Kinetic Evidence for Three Photolyzable Taxonomic Conformational Substates in Oxymyoglobin

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ABSTRACT The kinetics of oxygen geminate binding with the taxonomic substates of MbO₂ are reported. The maximum entropy method was used to analyze the rebinding kinetics of MbCO and MbO₂ monitored in the Soret. The resulting rate distributions were found to consist of a small number of overlapping bands. A global parametric fit of a series of rate distributions recorded at several temperatures was performed using a Gaussian basis set to resolve the individual enthalpy distributions P(H). This approach was first validated by showing that the well-documented taxonomic substates of MbCO could be recovered. The method was then applied to MbO₂. Three taxonomic substates were identified at pH 4.8, whereas only two of them contribute to oxygen geminate rebinding at pH 7.0. These findings show that, similarly to MbCO, MbO₂ also exists as three photolyzable and kinetically different taxonomic substates and suggest reconsidering the issue of the photolysis quantum yield of MbO₂.

INTRODUCTION

The carbon monoxide complex of sperm whale myoglobin (sw MbCO) presents three well-documented taxonomic conformational substates A₀, A₁, and A₃ characterized by different CO stretch frequencies in the near infrared (Alben et al., 1982; Ansari et al., 1987). Their relative occupancy depends on a number of external parameters such as pH, temperature, pressure, and crystal form (Ansari et al., 1987; Iben et al., 1988; Frauenfelder et al., 1990; Hong et al., 1990; Müller et al., 1999). In the absence of conformational fluctuations, e.g., in hydro-organic solvents below 180 K, the taxonomic substates (CS₀) are frozen in a nonequilibrium distribution with invariant proportions. Flash photolysis experiments monitoring the CS₀ individually in the infrared band showed that the kinetics are nonexponential, indicating that each CS₀ consists of a wide distribution of CS₁ conformational substates (Ansari et al., 1987; Johnson et al., 1996). The overall CO rebinding rates decrease in the order A₀ > A₁ > A₃.

The taxonomic substates of MbO₂ are more difficult to characterize because the O—O stretch bands are intrinsically weak and appear in a spectral region in which absorbance of water, protein, and heme are predominant (Potter et al., 1987). In addition, the photolysis quantum yield of MbO₂ is significantly smaller than that of MbCO (Chance et al., 1990; Miller and Chance, 1995). As a consequence, nothing is known concerning the rebinding kinetics of O₂ with the taxonomic substates of MbO₂ individually.

In the present work we attempted to circumvent the difficulties associated with infrared kinetic spectroscopy of MbO₂. The electronic Soret absorption band in the visible is specific of the heme group and kinetic experiments monitored in this region measure the total amount of unbound ligand. It has been argued that such measurements do not distinguish among different taxonomic substates and yield information only about the average rebinding behavior (Johnson et al., 1996). In fact the Soret absorption band of the CS₀ are not exactly identical. Hole burning experiments with MbCO have shown that, compared with A₁, the Soret band of A₀ is shifted by 0.4 nm to the blue and that of A₃ by 0.6 nm to the red (Ormos et al., 1990). Thus, upon ligand photodissociation at low temperature, the transient absorption change in the Soret displays inhomogeneous kinetics corresponding to a superposition of the rebinding rate distribution of all CS₀.

We have reinvestigated the kinetics of geminate CO rebinding with Mb in the Soret using the maximum entropy method (MEM) to obtain the rate distribution P(log k) of the nonexponential kinetics between 77 and 170 K. These distributions indicate that several kinetic species are contributing to rebinding. A global analysis of the rate distributions provided evidence that these species may be assigned to the taxonomic conformational substates of MbCO. The method was then applied to oxygen geminate rebinding in MbO₂. We found that MbO₂ also exists in three different photolyzable CS₀, each of them being characterized by a photolysis yield smaller than unity.

