visible bands of sperm whale myoglobin as a function of distal pocket mutation and temperature. A small blue shift in the 560-nm deoxy absorption peak marked water entry several hundred nanoseconds after CO photodissociation. The observed rate suggests that water entry is rate-limited by the escape of internal dissociated CO. The heme pocket hydration and geminate recombination yields were found to be the primary factors controlling the overall bimolecular association rate constants for CO binding to the mutants studied. The kinetic analysis provides estimates of 84%, 60%, 40%, 0%, and 99% for the steady-state hydrations of wild-type, H64Q, H64A, H64L, and V68F deoxymyoglobin, respectively. The second-order rate constants for CO and H₂O entry into the empty distal pocket of myoglobin are markedly different, 8×10^7 and $2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$, respectively, suggesting that hydrophobic partitioning of the apolar gas from the aqueous phase into the relatively apolar protein interior lowers the free energy barrier for CO entry.

distal water occupancy | spectrokinetic assay | Mb mutants | rebinding kinetics

X-ray crystallography of myoglobin (Mb) reveals no major open pathways for exogenous ligands such as O₂, NO, and CO to reach the heme-binding site, implying that dynamical fluctuations are required for ligand entry and escape between the heme pocket and the solvent (1). Kinetic studies have focused on the barriers to ligand entry and escape to shed light on the associated protein conformational changes and their role in regulating ligand binding (2–17). An electronic barrier prevents CO from participating in the ultrafast internal rebinding processes observed for O₂ and NO (3, 4), limiting MbCO to a nanosecond geminate process of modest amplitude. Photodissociated CO docks within picoseconds at distal site B, lying parallel to the heme plane just over pyrrole C (5–8). Ligands then may escape from the distal pocket further into the interior protein matrix or out to the solvent (9, 10) but appear to remain largely localized to the heme pocket region for roughly a microsecond, becoming equilibrated by 100–300 ns between the B site and several adjacent Xe-binding cavities (mainly the proximal Xe1 site) to form the kinetic C state (2, 11, 12, 18). Two mechanisms for the eventual escape of ligands to solvent have been proposed as follows: (i) a direct channel through the distal histidine “gate” (13), and (ii) multiple indirect hydrophobic pathways (14). The former mechanism seems to dominate at ambient temperatures, whereas escape via Xe1 may occur at <250 K (15).

Combined mutagenesis and photodissociation experiments (18, 19) have yielded kinetic evidence that an internal solvent water molecule hinders ligand access to the distal pocket (20, 21).

DeoxyMb crystal structures show that a water molecule hydrogen bonds (H-bonds) to the N ϵ atom of His-64 (E7) (Fig. 1; refs. 22–24). Although not coordinated to the heme, its displacement is required for heme to bind exogenous ligands. Water may “close” the histidine gate by increasing the effective size of the imidazole side chain and by weak transient interactions with the heme iron (25). Protonation of the distal histidine speeds ligand binding by causing the imidazolium cation to swing out into the solvent (16). Distal water also appears to slow ligand return from the Xe cavities (12, 26). Accordingly, the ligand-binding properties and hydrophobicity of the distal pocket are strongly correlated. The insertion of apolar residues at position 64 increases the CO association rate and equilibrium constants, presumably due to lower deoxy water occupancies (27). The size of residue 64 also can affect sterically the “inner” barrier height when iron binds O₂ or CO within the pocket (2), modulating directly the overall association rate constant.

