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REFERENCES

Banville, D. L., Marzilli, L. G., & Wilson, W. D. (1983) *Biochem. Biophys. Res. Commun. 113*, 148–154.
Carvlin, M. J., & Fiel, R. J. (1983) *Nucleic Acids Res. 11*, 6121–6139.
Carvlin, M. J., Mark, E., & Fiel, R. (1983) *Nucleic Acids Res. 11*, 6141–6154.
Edmondson, S. P., & Curtis Johnson, W., Jr. (1985) *Biochemistry 24*, 4802–4806.
Fiel, R. J., & Munson, B. R. (1980) *Nucleic Acids Res. 8*, 2835–2842.
Fiel, R. J., Howard, J. C., & Datta Gupta, N. (1979) *Nucleic Acids Res. 6*, 3093–3118.
Gabbay, E. J., Adawadkar, P. D., & Wilson, W. D. (1976) *Biochemistry 15*, 146–151.
Geacintov, N. E., Gagliano, A., Ivanovic, V., & Weinstein, I. B. (1978) *Biochemistry 17*, 5256–5262.
Geacintov, N. E., Yoshida, H., Ibanez, V., & Harvey, R. G. (1982) *Biochemistry 21*, 1864–1869.
Houssier, C. (1981) in *Molecular Electro-Optics* (Krause, S., Ed.) pp 363–398, Plenum, New York.
Kelly, J. M., & Murphy, M. J. (1985) *Nucleic Acids Res. 13*, 167–184.
Lee, C.-H., & Charney, E. (1982) *J. Mol. Biol. 161*, 289–303.
McGhee, J. D., & Von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.
Neidle, S. (1979) *Prog. Med. Chem. 16*, 151–221.
Neta, P. (1981) *J. Phys. Chem. 85*, 3678–3684.
Norden, B. (1978) *Appl. Spectrosc. Rev. 14*, 157–248.
Norden, B., & Tjerneld, F. (1976) *Biophys. Chem. 4*, 191–198.
Pasternack, R. F., Gibbs, E. J., & Villafranca, J. J. (1983a) *Biochemistry 22*, 2406–2414.
Pasternack, R. F., Gibbs, E. J., & Villafranca, J. J. (1983b) *Biochemistry 22*, 5409–5417.
Pasternack, R. F., Antebi, A., Ehrlich, B., Sidney, D., Gibbs, E. J., Bassner, S. L., & Depoy, L. M. (1984) *J. Mol. Catal. 23*, 235–242.
Pasternack, R. F., Gibbs, E. J., Gaudemer, A., Antebi, A., Bassner, S., DePoy, L., Turner, D. H., Williams, A., Laplace, F., Lansard, M. H., Merienne, C., & Peree-Fauvet, M. (1985) *J. Am. Chem. Soc. 107*, 8179–8186.
Pasternack, R. F., Garrity, P., Ehrlich, B., Davis, C. B., Gibbs, E. J., Orloff, G., Giartosio, A., & Turano, C. (1986) *Nucleic Acids Res. 14*, 5919–5931.
Patel, D. J. (1979) *Acc. Chem. Res. 12*, 118–125.
Ramstein, J., Houssier, C., & Leng, M. (1973) *Biochim. Biophys. Acta 335*, 54–68.
Wada, A. (1964) *Biopolymers 2*, 361–380.
Wada, A., & Kozawa, S. (1964) *J. Polym. Sci. 2*, 853–864.
Weiss, C. (1972) *J. Mol. Spectrosc. 44*, 37–80.
Wilson, R. W., & Schellman, J. A. (1978) *Biopolymers 17*, 1235–1248.

Time Dependence of Near-Infrared Spectra of Photodissociated Hemoglobin and Myoglobin

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ABSTRACT: The near-infrared charge-transfer transitions at ~760 nm in photodissociated hemoglobin and myoglobin display very different time dependences. In photodissociated myoglobin at room temperature the transition has fully relaxed to its deoxymyoglobin value by 10 ns. In photodissociated hemoglobin, the transition is shifted by 6 nm to longer wavelengths at 10 ns. It relaxes about halfway back to the deoxyhemoglobin value by about 100 ns but subsequently changes very slowly out to about 100 μs when the signal intensity becomes too small to follow any further. The intensity of this transition, present in only five-coordinate hemes, is found to follow the same time dependence as the wavelength change. Consequently, there appears to be a correlation between a structural property of the heme (as inferred from the wavelength of the charge-transfer transition) and a functional property (the CO recombination) of the protein (as inferred from the intensity of the transition). Possible origins for this correlation are considered.