MATERIALS AND METHODS

Materials

A sample of lyophilized sperm whale Mb (Serva, Heidelberg, Germany) that had been kept in liquid nitrogen was freshly purified by passage through a Sephadex G-75 column. The required amount of Mb was diluted with glycerol, buffer, and water to reach a final protein concentration of 10 to 20 μM in 50 mM buffer. Samples at pH 4.8 and 7.0 were prepared by using a potassium citrate/phosphate or a potassium phosphate buffer, respectively. (At low temperature the pH is expected to increase by ~1 unit.) Experiments were performed in 79% (w/w) glycerol (glass transition...
temperature $T_g \approx 173$ K. The ferrous CO complexes were prepared by passing a stream of CO above the protein solution submitted to gentle stirring, and adding a few microliters of a concentrated deaerated dithionite solution. The oxygenated complexes were prepared by passing a stream of argon above the solution and adding oxygen after reduction with sodium dithionite. Ligand binding was controlled by following the absorption change in the Soret.

**Methods**

**Data collection**

Sw Mb solutions in gas tight square cuvettes were cooled in a liquid nitrogen cryostat (Oxford Instruments DN704). Rebinding kinetics were recorded at 10- to 20-K intervals. The cooling rate was $\sim 2$ K/min, and the protein was allowed to equilibrate for further 15 min once the desired temperature was reached. Photodissociation was achieved by the 10-ns pulse of the second harmonic (532 nm) of a Q-switched Nd-YAG laser (Quantel, France). Transient absorption changes, monitored at the peak of the Soret band of the hexa-coordinated complex (423 nm for CO and 418 nm for O$_2$) or of the deoxy Mb (434 nm), were recorded over two decades in amplitude and six to seven decades in time using our fast kinetic spectrometer set-up (Tetreau et al., 1997).

**Distribution of rate parameters and of activation enthalpy**

Geminate rebinding is a first-order process. Due to the wide distribution of conformational substates (CS$^i$) in the rigid, glassy solvent, kinetics are highly nonexponential. The overall survival fraction $N(t)$ of uncombined proteins is given by:

$$N(t) = \int_0^\infty P(\log k) e^{-kt} d(\log k)$$

The rate distribution (or rate spectrum), $P(\log k)$, was obtained from the observed kinetics $N(t)$ by Laplace inversion of Eq. 1 using the MEM starting from a set of logarithmically spaced and equiprobable $k$ values (Lavalette et al., 1991; Steinbach et al., 1991). This probability density function provides an equivalent description of the rebinding kinetics but is more powerful for analyzing complex processes.

Within one given taxonomic substrate (CS$^i$), the rate distribution is caused by the distribution of the activation enthalpy $P(H, T)$ among CS$^i$, the preexponential factor $A$ remaining sharp. Below the solvent’s glass transition temperature, $T_g$, interconversion among CS does not take place, the protein ensemble is frozen, and the enthalpy distribution remains equal to $P(H, T_g)$ at any $T < T_g$. The enthalpy distribution is usually obtained by simple transformations from a set of $P(\log k)$ determined at different temperatures (Tetreau et al., 1997). Recent developments of our software now also permit to determine unimodal $P(H)$ by a global maximum entropy fit of a series of kinetics recorded at different temperatures. For multimodal $P(H)$, such as those observed in P450 (Tetreau et al., 1997) and in this work, this approach is not sufficient. Characterizing each mode (or subset) by an independent $\{A_i; P_i(H)\}$ pair is easy when the modes are well separated, but additional assumptions are needed when several $P(\log k)$ bands are overlapping and shift with respect to each other with temperature. In the absence of a theoretical model, we approximate the shape of the individual bands by a Gaussian. We consider the series of rate spectra $P(\log k)$ obtained at different temperatures below $T_g$ as secondary data and perform a global parametric fit of all $P(\log k)$ by developing the enthalpy distribution:

$$P(H) = \sum_i^3 \alpha_i G_i(H)$$

(2)

in which the $G_i$ are Gaussians with temperature independent mean and standard deviation. Their weighting factors $\alpha_i$ remain constant at all temperatures lower than $T_g$. Each $G_i$ is associated with a preexponential factor $A_i$. The $T$-independent global $P(H)$ is linked to individual $P(\log k)$ by:

$$k = A(T/T_g) \exp(-H/RT)$$

(3)

which yields the simple Arhennius relation if $T_g$ is permanently equal to $T$. However, to compare our results with those of the literature, we used the transition state-like relation obtained by assigning to $T_g$ the constant value $T_g = 100$ K (Dlott et al., 1983).