An assessment of the distal water’s influence on ligand association rates and affinities in native and mutant Mbs requires quantitative information about its occupancy in the deoxygenated form. The high-resolution (1.4–2.0 Å) crystal structures of Phillips (24) and Quillin *et al.* (22) have been interpreted in terms of “full” (0.84) occupancy of this water molecule in the native protein. However, a recent 1.15-Å-resolution x-ray study reported an occupancy (0.6) that was significantly less than complete because of multiple conformations of the His-64 side chain, one of which precludes interaction with distal pocket water by proximity of the N ϵ to the porphyrin ring (28). Determining the water occupancy is important because small differences can have large implications for the water displacement model. As explained further below, the observed association rate constant is expected to be directly proportional to the nonoccupancy of the distal water molecule, i.e., the fraction of empty distal pockets in deoxyMb. Thus, 99% distal water occupancy will slow the observed rate of binding by a factor of 0.01, whereas for 90% occupancy the decrease would be only 0.1. Several lines of kinetic evidence have suggested previously that the occupancy is close to the latter value in wild-type (WT) Mb (21).

The insertion of apolar E7 residues appears to reduce or eliminate water from the distal pocket in the crystal structures of the corresponding deoxyMb mutants (i.e., H64L deoxyMb; Fig. 1) (22). However, quantification of this trend has been impeded by the susceptibility of deoxy crystals to autooxidation and the inherent ambiguity of the crystallographic occupation

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Abbreviations: Mb, myoglobin; SVD, singular value decomposition.

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Fig. 1. X-ray electron densities for the distal pocket residues, noncoordinated water, and the proximal histidine in WT, V68F, H64Q, and H64L deoxyMb (22, 31). Water occupancy factors shown refer to the x-ray densities.

factors, which reflect only those noncoordinated waters that are ordered sufficiently to be resolved by x-ray diffraction. The distal water occupancies of metMb mutants provide a useful surrogate. Coordination to the ferric heme helps to resolve the water electron density and shifts the solvent-to-distal-pocket water equilibrium constant, increasing the observed occupancies. Quillin *et al.* (22) observed distal water occupancies to be full in WT and H64G, partial in H64T, and absent in H64V and H64L metMb. This trend correlates with the oxygen affinities of the

ferrous Mb series and is expected to correlate roughly with the relative distal water occupancies in the deoxy forms of these mutants. In contrast to the CO affinities, the O₂ association equilibrium constants increase markedly, up to 1,000-fold, with increasing polarity of residue 64 due to H-bonding interactions with the partial negative charge on the bound O atoms (21).

Given the strong evidence that distal pocket water hinders ligand return after photodissociation, the question naturally arises how rapidly this water takes up residence. Simple estimates based on the bimolecular rate constants for NO entry into apolar mutants and the concentration of water (55 M) suggest a subnanosecond rehydration time (21). However, previous time-resolved magnetic optical rotatory dispersion evidence suggests that rehydration requires several hundred nanoseconds (29). Given that water and ligand cannot occupy the distal pocket simultaneously, the latter time scale would be consistent with the observed rate of water entry being limited by the speed of ligand escape. Striking support for this proposal comes from the picosecond x-ray crystallographic “movie” obtained by Schotte *et al.* (30) showing water entering the distal pocket of WT MbCO between 0.3 and 3 μ s after photolysis, when the photodissociated CO molecule has either escaped from the protein or is in the proximal Xe1 pocket.

In this work, we use a small spectral shift in the visible absorption band of photodissociated MbCO (29) to investigate the time course and extent of distal water entry in a series of mutants with differing distal heme pocket polarities and sizes, and the speed of water entry into WT MbCO at different temperatures. A distal water-displacement model for ligand binding was applied to the spectrokinetic data in a previously undescribed method for assessing water occupancy. This spectrokinetic “window” into the distal pocket hydration barrier as a mechanism for regulating ligand binding in heme proteins reveals that water entry into ferrous Mb competes directly with geminate CO rebinding and that this water may be present even when not seen crystallographically. Its rate of appearance in the

Fig. 2. First two SVD components of time-resolved photolysis difference spectra of MbCO WT (*a* and *f*), H64Q (*b* and *g*), H64A (*c* and *h*), H64L (*d* and *i*), and V68F (*e* and *j*) in the visible region. (*a–e*) $U_1S_1^{1/2}$ (solid line) and $U_2S_2^{1/2}$ (dashed line). (*f–j*) $V_1S_1^{1/2}$ (solid line) and $V_2S_2^{1/2}$ (dashed line).