Hemoglobin and myoglobin have a well-known charge-transfer transition in the near-infrared at 759 and 761 nm, respectively (Eaton & Hofrichter, 1981). Several years ago Iizuka et al. (1974) studied the properties of this transition in the photoproducts obtained by photodissociating the CO-bound complexes of these proteins at 4 K. In both hemoglobin and myoglobin they found a substantial shift of this line to lower energy in the photoproducts as compared to the deoxy preparations under the same conditions. Recently, other groups have confirmed these low-temperature differences (Ansari et al., 1985; Fiamingo & Alben, 1985). Moreover, since these seminal studies of the low temperature stabilized photoproducts, studies of other spectroscopic (Ondrias et al., 1983b; Rousseau & Ondrias, 1985; Rousseau & Argade, 1986) and structural (Chance et al., 1983; Powers et al., 1984) properties have been reported. In addition, many studies of the properties of the photoproducts generated at ambient temperatures with transient techniques have been carried out (Henry et al., 1983a,b; Hofrichter et al., 1983; Scott & Friedman, 1984; Findsen et al., 1985a,b; Friedman, 1985; Dasgupta et al., 1985; Rousseau & Argade, 1986). From these many studies it has been found that at room temperature the

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optical spectrum and the resonance Raman spectrum of the photoproduct of myoglobin are fully relaxed to the equilibrium deoxy values within 10 ns. In contrast, the 10-ns time-resolved photoproduct of hemoglobin has optical absorption and resonance Raman spectra that are very distinct from those of deoxyhemoglobin.

At low temperature, substantial differences between the resonance Raman spectrum of the carbon monoxide photoproduct and the deoxy preparation have been observed in both hemoglobin (Ondrias et al., 1983b; Rousseau & Ondrias, 1985) and myoglobin (Rousseau & Argade, 1986; Sassaroli et al., 1986). Ondrias et al. (1983a) demonstrated that these differences in hemoglobin at cryogenic temperatures are the same as those reported at room temperature using transient techniques. More recently, Rousseau and Argade (1986) found that changes in the region of the Raman spectrum that is sensitive to the porphyrin core size between the carbon monoxide myoglobin photoproduct and the deoxy preparation were the same at 4 K as those found by Dasgupta et al. (1985) at room temperature with 30-ps pulses. Sassaroli et al. (1986) have examined the low-frequency region of the resonance Raman spectrum of the photoproduct at cryogenic temperatures and have found substantial differences between it and the deoxy preparation, especially in the line that is assigned as the iron–histidine stretching vibration (Argade et al., 1984). No corresponding changes have been reported at room temperature by transient techniques on a time scale as fast as 30 ps (Findsen et al., 1985b).

In spite of the large number of spectroscopic studies that have been published on the photoproducts of myoglobin and hemoglobin at low temperature and at room temperature, there have been no reports of the near-infrared optical spectrum of the photoproduct at room temperature by time-resolved techniques. The only measurement of any time dependence of the 760-nm transition was that reported recently by Ansari et al. (1985) on myoglobin at low temperature. An examination of the behavior of this charge-transfer transition at room temperature with time-resolved optical absorption is important in order to fully characterize the properties of the hemoglobin and myoglobin photoproducts. This will lead to a better understanding of the differences between the far from equilibrium photodissociated molecules and the equilibrium deoxy proteins. Such information will also lead to a better understanding of the relationship between the low-temperature metastable species and the room-temperature transients. Finally, the relation between the spectroscopic parameters of the photoproducts and the ligand rebinding processes (Austin et al., 1975) must be determined. With these objectives in mind we have generated the photoproduct of carbon monoxide hemoglobin and myoglobin with 10-ns pulses from a Nd-YAG laser and compared the near-infrared absorption spectra to those of the deoxy preparations. We were able to monitor the wavelength of the transition, which is a property of the heme structure. We were also able to measure the intensity of the transition, which depends on the deoxyheme population, which in turn depends on the amount of carbon monoxide recombination. The intensity variation is thereby a monitor of a functional property of the proteins.

Experimental Procedures

For the experiments reported here, the hemoglobin and myoglobin samples were maintained at concentrations of 0.75 mM and were buffered with 0.1 M phosphate at pH 7. Myoglobin was Sigma type II (sperm whale). It was filtered with a 2-μm Millipore filter prior to use. Hemoglobin was isolated from human, and after purification, it was stored as the CO form under liquid nitrogen until ready for use. The samples of both hemoglobin and myoglobin were reduced and deoxygenated in an anaerobic atmosphere with minimal dithionite. Following reduction they were divided in half. Half (the deoxy preparation) was placed in a 1 cm path length cuvette, which was sealed for the experimental run. The other half was flushed with carbon monoxide and then also sealed in a cuvette.

