Reactive Line-Shape Narrowing in Low-Temperature Inhomogeneous Geminate Recombination of CO to Myoglobin

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ABSTRACT: The temporal shift in the near-IR absorption peak of myoglobin (Mb) following flash photolysis of MbCO at cryogenic temperatures appears to be due largely to an inhomogeneous reactive process rather than to relaxation. This conclusion, which follows from a new analysis of the experimental data, is based on the following three points: First, at very low temperatures (60 K) a transient line-narrowing effect can be detected. Second, there is a universal, temperature-independent, correlation between spectral shift and survival probability in the rebinding kinetics, and third, the same quantitative model which accounts for rebinding accounts semiquantitatively for the temporal shift in the peak. A fit to the model indicates that the inhomogeneous broadening of the near-IR peak in myoglobin is 15–20% of the total width. The same rebinding process which governs the loss of intensity of this peak is therefore most likely responsible for the shift in its center wavelength.

The low-temperature rebinding kinetics of CO and O_2 to myoglobin (Mb) has been studied in detail over wide temperature and time domains (Austin et al., 1975). By monitoring the Soret absorption subsequent to laser-pulse photolysis of ligand-bound myoglobin, it was discovered that below the glass transition of the solvent (ca. 220 K) ligand recombination is geminate, and nonexponential in time. This power-law kinetics has been attributed to inhomogeneity in the frozen protein population, resulting in a distribution of recombination barrier heights and rate coefficients.

Subsequently, models have been suggested (Agmon & Hopfield, 1983; Agmon, 1986; Young & Bowne, 1984) for these distribution functions, by extending the traditional picture of an energy profile along the reaction (iron–ligand) coordinate. This was done via an additional degree of freedom (the "protein coordinate") representing the protein conformations explicitly. It has been suggested (Agmon & Hopfield, 1983) that not only the temporal variation in intensities but also those of spectral line shapes should reflect the inhomogeneity in the recombination process.

The optical spectrum of hemoglobins (Eaton & Hofrichter, 1981) contains, in addition to the very intense Soret band, some weaker bands which may be used to monitor ligand recombination. A weak charge-transfer band around 760 nm, which appears only for the deligated form, shifts to the blue subsequent to dissociation (Iizuka et al., 1974). An extensive study of this band has been initiated (Bowne, 1984; Ansari et al., 1985; Frauenfelder, 1985) in the temporal range of microseconds to seconds and in the temperature range of 60–180 K. It was found that this shift is also nonexponential in time.

The above-mentioned behavior has been attributed (Ansari et al., 1985) to a relaxation process with a distribution of relaxation rates. The discrepancy with the notion of frozen protein conformations at cryogenic temperatures (Austin et al., 1975) has been resolved by assuming that this relaxation is of a smaller spatial extent as compared to that which is responsible for rebinding. Hence, different levels (tiers) of "functionally important motions" were postulated to play a role in myoglobin and hemoglobin dynamics (Frauenfelder, 1985).

An alternative mechanism for the temporal line shift may operate when a line is (at least partially) inhomogeneously broadened, e.g., by different protein conformations. Such a broadening effect has recently been detected in the Soret band (Šrajer et al., 1986). If there exists a simple relation between protein conformation and absorption wavelength, and some conformations react faster than others as in myoglobin (Austin et al., 1975), one expects the line shape to diminish faster on one side, resulting in a shift which is correlated with the decrease in intensity (Agmon et al., 1986). At the time, this possibility has not been seriously considered. However, a recent experiment (Campbell et al., 1987) has proven that at 60 K there is no detectable relaxation which is independent of rebinding. The frequency shift should therefore be attributed to a "kinetic hole burning" mechanism.

This work undertakes a reexamination of the temporal data (Bowne, 1984; Ansari et al., 1985) on the near-IR band at 760 nm. Evidence obtained for reactive line narrowing is described below. The results are divided into two: (i) results which are independent of any particular model for the distribution of barrier heights and (ii) a quantitative comparison with such a model (Agmon & Hopfield, 1983).

Experimental Evidence for Reactive Line Narrowing

The transient absorption of sperm whale myoglobin–CO (MbCO), dissolved in 75% glycerol–water and buffered to pH 7.2, has been measured by the Frauenfelder group in the range 700–820 mm following a 20-ns photolyzing pulse (Bowne, 1984; Ansari et al., 1985). Line shapes at different delay times have been obtained by rearranging the transient absorption at different wavelengths.