Scheme 1.

distal pocket is governed in part by the rate of CO migration out of the protein or inward into the Xe cavities, processes occurring with observed time constants on the order of 100–300 ns. Finally, we find an intrinsic bimolecular rate constant for water entry, $\approx 0.3 \mu\text{M}^{-1}\text{s}^{-1}$, that is unexpectedly slow compared with that for apolar diatomic ligands, $20\text{--}30 \mu\text{M}^{-1}\text{s}^{-1}$, a finding we discuss in terms of different entropic barriers for the two processes.

Results

The visible region spectrokinetic data for photodissociation of WT MbCO and the four distal pocket mutants H64Q, H64A, H64L, and V68F are presented in Fig. 2 as the first two singular value decomposition (SVD) spectral and temporal components (weighted by the square root of the corresponding singular value S_i). The first SVD spectral component for each species closely resembles the MbCO – Mb difference spectrum (Fig. 2 *a–e*), featuring in each case a central peak near the 556-nm absorption maximum of WT deoxyMb and troughs near the 542- and 579-nm absorption peaks of the CO complex. Accordingly, the first temporal SVD component, V_1 , reflects to a close approximation the time evolution of ligand recombination.

The distal pocket mutations affected both the geminate and bimolecular phases of ligand recombination. The WT MbCO time courses (Fig. 2*f*) feature a nearly negligible nanosecond geminate recombination phase and a dominant bimolecular phase. The fitted time constant for the latter process is $1,500 \mu\text{s}$ at 1 atm CO (1,000 μM ligand; 1 atm = 101.3 kPa). The bimolecular recombination phases for the more apolar distal histidine mutants H64Q, H64A, and H64L are increasingly faster than that observed for WT MbCO (Fig. 2*g–i*), and the fitted time constants are 560, 200, and $25 \mu\text{s}$, respectively. The geminate recombination phase ($\tau_g = 190 \text{ ns}$) of the H64L mutant is much more prominent than those observed for WT MbCO and the other distal histidine mutants and has an amplitude nearly equal to that of the bimolecular phase. Finally, the V68F MbCO mutant also has a large and rapid geminate recombination phase, but bimolecular rebinding is significantly slower in this case than in any of the other mutants.

The second spectral SVD components (Fig. 2 *a–e*) correspond to small shifts in the photodissociated deoxyMb absorption peaks. The amplitude of this secondary component is largest for WT and V68F Mb, becomes increasingly smaller for the less polar His-64 mutants, and vanishes for the H64L mutant, in which the distal pocket is completely anhydrous (Fig. 2*i*, where the second component represents random noise). The sign of V_2 generally changed from negative to positive with increasing time; thus, U_2 corresponded to a growing blue shift in the deoxy peak for the species in Fig. 2 with U_2 spectral structure.

Application of the global kinetic modeling procedure (Scheme 1) to the data in Fig. 2 yielded the microscopic rate constants shown in Table 1. Constrained values are shown in parentheses (see *Materials and Methods*). The rate constants for geminate ligand recombination and escape, $k_{\text{Fe}}^{\text{CO}}$ and $k_{\text{out}}^{\text{CO}}$, respectively (k_{CA} and k_{CX} or k_{bond} and k_{escape} in refs. 2 and 19, respectively), and geminate recombination yields, $\phi_g = k_{\text{Fe}}^{\text{CO}} / (k_{\text{Fe}}^{\text{CO}} + k_{\text{out}}^{\text{CO}})$, obtained here for WT, H64L, and V68F Mb are in good agreement with values reported by Carver *et al.* (2). The fraction of geminate recombination, ϕ_g , decreases the overall CO association rate constant, k' , from the microscopic rate constant for ligand entry into the heme pocket by accounting for those molecules that escape the protein before bonding to the iron atom. The other factor decreasing k' is distal water occupancy, $n_w = k_{\text{in}}^{\text{H}_2\text{O}} / (k_{\text{out}}^{\text{H}_2\text{O}} + k_{\text{in}}^{\text{H}_2\text{O}})$ in our model or $K_{\text{H}_2\text{O}} / (1 + K_{\text{H}_2\text{O}}[\text{H}_2\text{O}])$ in the equilibrium mechanism of Olson and Phillips (21). Accordingly, the overall pseudo-first-order ligand association rate is defined as $k'[\text{CO}] = \phi_g(1 - n_w)k_{\text{in}}^{\text{CO}}$ and agrees well with the observed pseudo-first-order recombination rate obtained from the phenomenological exponential fitting, τ_{obs}^{-1} in Table 1.