Two types of experimental setups were used to obtain the data reported here. In the first the frequency-doubled output (20–50 mJ/pulse at 532 nm) from a Nd-YAG laser was split into two beams. One beam was focused on a quartz capillary tube through which a mixture of dyes [oxazine, DOTC (3,3′-diethyloxatricarbocyanine), HITC (1,1′,3,3,3′,3′-hexamethyl-2,2′-indotricarbocyanine), and DODC (3,3′-diethyloxadicarbocyanine)] was circulated. The fluorescence output from the dyes was focused through the 1-cm optical cell containing the samples. A red filter was placed between the capillary containing the dyes and the heme protein sample to remove light emitted in the visible and scattered laser light, both of which contributed to some photodissociation. The other Nd-YAG beam at 532 nm was partially focused with a cylindrical lens on the sample crossing the probe beam illumination. In these experiments the photolysis and the probe light pulses were temporally coincident and had a 10-ns width. The dyes were chosen to give high intensity in the 700–800-nm region with 532-nm excitation. The mixture was found to be useful both to fill in the valleys between individual dyes and also to transfer energy from the dyes with outputs in the ~600-nm range to the near-infrared dyes. The optimum concentrations and proportions were determined empirically by obtaining as flat an output as possible in the 760-nm range.

In the second arrangement used to obtain the time evolution data, the probe pulse was generated by a Xenon Corp. Nanopulser arc lamp that was modified to allow control of the gas atmosphere. In the experiments reported here the lamp was operated in an atmosphere of pure xenon to yield a spectrum with little structure in the 760-nm region. The remaining optical path was the same as that in the time-synchronized experiments. The pulse from the Nanopulser lamp is asymmetric with a primary pulse width of ~50 ns and a tail of ~200 ns. The time delay between the lamp and the photolysis laser beam could be varied continuously with a delay generator and was measured before each experiment with a fast photodiode. The broad time spectrum of the output from the Nanopulser restricted its time-resolution capabilities to the 100-ns range.

Samples of the CO-bound preparations were examined at the start of each run with the photolysis pulse blocked to assure that the probe lamp was not photodissociating any of the ligand-bound protein. If it was, the intensity was adjusted to minimize this effect. In all experiments the contribution from the probing light was less than 5% of the signal from the photoproduct at zero time delay.

The transmitted light from the probe pulse was focused on the entrance slit of a 1.25-m monochromator and detected with a Princeton Applied Research intensified reticon array. Integration times were typically 15 min. The absorbance was calculated by taking the natural log of the blank divided by the sample transmittance. For the deoxy preparations the blank was a water sample. For the photodissociated samples the blank was the sample without the photolysis pulse.

The absorbance spectrum obtained from the procedure described above generally had a sloping background. A multipoint polynominal fit to this background was made, and

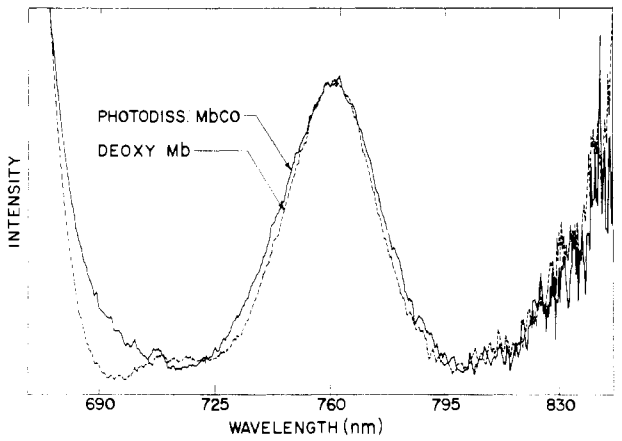

FIGURE 1: Comparison of absorbance at ~760 nm between deoxymyoglobin and photodissociated carbon monoxide myoglobin (MbCO) obtained with 10-ns pulses. The center of both lines is at 761 nm.