In this work, the wavelength (λ) dependence of the original (see Acknowledgments) absorption line shapes, $I(\lambda,t)$, at different times has been fitted to a sum of a Gaussian (the desired line shape) and a parabola (base line due to other peaks in the spectrum). Six parameters were determined by the multidimensional least-squares computer program:

$$I(\lambda,t) = c_0 + c_1\{c_2(\lambda - \lambda_{min})^2 + \exp[-(\lambda - \lambda_p)^2/\sigma^2]\} \quad (1)$$

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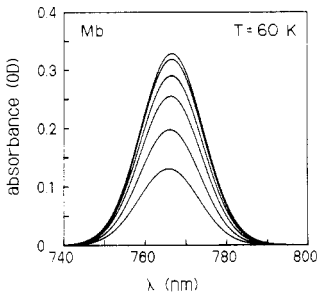

FIGURE 1: Temporal behavior of the near-IR peak following laser dissociation of myoglobin–CO. Lines show the best fit to the experimental line shapes, after subtraction of the (parabolic) base line, at (top to bottom) 1×10^{-5}, 1×10^{-4}, 1×10^{-3}, 1×10^{-2}, 0.1, and 1 s after the laser flash.

In order to get the program to work, all parameters must be of a similar order of magnitude. Wavelengths were therefore entered in units of 100 nm.

The quality of such a fit was demonstrated by Ansari et al. (1985), though often the agreement is not as good as that shown in their Figure 3, especially in the wings of the peak. Typical values for the time-dependent parameters are as follows: λ_{min} = 780–820 nm, $c_2 = 10^{-5}$–10^{-4} nm^{-2}, λ_p = 760–766.5 nm, and σ = 10–12 nm.

The uncertainties in the peak wavelength, λ_p, are estimated to be ±0.2 nm (this does not include possible systematic errors). This error is small enough to allow one to observe systematic temporal changes in λ_p, which are 1–3 nm in magnitude. In contrast, the uncertainty in σ, which may be as large as 10%, generally prevents one from resolving systematic changes of only 1 or 2 nm. Hence, unfortunately, the observed variations in σ are mostly statistical noise. An exception is the measurement at the lowest temperature, 60 K (see below).

Figure 1 shows the Gaussian line shape at 60 K with the best-fit parameters. This peak, which is typical of the deoxy state only, diminishes in amplitude (area) due to rebinding. In addition, there is a small blue shift for increasing time delays. As can be seen, this shift is very small (<1 nm at 60 K) compared to the line width (which is ca. 20 nm at $1/e$ height), and it increases with increasing temperature.

The area under the line shape is proportional to the total population of the deligated state. In order to compare the present results with the deoxymyoglobin survival probability, as obtained (Austin et al., 1975) by monitoring in the Soret (430 nm), the present results have to be normalized to unity at $t = 0$. This is especially important since the experimental results (Ansari et al., 1985) were obtained from four different preparations. For two of these (used for measurements at T = 60, 80, and 100 K) the normalization was determined from the area of the peak obtained at 60 K and $t = 1 \times 10^{-5}$ s after dissociation. For the other two preparations (the 120, 140, and 160 K data) no low-temperature experiments were performed, so the normalization was determined from comparison with the previous measurements in the Soret (Austin et al., 1975).

The symbols in Figure 2 show the temporal behavior of the survival probability, $Q(t)$, as calculated from the 760-nm data. The lines were generated from a fit of a model (Agmon & Hopfield, 1983) to the results (Austin et al., 1975) obtained previously by monitoring the Soret. The deviation of the lines from the Soret data is smaller than the difference between various compilations (Austin et al., 1975; Young & Bowne, 1984) of the experimental data. From Figure 2 it can be seen that there is fair agreement between monitoring at 760 nm

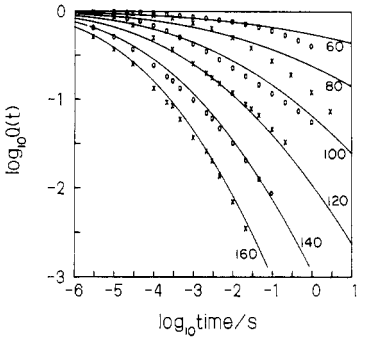

FIGURE 2: Temporal dependence of the deligated myoglobin survival probability. Symbols are from the total area of the experimental near-IR line shapes fitted to eq 1, after the subtraction of the (parabolic) base line. Normalization factors for the experimental data (Ansari et al., 1985) were 3.05, 2.34, 3.05, 2.5, 2.5, and 1.4 for T = 60, 80, 100, 120, 140, and 160 K, respectively. Lines are the results of fitting the quantitative model, eq 3 and 6–8, with the parameters shown in Table I of Agmon and Hopfield (1983) to the experimental data (Austin et al., 1975) obtained by monitoring in the Soret.