By definition the MEM yields the “optimum,” or most likely, rate distribution corresponding to the experimental kinetics. Therefore, it is not surprising that the backward calculation of the kinetics by Laplace transformation of the Gaussian enthalpy model departed somewhat from the MEM fit, due to the approximation of the Gaussian bandshape. However, the overall agreement was generally quite satisfactory (Fig. 1). For this reason, we prefer to fit the rate spectra rather than using the approximate model of Eq. 2 to fit the kinetics directly.

**Wide-band ultraviolet-visible monitoring**

The more details are present in the rate spectra, the more accurate their decomposition into individual components will be. The rate profile was found to change with the monitoring wavelength as well as with the spectral bandwidth used, as expected for the inhomogeneous Soret. In addition, we observed that rate distributions tended to exhibit a more pronounced structure when the kinetics were recorded using a large spectral bandwidth. To see how kinetic information is recovered under condition of wide-band ultraviolet-visible monitoring, we first consider for simplicity an inhomogeneous absorption band resulting from the presence of only two species, $P$ and $Q$, in the concentration ratio: $c_P/c_Q$. Assuming
two kinetically distinct photoproducts, \( P^* \) and \( Q^* \), the overall survival (rebinding) function is:

\[
N(t) = c_{P^*}(t)n_{P^*}(t) + c_{Q^*}(t)n_{Q^*}(t) \tag{4}
\]

in which \( n_{P^*}(t) \) and \( c_{P^*}(0) \) are the normalized survival probabilities and the initial concentrations of the photoproducts respectively. The latter depends on the ratio of the extinction coefficient \( \epsilon_i \) of \( P \) and \( Q \) of and of the photolysis quantum yield \( \phi_i \) at the excitation wavelength. With monochromatic laser excitation:

\[
c_{P^*}(0)/c_{Q^*}(0) = (\phi_P\epsilon_Pc_{P^*}/\phi_Q\epsilon_Qc_{Q^*})_{\lambda_i} \tag{5}
\]

If the spectral width \( \Delta \lambda \) of the monitoring light is relatively large (e.g. \( \sim 10 \) nm or more), Beer-Lambert’s law must be integrated over the whole spectral interval, yielding for the apparent transient absorbance change \( \delta D_{app} \) of the impinging light beam and of the decimal molar extinction coefficients \( \epsilon_i(\lambda) \). If the wavelength dependence of \( I_{\lambda}(\lambda) \) can be neglected in the observation range \( \Delta \lambda \) then \( \epsilon \) becomes equal to \( \epsilon(\lambda) \), the average of the extinction coefficient over the wavelength interval. With monochromatic monitoring \( \epsilon = \epsilon(\lambda) = \epsilon \) and \( \delta D_{app}(\Delta \lambda; t) = \frac{\delta D_{app}(\Delta \lambda)}{D_{app}(\lambda, t)} \).

Eq. 5 indicates that the initial kinetic information is recovered except for a change of the amplitudes. This is not a limitation because in flash photolysis amplitudes are never directly representative of the concentration ratio of the species initially present (because of different yields and extinction coefficients).