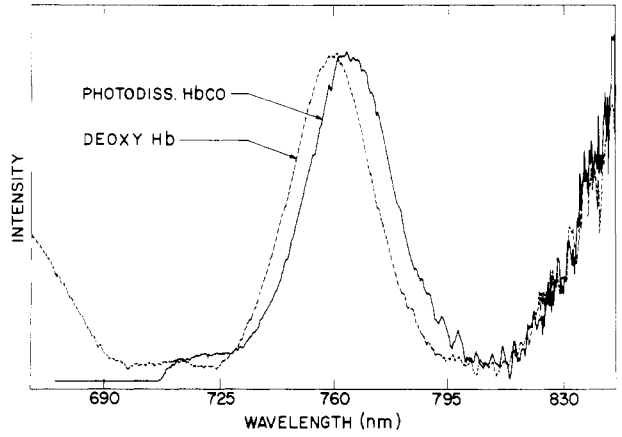

FIGURE 2: Comparison of absorbance at ~760 nm between deoxyhemoglobin and photodissociated carbon monoxide hemoglobin (HbCO) obtained with 10-ns pulses. The value of the transition is 759 nm in deoxyhemoglobin and 765 nm in the photoproduct.

it was subtracted from the absorbance spectrum, leading to a flat spectrum over the absorbance line. Peak positions and wavelength shifts were determined from these reduced data by calculating the deoxy photoproduct difference spectra, locating the line center by taking the first derivative of the line, or measuring the midpoint of the full width at half-maximum (FWHM). By these techniques we obtained a sensitivity of better than ±1 nm.

RESULTS

Zero Time Delay. In experiments with the synchronized 10-ns dye lamp and Nd-YAG pulses, both hemoglobin and myoglobin were examined. The results for myoglobin are shown in Figure 1. We place the peak of the infrared transition at 761 nm for both the deoxy preparation and the carbon monoxide photoproduct. Within our sensitivity, no difference in the wavelength of the transition between the photoproduct and the deoxy species could be found. The measurements were repeated several times to verify their validity. The intensity of the 761-nm line in the photoproduct was found to be the same as that in deoxymyoglobin.

For hemoglobin, the charge-transfer transition of the photoproduct is at 765 nm at 10 ns compared to deoxyhemoglobin, which has the transition at 759 nm (see Figure 2). As in the myoglobin, the intensity of the photoproduct in the hemoglobin was also approximately the same as that in deoxyhemoglobin. To assess the frequency shift quantitatively, we have also obtained the difference spectrum (Figure 3) between the

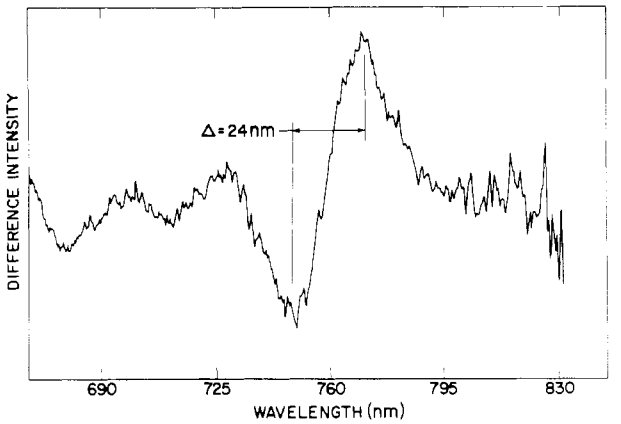

FIGURE 3: Difference spectrum of photodissociated HbCO minus deoxyhemoglobin. The 6-nm separation between the peaks in the two spectra (Figure 2) yields a 24-nm separation between the peak and valley in the difference spectrum.

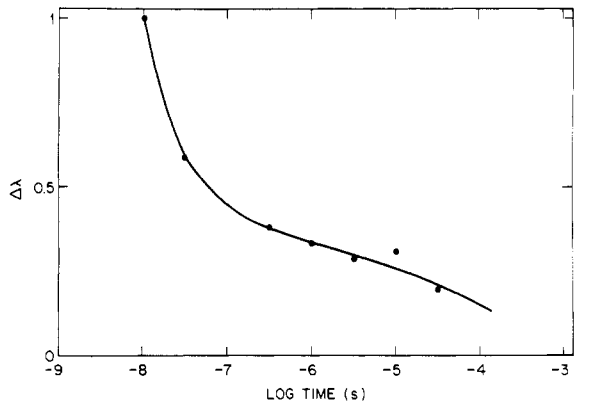

FIGURE 4: Change in wavelength vs. time of the "760-nm" absorbance in hemoglobin. The curve is only to guide the eye.

photoproduct and the deoxy preparation. The separation between the peak and the valley in the difference spectrum is ~24 nm. This separation, which is much greater than the peak-to-peak separation in the indiviaul spectra, is the expected result for a difference spectrum. The line width for the infrared absorption line in hemoglobin is ~30 nm. The peak-to-peak separation in the difference spectrum is expected to be $(1/3^{1/2})\Gamma$ for a Lorentzian line (Γ is the full width at half-height) and to be $\Gamma/(2 \ln 2)^{1/2}$ for a Gaussian line (Rousseau, 1981). For a 30 nm wide line these give expected separations of 17 nm for a Lorentzian line and 25 nm for a Gaussian line. Thus, the 760-nm line with its 24-nm separation in the difference spectrum has a Gaussian shape.