The effects of the two factors, ϕ_g and $(1 - n_w)$, in decreasing the overall association rate constant k' from the diffusion limit represented by $k_{\text{in}}^{\text{CO}}$ varies considerably for the mutants studied. WT Mb shows the largest ϕ_g -associated decrease, its ϕ_g value slowing the overall rate by a factor of ≈ 20 . H64L and V68F Mb show the least effect, with ϕ_g values of 0.3–0.4 that slow bimolecular binding by factors of only 2–3. The small value of ϕ_g in WT MbCO is due in part to steric restriction of access to the iron atom by the large His-64 side chain and the C γ 2 methyl group of Val-68 (2, 18, 19, 21, 31). In H64L Mb, steric restriction to internal rebinding is reduced by the smaller isobutyl side chain of Leu-64, and in V68F Mb, geminate rebinding is facilitated by both loss of the C γ 2 methyl group at position 68 and sequestering of the dissociated ligand in the vicinity of the iron atom by the large phenyl side chain (Fig. 1; ref. 31).

The effect of water occupancy is largest in V68F Mb, wherein k' is slowed by a factor of ≈ 80 by the $(1 - n_w)$ term (Table 1).

Table 1. Kinetic parameters for MbCO WT and heme pocket mutants

Species	$k_{\text{Fe}}^{\text{CO}}$, μs^{-1}	$k_{\text{in}}^{\text{CO}} [\text{CO}]$, μs^{-1}	$k_{\text{out}}^{\text{CO}}$, μs^{-1}	$k_{\text{in}}^{\text{H}_2\text{O}} [\text{H}_2\text{O}]$, μs^{-1}	$k_{\text{out}}^{\text{H}_2\text{O}}$, μs^{-1}	n_w	ϕ_g	$1 - n_w$	$k' [\text{CO}]$, μs^{-1}	τ_{obs}^{-1} , μs^{-1}
WT	0.11	(0.08)	2.2	9.0	1.7	0.84	0.051	0.16	0.00064	0.00066
WT*	0.24	0.07	5.3	—	—	0.84 [†]	0.043	0.16	0.00051	—
H64Q	0.11	(0.08)	1.5	59	27	0.68	0.065	0.32	0.0017	0.0018
H64A	0.24	(0.08)	(2.2)	15	24	0.40	0.097	0.60	0.0046	0.0050
H64L	2.0	0.078	2.2	—	—	(0)	0.48	(1)	0.037	0.040
H64L*	4.1	0.078	8.2	—	—	0*	0.33	1.0	0.026	—
H64L [§]	5.6	0.13	14	—	—	—	0.29	—	0.038	—
V68F	2.2	(0.08)	4.0	27	0.33	0.99	0.36	0.012	0.00034	0.00034
V68F*	2.2	0.03	4.8	—	—	0.97 [¶]	0.32	0.03	0.00025	—

Rate constants and inverse lifetimes reported in μs^{-1} ; $[\text{CO}] = 1.0 \text{ mM}$.

*Kinetic parameters from ref. 2. In these cases, $k_{\text{in}}^{\text{CO}} [\text{CO}]$ was calculated from $k' [\text{CO}] / (\phi_g (1 - n_w))$.

[†]X-ray occupancy factor for HOH 261 in Protein Data Bank (PDB) ID code 2MGL (22).