In this work the detection system comprised a monochromator whose slits were adjusted to a fixed value corresponding to a spectral bandwidth \( \Delta \lambda = 10 \) nm compared with the Soret one-half width of \( 16 \) nm. The intensity of the monitoring light beam was maintained at the nominal value suitable for the photoreceptor by means of a variable neutral density filter. At low temperature, where rebinding is slow, pumping by the monitoring light may possibly represent another cause of distortion of the rate distribution because long-lived components will be predominantly affected. We therefore favored an observation in the Soret of the penta-coordinated (photodissociated heme) at \( 434 \) nm. In this way, illumination within the Soret of the dissociable hexa-coordinated was minimized by using a low pass, broadband interference filter.

**RESULTS AND DISCUSSION**

The kinetics of CO and \( O_2 \) rebinding with sw Mb were determined at low spectral resolution in the Soret absorption band of the deligated (penta-coordinated heme) species at two different pH values. Although they were quite similar to those repeatedly reported in the literature by monitoring the Soret of the hexa-coordinated heme with monochromatic resolution (Austin et al., 1975; Doster et al., 1982; Steinbach et al., 1991; Johnson et al., 1996), subtle details could be appreciated only after computing the rate distributions. Rate distributions have been occasionally reported for Mb (Steinbach et al., 1991, 1992; Lambright et al., 1993; Johnson et al., 1996), but no systematic MEM analysis covering the whole temperature range has been published. The rate distributions \( P(\log k) \) are shown in Figs. 2 and 4. The irregular band shape suggests that several kinetic components are overlapping and that their contributions are redistributed between pH 4.8 and 7.

The analysis of the complex rate spectra depends on the reliability of the band shape recovered by the MEM. The principal advantage of the inversion of Eq. 1 by the MEM is that this method, contrary to parametric fitting, does not require postulating any analytical model. It yields the most general rate distribution that is consistent with inherently noisy data. An incorrect estimation of noise is therefore a

![FIGURE 2](image-url)
potential source of difficulties. If the estimation is too low, noise may be partially fitting as signal. The rate spectrum may then overinterpret the data by showing unnecessary details. If, on the contrary, the noise estimate is too high, genuine kinetic details may be blurred or lost. Although an automatic noise estimator of the signal immediately before photolysis is part of our data processing software we apply the precautionary rule according to which only those details in the rate spectra that persist after arbitrarily increasing twofold the noise values are considered as genuine. This ensures that the data will never be overinterpreted. In this work, the reproducible effects of pH upon the band shape of the rate spectra further confirm that details in band profiles are significant.

**CS\textsuperscript{0} substrates of sw MbCO**

The rate distributions of MbCO (Fig. 2) could be fitted equally with a sum of two or three Gaussians, but doing so did not automatically warrant the temperature invariance of the enthalpy peak and width values nor that of their relative weight. Infrared monitoring indicates that only the relative populations of the taxonomic substrates are affected by pH, whereas the rebinding enthalpy and preexponential factor of the individual CS\textsuperscript{0} remain unchanged (Ansari et al., 1987; Mourant et al., 1993). We therefore performed a global fit of the rate distributions in which, at a given pH, all spectra were fitted assuming the same number of Gaussians under the constraint that their weighting factors remain constant at all temperatures.

In line with previous observations in the infrared bands indicating that only two CS\textsuperscript{0} \((A_\text{I} \text{ and } A_\text{II})\) of MbCO have significant contributions at pH 7.0, whereas the contribution of \(A_0\) becomes important at pH 4.8 (Ansari et al., 1987; Hong et al., 1990) we first fitted the rate distributions at pH 7 using a two-Gaussians model for the enthalpy distribution. This resulted in a satisfactory decomposition of the rate spectra, as shown in Fig. 2 (right). We then imposed the same \(H_\text{i}\) of the enthalpy bands obtained at pH 7 to fit the data at pH 4.8, leaving their relative weights as well as their preexponential factor free to vary. No satisfactory global fit could be obtained in this way. Only the addition of a third unconstrained Gaussian yielded the satisfactory decomposition shown in Fig. 2 (left). The data at pH 4.8 could be fitted with 3-Gaussian enthalpy bands as well without imposing the enthalpies derived from pH 7. The resulting enthalpy spectrum was very similar, except for some variation of the \(H_\text{i}\) compared with their values at pH 7. However, there is evidence from the literature for the invariance of the enthalpy with pH (Ansari et al., 1987; Mourant et al., 1993). Deviations may be expected with the choice of the fitting procedure, because a different question calls for a different answer. As we shall show below, the pH invariance was found to emerge naturally with MbO\textsubscript{2}.