Time Evolution of Hemoglobin Photoproduct. A series of measurements were made on hemoglobin to follow the time evolution from zero delay (10 ns) to 1 ms. No time decay measurements were made in myoglobin since no shifts were detected at the shortest times examined. By maintaining an identical optical arrangement for each of the different time slots and by randomizing the order in which the time delays were chosen, we were able to follow both the wavelength change and the intensity change as a function of time after photolysis. A plot of the wavelength change over the range examined is shown in Figure 4. At "zero" delay time the wavelength of the infrared transition in the photoproduct has already decayed from 765 nm (seen with the 10-ns dye lamp) to about 763 nm. We attribute this shift at zero delay as being due to the broad time width of the Nanopulser probe lamp. At longer times there is a gradual relaxation of the peak position toward the deoxy value. Reliable peak wavelength

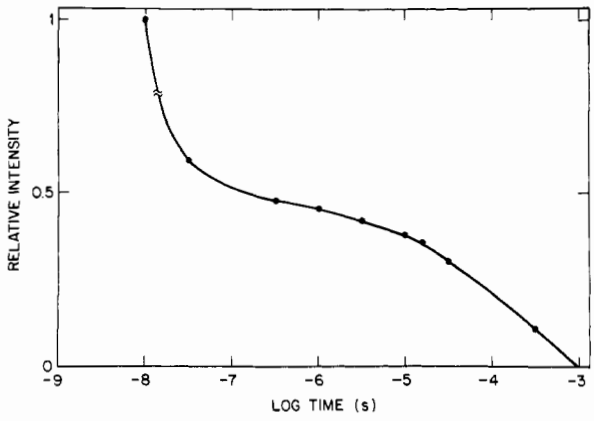

FIGURE 5: Change in intensity vs. time of the 760-nm transition in hemoglobin. The curve is only to guide the eye. The break in the curve is included to indicate that the 10-ns point was obtained with the dye emission and the remaining points with the xenon arc.

assignments could not be made for delay times beyond 50 μs, due to the lowered intensity of the transition.

The relaxation of the spectral position of the infrared line with time shows a large change between 10^{-8} and 10^{-7} s to a value just below half of the maximum shift, followed by a plateau region with only a slight decrease in frequency until times longer than 10^{-5} s. During the relaxation of the position of the infrared peak, no change in its width was detected. A value of 30 ± 2 nm for the line width (FWHM) was measured throughout the time decay. However, the actual width change anticipated for the sum of two Gaussian lines of equal intensity is substantially less than the separation between them. Specifically, the addition of two lines 6 nm apart and 30 nm wide yields a composite line less than 1 nm wider than the individual lines. Thus, within our sensitivity, we cannot distinguish between the case in which the line shifts continuously from its wavelength in the photodissociated protein to its wavelength in the deoxygenated protein from the case in which there are only two wavelengths for the line, one at the photodissociated value and one at the deoxy value.

The integrated intensity of the infrared transition was also followed as a function of time. As shown in Figure 5, the decay of the intensity appears to take place with the same general biphasic behavior as the wavelength decay. For the intensity data, reliable measurements could be made out to longer time delay values (between 10^{-4} and 10^{-3} s), allowing a more complete relaxation curve to be determined. In the data in Figure 5, the curve between the first point, obtained with the 10-ns light source, and the following points, obtained with the Nanopulser, is broken because the different light sources could result in intensity errors due to alignment differences. To minimize such errors, the sample volume projected on the entrance slit of the spectrometer was substantially smaller than both the probe beam size and the photolysis beam size.