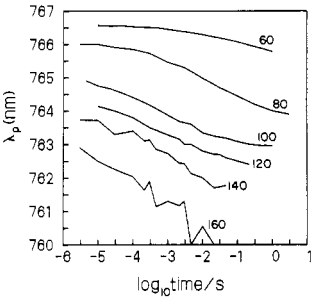

FIGURE 3: Temporal shift in the peak wavelength of the near-IR band following flash photolysis of MbCO. The values of λ_p were obtained by fitting eq 1 to the experimental data. Uncertainties in the fitting procedure are not larger than 0.2 nm.

and monitoring at 430 nm. The only large deviations are for the 80 K results. At low temperatures it takes a long time for the sample to recover between laser flashes; hence in the multiple-flash technique used, kinetics for $T < 120$ K cannot be expected to agree quantitatively with the single wavelength measurement in the Soret.

Figure 3 shows the temporal shift of the peak wavelength at the six temperatures. The curves generally tend to become steeper with increasing temperature. An important question for the analysis is their asymptotic value for $t \rightarrow 0$. Bowne (1984) and Frauenfelder (1985) have assumed that the difference between the initial and deoxy peaks is temperature independent. [The temperature dependence of the corresponding deoxy-Hb peak has been reported by Cordone et al. (1986).] At cryogenic temperatures, this has led to the conclusion that all curves have a common temperature-independent asymptotic value, similar to that of the 60 K curve.

A closer examination of the data indicates that at the onset of recombination the peak becomes blue shifted with respect to T: (i) From an extrapolation of the rebinding data in Figure 2 it appears that at 0.1 μs rebinding is negligible for $T \leq 160$ K. In contrast, an extrapolation of λ_p (Figure 3) to 0.1 μs gives temperature-dependent shifts. (ii) The data at 60 and 80 K show negligible recombination already for $t < 10$ μs. The shifts for these two temperatures seem to reach different (by ca. 0.5 nm) asymptotic limits at short times. The "initial wavelength" λ_0 was therefore chosen to correspond to the peak position at the onset of the recombination process. This is shown in Figure 4, which collects different values of λ_p from various samples at times between 2 and 10 μs. The temperature dependence of λ_0 shown in Figure 4 is also the upper bound for all available

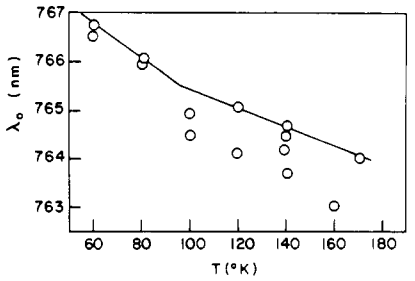

FIGURE 4: Peak wavelength of the near-IR band in photodissociated MbCO at times between 2 and 10 μs after photolysis at various temperatures. Circles represent a compilation of different experimental runs which were fitted to eq 1. The line, drawn as an upper bound to those results, is assumed to be the temperature dependence of the initial peak wavelength, λ_0. This gives λ_0 = 766.7, 766.1, 765.4, 765.05, 764.7, and 764.3 for T = 60, 80, 100, 120, 140, and 160 K, respectively.

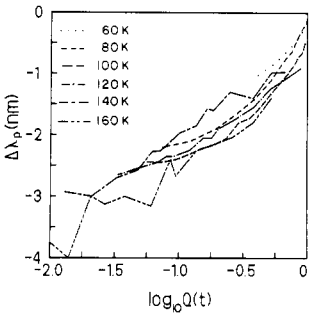

FIGURE 5: Dependence of the shift in the peak wavelength on survival probability. $\Delta\lambda_p = \lambda_p - \lambda_0$. $\lambda_p(t,T)$ and $\lambda_0(T)$ are given in Figures 3 and 4, respectively. $Q(t,T)$ is taken from the experimental points in Figure 2. A legend for the six different temperatures is given in the figure.

experimental data (circles). Extrapolation to room temperature gives λ_0 (300 K) = 761.5 ± 0.5 nm. Indeed, Sassaroli and Rousseau (1987) have found that at room temperature $\lambda_p \approx 761$ nm for both deoxy–Mb and the photoproduct of MbCO 10 ns after photodissociation.