Within the experimental uncertainties, it can be concluded that three species are necessary to account for the rate distributions in the Soret of MbO\textsubscript{2}; they all appear at pH 4.8, whereas only two of them are present at pH 7. The enthalpy distributions \(P(H, T_\text{g})\) at pH 4.8 and 7 are displayed in Fig. 5. The preexponential factors and \(P(H)\) parameters are compared in Table 1 with those obtained by monitoring each CS\textsuperscript{0} individually in the infrared (Ansari et al., 1987; Berendzen and Braunstein, 1990; Steinbach et al., 1991; Johnson et al., 1996; Nienhaus et al., 1998). Despite some variability, agreement is good enough to provide evidence that the kinetic species resolved in the Soret indeed correspond to the three taxonomic substrates of MbCO.

The difficulty in recovering precise parameters for the CS\textsuperscript{0} even with direct monitoring in the infrared is probably due to spectral overlap and small absorbance. More generally, experimental uncertainties lead to apparent entropy-enthalpy compensation because preexponential factor and enthalpy are linked parameters (via Eq. 3). In addition, the mathematical approach varies with authors. Using an singular valve decomposition approach Hagen et al. (1996) attempted to account for kinetic differences exhibited by MbCO in glycerol and in a trehalose glass by fitting their global \(P(H)\) obtained at pH 8.0 with three Gaussians of identical width.

To appreciate the variability of the data we consider the Arrhenius plot of \(k_\text{peak}\) values computed using the parameters reported by different authors (Fig. 3). Agreement is
excellent for $A_1$, but the parameters of the other taxonomic substates are more scattered, presumably in view of the experimental difficulties mentioned above. The present work yields results that are representative of the average among the determinations made by others.

Thus, although the present approach is indirect, its reliability is comparable with that of specific observations at the infrared wavelengths. Its main interest is to provide a straightforward alternative when infrared measurements are not possible, such as in the case of MbO$_2$ that we shall now examine.

**CS$^0$ substates of sw MbO$_2$**

Although their origin has long been debated, it is now commonly accepted that the taxonomic substates of liganded Mb are due to differences in the local electric field that result from alternative conformations of the distal histidine His-64. Their structure has been recently established at near atomic resolution (Vojtechovsky et al., 1999; Yang and Phillips, 1996). In MbCO, $A_0$ was identified with a doubly protonated histidine conformation, which is swung out of

![Graph showing rate distributions of geminate O$_2$ rebinding with sw Mb at pH 4.8 (left) and 7 (right) monitored at low spectral resolution in the Soret ($\Delta \lambda = 10$ nm). The decomposition resulting from the global fit of the rate distribution according to the sum of Gaussians enthalpy distribution (Eq. 6) is shown in dotted lines with the proposed assignments to the CS$^0$. Other remarks are as in Fig. 2.](image1)

![Graph showing normalized enthalpy distributions of the three CS$^0$ of sw MbCO (left) and sw MbO$_2$ (right) at pH 4.8 (top) and pH 7.0 (bottom).](image2)

**TABLE 1** Thermodynamic parameters for CO rebinding with the CS$^0$ substates of sw Mb

<table>
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<th>$A_0$</th>
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The widths are the full widths measured at one-half maximum of the enthalpy distribution. Mean standard deviations are estimated as 0.8 for log $A$ and 1.5 for $H_{\text{peak}}$.