DISCUSSION

Assignments. The 760-nm line is present in deoxyhemoglobin and deoxymyoglobin but absent in the six-coordinate forms of these proteins, in which there is no spectral structure in this region (Eaton & Hofrichter, 1981). Thus, it serves as a reliable marker of the change from six- to five-coordination. In a study of the optical properties of hemoglobin, Eaton et al. (1978) assigned the 760-nm line as a charge-transfer transition between the porphyrin π system and the iron π system [$a_{2u}(\pi) \rightarrow d_{yz}$]. This assignment was based on circular dichroic, magnetic circular dichroic, and single-crystal polarized absorption data. In a later study by Makinen and Churg (1983) on myoglobin, the line was assigned as a $d_{x^2-y^2} \rightarrow e_g(\pi^*)$ transition on the basis of polarization data. Resolution between these different interpretations will be important for the eventual determination of the molecular mechanism of the shifts that are seen under certain conditions in the photoproducts. At present it is sufficient to simply note that the line is a charge-transfer transition.

Hemoglobin vs. Myoglobin. The behavior of the 760-nm line at cryogenic temperatures in both hemoglobin (Hb) and myoglobin (Mb) was originally described by Iizuka et al. (1974). They reported that the line shifted from 758 nm in deoxymyoglobin to 772 nm in its photoproduct and from 750 nm in deoxyhemoglobin to 772 nm in its photoproduct. Shifts in the 760-nm line in myoglobin at low temperature have more recently been confirmed by Ansari et al. (1985) and by Fiamingo and Alben (1985), although in both cases the shifts were somewhat smaller than originally reported by Iizuka et al. (1974).

The 6-nm shift that we detect in hemoglobin with 10-ns pulses is of the same order as that observed in myoglobin in recent low-temperature studies (Ansari et al., 1985; Fiamingo & Alben, 1985). In addition, for this size of a shift in Gaussian lines that are ~30 nm wide, we would predict a separation between the peak and the valley in the difference spectrum of ~25 nm, which is the same as our observed value within the experimental uncertainty. The room temperature values for hemoglobin thereby suggest that the same unrelaxed structure is present in the room temperature photoproduct as in the low-temperature photoproduct.

Our observation that there is no shift in the 760-nm line in myoglobin but a significant shift in hemoglobin in the comparison between the photoproducts and the corresponding deoxy preparations at 10 ns is consistent with a large body of other spectroscopic data. The optical absorption spectra (Henry et al., 1983b) and the resonance Raman spectra (Findsen et al., 1985b; Rousseau & Argade, 1986) of the myoglobin photoproduct also demonstrate that on the 10-ns time scale the photoproduct has fully relaxed. In contrast, by the same techniques (Henry et al., 1983a; Hofrichter et al., 1983; Scott & Friedman, 1984; Friedman, 1985), it has been found that the hemoglobin photoproduct does not relax on this time scale. This difference in relaxation behavior of these two similar proteins has been rationalized as a consequence of the difference in the F-helix displacement upon going from the six-coordinate state to the five-coordinate deoxy state in these two proteins (Dasgupta et al., 1985; Sassaroli et al., 1986). The displacement is ~1 Å in hemoglobin (Baldwin & Chothia, 1979) but is smaller in myoglobin (Takano, 1977; Phillips, 1980). Since the heme is linked to the F-helix by the proximal histidine, such a difference in the extent of F-helix motion between the two proteins is expected to result in significant differences in the time scales of relaxation at the heme.

Alternatively, there may be a significant change in the protein dynamic freedom in comparing monomeric myoglobin to tetrameric hemoglobin, or tertiary structure differences in the heme pockets could be significant. One or both of these factors could make it much more difficult for the hemoglobin to relax from its ligand bound to its deoxy structure. Experiments on isolated hemoglobin subunits should clarify this point.

Origin of Wavelength Changes. The above arguments imply that the histidine–heme conformation is involved in the optical changes reported here. Since the proximal histidine links the F-helix to the heme, this is a reasonable possibility in that the 760-nm optical transition is a charge-transfer transition be-

tween the porphyrin orbitals and the d orbitals of the iron atom. Changes in the properties of the iron-histidine bond would affect the energies of the iron d orbitals.

Changes in the histidine–heme conformation have been invoked to explain the frequency shifts in certain modes of the resonance Raman spectra of both the hemoglobin (Freidman et al., 1982; Freidman, 1985) and the myoglobin (Sassaroli et al., 1986) photoproducts. In the resonance Raman data at cryogenic temperatures, it has been found that in both hemoglobin (Ondrias et al., 1983b; Rousseau & Ondrias, 1985) and myoblogin (Sassaroli et al., 1986) the iron–histidine stretching mode is shifted to higher frequency in the photoproduct as compared to the deoxy preparation. A similar shift has been detected with 10-ns pulses at room temperature in hemoglobin (Friedman, 1985), but no shifts were detected in the frequency of this mode in myoglobin in 10-ns and in 35-ps experiments (Findsen et al., 1985b).