The present experimental data do not enable one to determine whether a temperature-dependent blue shift is the rule for very short times or else that an initial temperature-independent wavelength of this peak is followed by very fast T-dependent relaxation. If such a relaxation process exists, it is expected to be localized around the heme without affecting the globin's conformation or its nonexponential rebinding kinetics. For example, the initial subpicosecond out-of-plane motion of the iron may be larger at higher temperatures, when the proximal side of the heme pocket becomes more flexible. The distance of the iron from the prophyrin plane is in turn expected (Šrajer et al., 1986) to correlate with the blue shift in the near-IR band.

We are now in a position to consider the evidence for the kinetic line narrowing. Imagine, for simplicity, a square line shape whose width is due to inhomogeneous broadening and a monotonic wavelength-dependent rate coefficient $k(\lambda)$. By time t, the recombination reaction would deplete all values of λ for which $k(\lambda) > 1/t$. The peak would then become progressively narrower from one side. The crucial point to note (J. J. Hopfield, private discussion) is that, irrespective of the exact functional form of $k(\lambda)$, a given decrease in the area would always correspond to the *same* shift in the center (peak) wavelength. This means, that for reactive hole burning, a plot of the spectral shift as a function of rebinding should reveal a universal behavior, which is independent of temperature.

Figure 5 shows that this is indeed the case. Plotted is the spectral shift $\Delta\lambda_p \equiv \lambda_p - \lambda_0$, as a function of the survival probability $Q(t)$. The data for $Q(t)$ are the same as in Figure 2; λ_p is taken from Figure 3 and λ_0 from Figure 4. The data for all six temperatures fall in a strip of 0.6-nm width. Given the accuracy in determining λ_p, ±0.2 nm, and the uncertainties in λ_0 and $Q(t)$, we conclude that all data do seem to fall on a universal curve to within the experimental error bars.

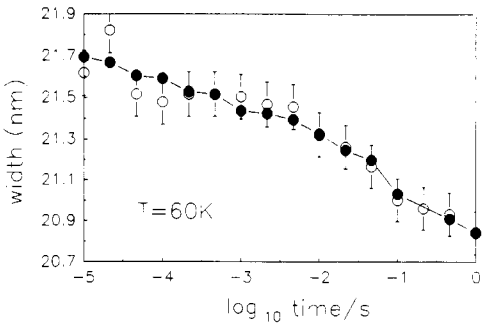

FIGURE 6: Temporal dependence of the width of the Gaussian absorption profiles of the near-IR band at 60 K (Figure 1). Open circles denote values of 2σ obtained by fitting the experimental data to eq 1. Error bars are deduced from the sensitivity of the fitting parameters to their initial values and do not include possible systematic errors. Closed circles connected by lines are obtained after smoothing the spectra with a singular value decomposition (SVD) routine, similar to that used by Hofrichter et al. (1985).

As discussed above, it is much more difficult to determine systematic changes in line width as compared to shifts of its peak. At the higher temperatures, changes in σ seem to be due mainly to statistical noise. At 60 K, however, a systematic decrease in width (2σ) with time is observed (Figure 6). Although errors in the fitting parameters here are small, possible systematic errors (introduced by the choice of the fitting function, eq 1, or by the experimental procedure) enable one to make only the qualitative statement that, as the recombination reaction proceeds, the peak does become *narrower*. This is a clear indication for the operation of a coordinate- (wavelength) dependent rate process.

A Quantitative Model

In treating the low-temperature data quantitatively, we assume (Agmon & Hopfield, 1983; Young & Bowne, 1984) that at each instant t there is a probability distribution function $p(x,t)$ for finding a deligated heme protein in a given conformation, namely, with a given value of its "protein coordinate" x. At low T, when all protein motion is frozen, $p(x,t)$ is determined by two factors: the initial distribution function $p^0(x) = p(x,0)$ and a recombination rate coefficient $k(x)$, which is assumed to increase monotonically with x.