[‡]From PDB ID code 2MGD (22).

[§]Kinetic parameters from ref. 26.

[¶]X-ray occupancy factor for HOH 214 in PDB ID code 1MLK (31).

(33) measured an Arrhenius activation energy of ≈ 10 kcal/mol for CO escape from MbCO. Barboy and Feitelson (34) used triplet-state quenching of Zn-protoporphyrin-substituted Mb to measure the rate constant and activation energy for the diffusion of oxygen through the protein and into the heme pocket, reporting values of $1.0 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and 6 kcal/mol, respectively. The former corresponds to a pseudo first-order rate constant of $0.1 \mu\text{s}^{-1}$ under the present conditions (1,000 μM free CO), in excellent agreement with our reported value for $k_{\text{in}}^{\text{CO}}[\text{CO}]$ in Table 1. Cao *et al.* (33) also reported a rate constant of $6 \times 10^6 \text{ s}^{-1}$ and an activation energy of 10 kcal/mol for the entry and binding of a water molecule to ferric heme after photodissociation of NO from metMb. However, it is clear from Scheme 1 and our results in Figs. 2 and 3 that water entry is limited by the rate of ligand escape. In the case of the experiments of Cao *et al.* (33), the rate of water entry would be $k_{\text{out}}^{\text{NO}}$, $\approx 6 \mu\text{s}^{-1}$, which is very similar to the values ($2\text{--}5 \mu\text{s}^{-1}$) we have estimated for $k_{\text{out}}^{\text{CO}}$. This correspondence and the similarity to $k_{\text{out}}^{\text{O}_2} \approx 6 \mu\text{s}^{-1}$ reported by Scott *et al.* (19) indicates that there are no significant differences between the rates of escape from ferric and ferrous Mb and between the diatomic ligands.

Discussion

The spectrokinetic assay method presented here promises to provide a quantitative measure of distal pocket water occupancy factors in deoxyMb and its mutants, information that can be difficult to obtain from structural studies. The photolyzed deoxyMb spectrum shifts on the same time scale as ligand escape ($0.1\text{--}1 \mu\text{s}$ after photolysis), due to the very fast intrinsic rate constant for water entry into the distal pocket, $k_{\text{in}}^{\text{H}_2\text{O}}[\text{H}_2\text{O}] \geq 10 \mu\text{s}^{-1}$ (Table 1). An alternative explanation is that the absorbance shift is due to a protein structural relaxation concomitant with ligand photodissociation and/or escape from the pocket. Two lines of evidence argue against such an assignment. First, previous time-resolved magnetic optical rotatory dispersion measurements show that the spectral shift is not associated with relaxation of the coordination geometry of the proximal histidine (29). Second, relaxation of the distal pocket structure could be triggered by ligand escape from the protein. The amplitude of a signal associated with such a distal relaxation should be proportional to the extent of ligand escape ($1 - \phi_g$). However, there is no correlation between the amplitude of the second component and $1 - \phi_g$. Both H64L and V68F MbCO show significant geminate recombination with $\phi_g = 0.3\text{--}0.4$, but the Leu-64 mutant shows no secondary component, whereas the Phe-68 mutant shows the largest amplitude for this secondary phase. In contrast, there is a strong correlation between the amplitude of the second spectral component and noncovalent water occupancy in the deoxyMb structures (Fig. 1).

A similar correlation between distal water occupancy and the visible band maxima of His-64 deoxy mutants was noted by Christian *et al.* (35). Fitting the dynamics and amplitude of this spectral change to Scheme 1 yields steady-state water-occupancy factors that are consistent with current kinetic, NMR, and crystallographic data. Most of this supporting x-ray and NMR structural information is static in nature; however, the recent time-resolved x-ray results of Anfinrud and coworkers (30) indicate that water does enter the distal pocket of WT Mb on time scales consistent with ligand escape, the mechanism in Scheme 1, and the secondary spectral changes shown in Figs. 2 and 3.