The observed shifts in the iron–histidine stretching mode in the resonance Raman spectra of hemoglobin and myoglobin have been attributed to the difference between the histidine tilt angle in the deoxy preparations and that in the six-coordinate species (Friedman et al., 1982; Ondrias, 1983b; Rousseau & Ondrias, 1985; Friedman, 1985; Sassaroli et al., 1986). When hemoglobin or myoglobin is six-coordinated, the heme is planar and the proximal histidine takes on an orientation perpendicular to the heme plane. In the five-coordinate case the iron moves to an out-of-plane position and the proximal histidine becomes tilted with respect to the heme plane normal. The motion of the histidine requires motion of the F-helix (to which the histidine is linked) with respect to the heme plane. Consequently, in the photoproduct studied under conditions in which the protein is not allowed to relax, an intermediate is generated in which the iron atom has moved to an out-of-plane position but the histidine has not yet tilted. This orientation is more favorable for the iron–histidine bond strength than the tilted case, where the bond is slightly destabilized by the repulsive interactions between the histidine and the heme. A higher frequency (stronger bond) is the consequence of the more favorable orientation. We propose that the change in the histidine–heme conformation, in addition to being responsible for the changes in the resonance Raman spectrum, is also responsible for the changes in the optical absorption spectrum reported here. Changes in the histidine–heme orientation would no doublt influence the iron d orbitals. Thus, this interpretation of the origin of the wavelength changes is compatible with either assignment of the charge-transfer transition, since both involve d orbitals.

Time-Dependent Recombination Rates and Structural Changes. There have been several reports of ligand rebinding in hemoglobin and myoglobin at both room temperature and cryogenic temperatures. The reported time dependence of ligand rebinding in both hemoglobin and myoglobin at room temperature is summarized in Figure 6. Two systems were well studied recently in hemoglobin: the rebinding of O_2 (Scott & Friedman, 1984) and the rebinding of CO (Hofrichter et al., 1983; Henry et al., 1983b). Several observations about these data may be made: (1) the qualitative features of O_2 and CO rebinding in hemoglobin are the same. (2) The time dependence of the rebinding in hemoglobin is characterized by rapid recombination (possibly exponential) between 10^{-8} and 10^{-7} s. This is followed by a time period with very little rebinding. At longer times, 10^{-2}–10^{-5} s, ligand binding resumes. (3) Myoglobin rebinding with CO does not have a significant amount of the early (10^{-8}–10^{-7} s) process that hemoglobin has. The early rebinding in hemoglobin has been

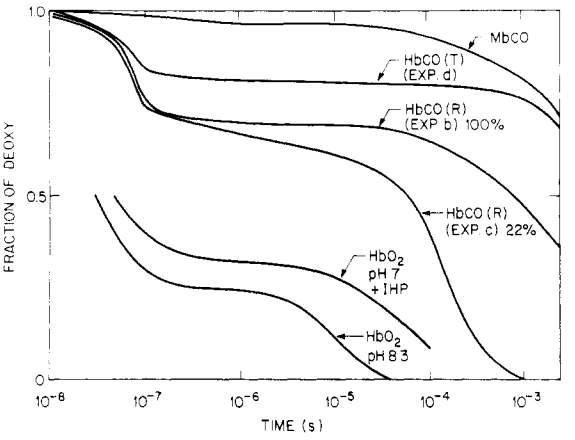

FIGURE 6: Ligand rebinding as a function of time for MbCO (Henry et al., 1983b), HbCO under various conditions (Hofrichter et al., 1983), and HbO_2 (Scott & Friedman, 1984). The labels for the photolyzed HbCO experiments were taken from the original reference (Hofrichter et al., 1983): experiment b, hemoglobin sample fully saturated and 100% photolyzed; experiment c, sample fully saturated but only 22% photolyzed; experiment d, sample only 10% saturated and 100% photolyzed. The partial saturation allows the hemoglobin to stay in the T (low-affinity) structure.

attributed to geminate recombination of ligand molecules that have not escaped from the heme pocket (Hofrichter et al., 1983). The fact that this binding process does not continue and lead to full saturation is presented as evidence for diffusion of the ligand away from the heme pocket. At longer times, bimolecular recombination accounts for the eventual resaturation of the protein.