* Parameters of the main Gaussian components.
the pocket toward the solvent whereas $A_1$ and $A_3$ correspond to conformations of His-64 with $N^e$ pointing toward the heme and $N^o$ pointing out toward the solvent. They differ slightly in the distance between CO and $N^o$. In MbO$_2$ only two conformations similar to those of the $A_1$ and $A_3$ substates of MbCO could be observed, the main difference being a hydrogen bond connecting the terminal oxygen atom with His-64-$N^e$ in $A_3$.

Because the structural features of the taxonomic substates of Mb imply essentially the protein and the protonation state of the distal histidine, one may reasonably expect to observe also the same CS$^0$ in MbO$_2$. Even though they are not spectroscopically resolvable in the infrared, their kinetic signature might be found by monitoring geminate rebinding in the Soret at low spectral resolution. The rate distributions for geminate oxygen binding and the decomposition resulting from a global fit using the Gaussian enthalpy model are presented in Fig. 4 and Table 2. Despite smaller signals, due to the reduced photolysis quantum yield of MbO$_2$, the structure of the rate distributions was slightly more pronounced than in MbCO. Again, three Gaussians were required to obtain good fits at pH 4.8, whereas only 2 were sufficient at pH 7.

Interestingly, the decomposition into three Gaussians at pH 4.8 gave almost identical results when $H_1$ and $H_3$ were fixed at the value derived from pH 7.0 or when they remained unconstrained. Therefore, the invariability of the enthalpy peaks with pH is more convincing with MbO$_2$. The absence of $A_0$ in the crystal structure of sw MbO$_2$ at pH 6 is not clear. It might result from a smaller occupancy (<20%), a fact that might possibly point to some influence of the ligand upon the populations of the CS$^0$. The Soret kinetic data are consistent with three distinct taxonomic substates. An assignment to the $A_1$ and $A_3$ conformers modeling diffraction data appears inescapable at pH 7. One additional, faster rebinding CS$^0$ resembling closely $A_0$ of MbCO is found at pH 4.8.

Comparing Tables 1 and 2, it can be noted that, for a given ligand, the preexponential factors vary very little among the taxonomic substates, but that the prefactors of CO tend to exceed those of O$_2$ by a factor of ~3 (0.5 log units). The enthalpy spectrum for O$_2$ binding (Fig. 5) is rather close to that of CO after a global shift of 2.2 to 3.2 kJ/mol, toward low enthalpies, presumably because of the naturally “bent” ligand experiences less steric hindrance. For both ligands, the peak enthalpies increase by ~4 kJ/mol between $A_0$ and $A_1$ and only by ~2.5 kJ/mol between $A_1$ and $A_3$. Again, this sequence is in agreement with the known ordering of the steric hindrance in the taxonomic substates.

To conclude this section, we believe that the evidence is strong enough that MbO$_2$, similarly to MbCO, may exist as three kinetically distinct taxonomic substates. With both ligands the activation enthalpy for binding can be regarded as being invariant upon a change of pH. Not only does sw MbO$_2$ appear to exist in the same CS$^0$ conformations as MbCO, but the fact that all of them are photolysable sheds new light on the intriguing issue of the photodissociation quantum yield of oxygen.

### Quantum yield of O$_2$ photolysis

The reason why, contrary to CO, the intrinsic quantum yield of O$_2$ photolysis appears smaller than unity (Chance et al., 1990) still remains a debated question. The difficulty with all reasoning about O$_2$ quantum yield may stem from the fact that it is often implicitly assumed that the photodissociation efficiency of one particular CS$^0$ can only be either 1 or 0. The present finding of three kinetic species implies that all CS$^0$ of MbO$_2$ are photolysable, at least to some extent. (We wish to stress again that the amplitudes of the Gaussian components in the decomposition of $P$(log $k$) in Figs. 2 and 4 should not be used to estimate the relative quantum yields, as described by Eqs. 5 and 6. Thus the total quantum yield should be written in the general form:

$$
\phi = \phi_0 A_0 + \phi_1 A_1 + \phi_3 A_3
$$

in which $A_i$ denotes the probability of the substates whose quantum yield $\phi_i$ may take any value between 0 and 1.