Once the two above-mentioned functions are known, the temporal evolution of protein conformations in the deoxy state is obtained by assuming that each conformation reacts unimolecularly and independently of all other conformations:

$$p(x,t) = p^0(x) \exp[-k(x)t] \tag{2}$$

Since the photolyzing laser pulse dissociates the entire heme population, $p^0(x)$ normalizes to unity. The survival probability $Q(t)$ is the total population in the deligated state:

$$Q(t) = \int_{-\infty}^{\infty} p(x,t)\, dx \tag{3}$$

In order to proceed, the protein coordinate must be related to the experimental variable, which is the spectral frequency or wavelength. The basic assumption of the proposed inhomogeneous-broadening mechanism is that each conformation absorbs in a slightly different wavelength. Since this variation is expected to be small, one need not bother about the most general $\lambda(x)$ relationship. Its linear approximation should

suffice, if the expansion is carried out around a point (e.g., x_0, see eq 7 below) inside the relevant range of x values. Hence, one may assume that

$$\Delta\lambda \approx \alpha\Delta x \tag{4}$$

where $\Delta\lambda \equiv \lambda - \lambda_0$ and $\Delta x = x - x_0$. Positive α implies that the red-shifted conformations react faster, which is plausible if indeed these conformations have the iron closer to the porphyrin plane, from where it has a shorter distance to move after rebinding.

Finally, one has to account for the homogeneous line broadening, which (see below) is the main contribution to the line width in the present case. Each protein conformation contributes a homogeneously broadened, Gaussian line shape, $g(\lambda)$, which is centered around the wavelength given by eq 4, and its width is similar to that of the experimental line shape. Summing up all these contributions leads to a convolution relation for the spectral intensity $I(\lambda)$:

$$I(\lambda) = \int_{-\infty}^{\infty} p(x,t)g[\lambda - \lambda(x)]\, dx \tag{5}$$

where $g(y) \equiv (\pi\sigma^2)^{-1/2} \exp(-y^2/\sigma^2)$. Note that since $g(y)$ normalizes to unity, $Q(t) = \int_0^\infty I(\lambda)\, d\lambda$.

The comparison with experiment now proceeds as follows: The functions $p^0(x)$ and $k(x)$ are determined by fitting the rebinding data to $Q(t)$, eq 3, as was previously done (Agmon & Hofield, 1983; Young & Bowne, 1984). σ is assumed equivalent to the experimental value because most of the broadening is homogeneous. This leaves us with the single parameter α to be determined from the temporal dependence of the shift of the peak frequency, $\Delta\lambda_p(t)$. This is not a void exercise, because data at all temperatures must fit with the same value of α.

In order to carry out the above scheme, one can use the functional forms for $p^0(x)$ and $k(x)$ given by Agmon and Hopfield (1983): The initial distribution is that of the ligated MbCO at the freezing temperature of the solvent, $T_f = 220$ K:

$$p^0(x) = (f/2\pi k_B T_f)^{1/2} \exp[-f(x - x_0)^2/2k_B T_f] \tag{6}$$

k_B being Boltzmann's constant and f a force constant. The x-dependent barrier height $V^*(x)$ is determined from the potential energy as a function of the protein coordinate and the iron–ligand distance:

$$V^{*2} - 3D_e V^* + (1/2)fx_0(x_0 - 2x) + \Delta = 0 \tag{7}$$

where $D_e - \Delta$ is the ligand's binding energy. The coordinate-dependent rate coefficient is finally given by

$$k(x) = A \exp[-V^*(x)/k_B T] \tag{8}$$

in which T is the actual temperature of the frozen sample.

The values for the parameters D_e, Δ, f, x_0, and A are given in Table I of Agmon and Hopfield (1983). Of these only Δ, fx_0, and A are important in the determination of the survival probability. The calculated survival probability is shown as the bold lines in Figure 2. Having determined the above parameters from $Q(t)$, and using an average experimental value, $\sigma = 11$ nm, for the half-width of the homogeneous line, a rough agreement with the transient experimental shifts in the near-IR peak can be obtained by adjusting α (Figure 7). The fit is quite insensitive to σ as long as it is large, but it is sensitive to α. The best value of $\alpha = 8 \pm 1$ nm/au is larger than the value of 5 nm/Å obtained by Šrajer et al. (1986) from an analysis of the Soret peak.

Having determined α, one can estimate the relative contributions of the two broadening mechanisms to the line width.