The early crystallographic evidence for WT deoxyMb was generally interpreted in terms of full occupancy of a noncoordinated water molecule in the distal heme pocket (22, 24). More recently, Kachalova *et al.* (28) reported a very-high-resolution structure with partial water occupancy, leading them to question the role of distal water in modulating the kinetics of ligand

binding (20, 21). The results in Table 1 add further independent support to previous kinetic and structural studies (2, 19, 21, 22, 31), which suggest that water is present in WT deoxyMb and that, although the occupancy is less than unity, it is still large enough to restrict the rate of ligand entry into the protein.

Both our spectrokinetic assay and the crystal structure of V68F deoxyMb (31) indicate that water occupancy in this mutant is close to unity and significantly higher than that in WT deoxyMb (Table 1). This increase in n_w is due to the conformation of the Phe-68 side chain, which allows the negative multipole of the phenyl ring face to accept a proton from the internal water and, at the same time, sequesters the water closer to the His-64 side chain and the heme iron atom (Fig. 1). Our fitted parameters for V68F MbCO attribute the slower bimolecular CO binding for the mutant almost entirely to a higher deoxy distal water occupancy. However, previous studies of NO and O₂ binding to V68F and V68W mutants have suggested that there is also a significant effect on the rate of ligand entry and the overall rate of ligand binding due to marked decreases in the inner-distal-pocket capture volumes of these mutants, including loss of the Xe4 cavity (2, 19, 31).

An important result of the analyses shown in Fig. 2 is the assignment of water occupancies in the H64Q and H64A mutants. Water has not been “seen” in the crystal structure of Gln-64 deoxyMb (Fig. 1), and the structure of Ala-64 deoxyMb has not been determined. Our observation of blue shifts in the visible bands of these mutants correlates with ligand escape and water entry. In particular, the fitted value of $n_w \approx 0.6$ for H64Q Mb provides a quantitative explanation for why $k'[\text{CO}]$ for this mutant is only 2- to 3-fold greater than that for the WT protein (Table 1). A similar interpretation of water binding explains why the bimolecular rate of CO binding to H64A Mb is less than that for the completely anhydrous H64L mutant.

Finally, the pseudo-first-order rate constant for water entry is ≈ 100 -fold greater than the rate of ligand entry in solutions equilibrated with 1 atm of CO. However, the calculated second-order rate coefficients are markedly different, $k_{\text{in}}^{\text{CO}} \approx 1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and $k_{\text{in}}^{\text{H}_2\text{O}} \approx 2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (Table 1). In both cases the activation energies are small. Thus, the unusually low bimolecular rate coefficient for water entry is due to a large entropic barrier. Sequestering a water molecule in the small distal pocket cavity is much more unfavorable than sequestering CO in the same space. The large and favorable entropy change for moving the apolar gas out of the aqueous phase compensates in part for confining the gas molecule in a small volume. Thus, hydrophobic partitioning facilitates CO, O₂, and NO entry, but no compensating entropic effect occurs for the polar water molecule. The large entropic barrier to H₂O entry has to be overcome by favorable internal electrostatic interactions with polar amino acids (e.g., His-64) in order for water to penetrate the internal active site of Mb, and presumably similar mechanisms apply to water movement into the internal cavities of most other proteins.

Materials and Methods

Preparation of Mutants. Recombinant Mb mutants were cloned, expressed, and purified as described by Carver *et al.* (36). WT sperm whale (SW) Mb was cloned from plasmid pMb221, a generous gift from Paul Ortiz de Montellano (University of California, San Francisco). MbCO samples were prepared for laser photolysis experiments at a final concentration of 75–100 μM by gel filtration into 0.1 M sodium phosphate buffer (pH 7.3), degassed under 1 atm of CO, and reduced with 1 mM sodium dithionite in a sealed cuvette.