Another feature of MbO$_2$ is the pH dependence of the oxygen photolysis yield that decreases from ~0.5 at high pH to 0.1 to 0.2 at low pH (Miller et al., 1996). A detailed investigation of the pH dependence of the infrared CO stretch bands has shown that $A_1$ and $A_3$ cannot be distinguished by a titration experiment because of an internal proton transfer that occurs without exchange with the solvent. Thus, $A^* = (A_1 + A_3)$ as a whole, is titrating against $A_0$. Moreover, the ratio $A_1/A_3 \approx 0.5$ remains nearly constant.
over the whole pH range (Müller et al., 1999). Expressing Eq. 8 in terms of $A^*$ and $A_0$, one obtains:

$$
\phi = \varphi_0 + \frac{A^*}{A^* + A_0} \left[ \alpha_1 \varphi_1 + \alpha_3 \varphi_3 - \varphi_0 \right]
$$

(9)
in which the $\alpha_i$ are the fractions of $A$ and $A_3$ constituting $A^*$.

Eq. 9 holds not only for sw Mb, but also for horse Mb in which $A_3$ is not observed. It is in agreement with the findings of Miller et al. (1996) who noticed that the pH dependence of the quantum yield of hh MbO$_2$ was following a tritration curve that parallels the pH-dependence of the $A_0$ subpopulation of MbCO.

Combining Eq. 9 with the above numerical data, one gets $\phi = (1/3)\varphi_1 + (2/3)\varphi_3 \approx 0.5$ and $\phi = \varphi_0 \approx 0.2$ at high and low pH respectively. Numerically, the first relation implies obviously that $\phi_3 < 1$ (in principle, no limit is obtained for $\phi_1$). Whatever mechanism may be responsible for the low photoproduct yield of MbO$_2$, it appears that two CS$^0$ at least are affected, in contrast to the idea that just one of them would not be photolyzable.

It is very difficult to discuss the quantum yield issue in terms of high-resolution crystal structures of the taxonomic substates because these data concerns ligand-bound states only, whereas kinetic experiments monitor dissociated states, and quite obviously only those that live longer than the photolyzing flash. As noted by Chance et al. (1990) any dissociated state rebinding on a subpicosecond time scale would appear as “unphotolyzable.”

The known crystallographic structure of photolysis intermediates in MbCO gives more relevant clues regarding the fate of the dissociated ligand. Immediately after photodissociation the ligand occupies a primary site above pyrrole C at 3.6 Å from the heme (Schlichting et al., 1994; Hartmann et al., 1996; Chu et al., 2000). In the crystal, this species is trapped at temperatures below 40 K. At higher temperature, the ligand leaves the heme proximity to reach a new docking site, now identified with the distal xenon-binding site (Xe$^4$) (Ostermann et al., 2000). Other migration paths occur above 160 K (Chu et al., 2000; Ostermann et al., 2000), but between 40 and 160 K, geminate rebinding corresponds to the return of the ligand from the secondary (Xe$^3$) docking site. The quantum yields of MbCO taxonomic substates are essentially unity, presumably because the relatively slow rebinding of this ligand cannot compete with escape toward (Xe$^4$).

Similar information is not available yet for the oxygen ligand, and the situation might be significantly different. If a primary docking site exists, its structure might not be related to that of CO, especially because O$_2$ can be involved in H bonding in many ways that might affect rebinding. (As an example, we may mention that the oxygenated complex of cytochrome P450cam, in which the oxygen ligand has been shown to take part in a H-bonding network (Schlichting et al., 2000) was found to be nonphotolyzable between 300 and 80 K (this laboratory, unpublished results). The fact that the photolysis yield of the oxygen CS$^0$ substates is smaller than unity removes some of the paradoxes that were pointed out previously (Vojtechovsky et al., 1999), but its understanding constitutes a new challenge requiring more information about the crystal structure of photolysis intermediates in MbO$_2$.

**REFERENCES**