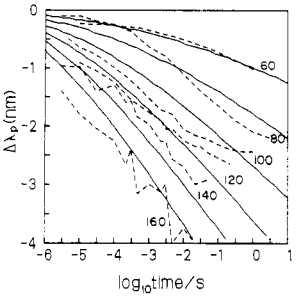

FIGURE 7: Comparison of the experimental shifts in the near-IR peak (dashed curves) with those calculated numerically from the quantitative model described in the sequel. σ in eq 5 was set equal to the average experimental value, 11 nm. The other parameters of the model, from Table I of Agmon and Hopfield (1983), are the same as those used to draw the lines in Figure 2. The new parameter in the present fit is α, which assumes the value 8 ± 1 nm/au. Convolution, eq 5, was performed on a grid of 400 points equally distributed in the interval $x = 0.4$–1.8 au centered at $x_0 = 1.1$ au (1 au = 0.569 Å).

The half-width of the inhomogeneous line can be determined from that of $p^0(x)$, eq 6, which is 0.25 au. Multiplying by α yields a value of 2 nm. Comparing with $\sigma = 10$–12 nm, we conclude that the homogeneous broadening is 5–6 times larger than the inhomogeneous one. This explains why systematic changes in width are much more difficult to observe than the shift in the peak. Evidently, the effect described in this work would be much more prominent in systems with less homogeneous broadening.

As for the line-narrowing effect, it is predicted by the model to be 2–4 times smaller than the results shown in Figure 6. Due to the qualitative nature of these results (see above) and the fact that the model fits the survival probability at 60 K only semiquantitatively (Figure 2), a quantitative comparison of experimental and theoretical line-narrowing effect is not yet feasible.

Conclusions

The new analysis presented above for the experimental data of Ansari et al. (1985) suggest that the temporal shift in the near-IR peak of myoglobin at cryogenic temperatures is due to an inhomogeneous reactive process rather than to relaxation. This corroborates the conclusion from the low-temperature spectra taken recently by Campbell et al. (1987). It verifies the prediction (Agmon & Hopfield, 1983) that "the observed line shape should be a faithful monitor of the shape of $p(x,t)$".

In the present analysis of the transient absorption data at 60 K a possible transient line-narrowing effect was observed, in line with a kinetic hole-burning explanation. In addition, it was demonstrated that the inhomogeneous reactive process which depletes this line shape is very likely the *same* process which is responsible for the nonexponential rebinding kinetics at low temperatures. This was concluded from the universal, temperature-independent, correlation between shift and rebinding and from the fact that the same quantitative model which accounts for rebinding can account semiquantitatively for the temporal shift in the peak. By fitting this model to the data, it was concluded that the inhomogeneous broadening for the near-IR peak in myoglobin is no more that 20% of its total width. More accurate experimental data are needed to check these conclusions.

The present kinetic hole-burning interpretation is not just a possible alternative interpretation to that of low-temperature conformational relaxation. It is actually an inevitable consequence of the second assumption: Relaxation on the "second tier" (Frauenfelder, 1985), if it exists, implies that different conformations on the second tier absorb at different wave-

lengths; otherwise, a shift in wavelength would not be detected. This should apply equally well to the different conformations on the first tier, which by assumption are even more widely separated than those on the second tier. But this means that the line shape is inhomogeneously broadened, and therefore at low temperatures, when a distribution of recombination rates is detected, one expects to see differential rebinding. Hence at low temperatures, the effect of inhomogeneous reactivity must always be the primary cause for the frequency shift, and only deviations from this mechanism should be attributed to relaxation.

At higher temperatures there is rapid conformational change, and the kinetics become exponential (Austin et al., 1975). This indicates that at high temperatures a spectral shift should be attributed mainly to conformational relaxation. Indeed, transient shifts of spectral lines after photolysis have been previously reported in transient Raman spectroscopy of hemoglobin at room temperatures (Friedman et al., 1982; Scott & Friedman, 1984; Sassaroli & Rousseau, 1987). It was suggested that the shifts are due to transient changes in the protein's tertiary and quaternary structure. Unfortunately, a quantitative analysis of these high-temperature data is not yet available.

Protein function was initially interpreted as structural, such as the "key and lock" model of enzyme action. Recent years have produced proofs for a protein dynamical role in biological processes (Welch, 1986). In the present work it was shown how a biologically important process, namely, ligand binding to heme proteins, is indeed closely linked to tertiary protein conformational change. The observation that protein conformations which rebind at different rates also absorb at different wavelengths means that one may be able to monitor spectroscopically the conformational changes controlling heme function. This increases our hope for collecting important experimental data on protein dynamics vis transient spectroscopy.

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