Time-Resolved Spectral Measurements. Time-resolved absorption data were collected as described in ref. 29 using a broadband probe, multichannel detection, and photolysis excitation with a

frequency-doubled Nd:YAG laser (8-ns 40-mJ pulses). Photolysis difference absorption spectra were collected over the spectral ranges 405–465 and 500–650 nm (Soret and visible bands, respectively) with a wavelength spacing of 0.3 nm, and 1,000 spectral measurements were collected at each of 41 logarithmically spaced time delays ranging from 20 ns to 20 ms after laser photolysis. Comparisons of before and after photolysis measurements of both steady-state UV-Vis spectra and 20-ns difference spectra were used to verify sample integrity.

Kinetic Analysis. Absorption difference spectra, represented as an $n \times m$ matrix $A(\lambda, t)$ (n = number of wavelengths and m = number of time points, $n > m$), were decomposed into three matrices by using the SVD expression $A(\lambda, t) = U(\lambda)SV^T(t)$, where U contains m orthogonal basis spectra as columns, the columns of V contain the orthogonal time evolutions of the corresponding columns of U , and the diagonal matrix S contains the size-ordered singular values weighting the contributions of U and V (37, 38). Only the first two SVD components were retained for analysis (i.e., the data were of rank 2).

A global SVD-based fitting procedure incorporating the three-step kinetic model shown in Scheme 1 was applied first to visible band photolysis data for the simplest case, the anhydrous heme pocket mutant H64L, optimizing k_{Fe}^{CO} , k_{in}^{CO} , and k_{out}^{CO} as free parameters and setting $k_{in}^{H_2O}$ to zero (39). {The states symbolized in Scheme 1 as [Fe–CO] and [Fe $\cdots$ CO] are often referred to in the literature as A and C, the bound and nanosecond photodissociated states, respectively (18).} The procedure was then applied to data for WT and the remaining mutants, freely optimizing all of the rate constants except k_{in}^{CO} . The maximum possible values for k_{in}^{CO} were capped at the value obtained for H64L, the variant with the fastest observed bimolecular recombination kinetics. The latter value, as reported above, was close to the diffusion-controlled limit for heme-ligand recombination, $\approx 10^8 \text{ M}^{-1}\text{s}^{-1}$ (33, 34), and

≈ 2 -fold smaller than the values suggested for ligand entry based on analyses of O_2 geminate rebinding and bimolecular NO binding, $\approx 2\text{--}3 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ (19). Finally, the procedure was applied globally to the Soret and visible region time courses of WT collected as a function of temperature. All rate constants in the kinetic scheme and their activation energies were optimized after accounting for the temperature dependence of the CO solubility and capping the maximum possible value for $k_{in}^{CO}[\text{CO}]$ at 20°C at $0.08 \mu\text{s}^{-1}$. Each Arrhenius activation energy, E_a , was optimized as a free parameter except for $E_a(k_{out}^{H_2O})$, which was constrained by the relation $E_a(k_{out}^{H_2O}) = E_a(k_{in}^{H_2O}) + \Delta H_w$, where ΔH_w is the enthalpy of heme pocket hydration. The latter quantity was estimated from the temperature dependence of the hydration constant values, $K_w = k_{in}^{H_2O}/k_{out}^{H_2O}$, obtained by first optimizing the fit for each temperature independently as described above for the ambient temperature WT. These $\ln K_w$ values were plotted vs. $(RT)^{-1}$ to give a ΔH_w value (van't Hoff plot), -8 kcal/mol , that was then used to constrain $E_a(k_{out}^{H_2O})$ in the global-fitting procedure over all temperature.

To obtain a unique fit of the kinetic parameters to the data, we needed to impose two constraints on the calculated spectra of the intermediates: the Scheme 1 species [Fe $\cdots$] and [Fe $\cdots$ CO] were given identical spectra, and the root of the sum of squares of the difference spectrum between these anhydrous intermediates and [Fe $\cdots$ H $_2$ O] was minimized (along with the residuals between the calculated and experimental temporal components of the SVD) in the optimization procedures (see ref. 40).

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