Attempts to establish a correlation between heme structural parameters and the rebinding processes have not met with success due to the difficulties associated with obtaining reliable time evolution data from structure-sensitive optical transitions. However, it appears also difficult to obtian a structural correlation from the resonance Raman data because the time dependences of the various structure-sensitive lines do not agree with each other (Rousseau & Friedman, 1987). The qualitative similarity of the two curves in Figures 4 and 5 to those in Figure 6 points to the need for additional experiments on the 760-nm line obtained with a higher density of points. A second laser currently being set up in our laboratory, replacing the xenon lamp, will make such measurements possible.

Ansari et al. (1985) studied the time dependence of the 760-nm line in the myoglobin photoproduct at cryogenic temperatures. They observed that the decay of the line position in the myoglobin photoproduct at low temperature was not exponential in time. The changes in frequency and intensity of the charge-transfer transition we report here for hemoglobin are also nonexponential. The processes are no doubt more complicated though because hemoglobin is known to have a more complex relaxation pathway than myoglobin. In addition to the study of the time dependence of the wavelength position of the 760-nm line after photodissociation as a function of temperature by Ansari et al. (1985), recombination data as a function of time and temperature have also been reported for MbCO (Ansari et al., 1986). In Figure 7, we present data for the time dependence of the wavelength shift of the 760-nm line for myoglobin subsequent to CO photolysis (Ansari et al., 1985) and also for the recombination as a function of time for various temperatures (Doster et al., 1982). It is clear from these data that the time constant for recombination is faster than the time constant for the structural relaxation. Thus, from these data there is no obvious relationship between the structural relaxation and the CO recombination.

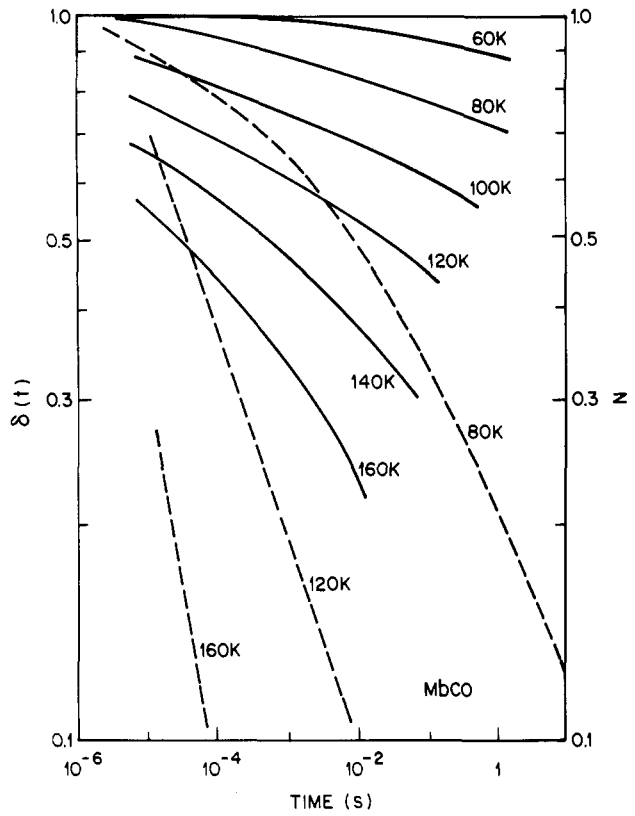

FIGURE 7: Structural change of the heme in low-temperature myoglobin after photodissociation determined from the position of the 760-nm line (solid lines, left axis) from Ansari et al. (1985) and recombination of CO with myoglobin after photodissociation (dashed lines, right axis) from Doster et al. (1982).

We must conclude from the low-temperature experiments on myoglobin that the processes which occur in myoglobin at cryogenic temperatures differ from those that occur in hemoglobin at room temperature. We do not find this especially surprising since the relaxation properties of hemoglobin and myoglobin are very different at room temperature (Sassaroli et al., 1986; Rousseau & Friedman, 1987). It would be interesting to follow the relaxation of the 760-nm line and the CO rebinding at cryogenic temperatures in hemoglobin in order to determine if the differences discussed above result from differences between hemoglobin and myoglobin or differences between room temperature and low-temperature relaxation processes.

Mechanism for Correspondence between Recombination and Relaxation. In the data reported here for hemoglobin at room temperature, there is a correspondence between the recombination and the conformational relaxation. It is also noteworthy that in myoglobin, in which the structural relaxation occurs very rapidly, as evidenced by no differences between optical properties of the photoproduct and the deoxy species at 10 ns, no significant early recombination takes place (in the 10^{-8}–10^{-6} s range as seen in hemoglobin). Taken together, these results indicate that there is a strong correlation between the conformational relaxation process and the CO recombination. There are three mechanisms by which this correlation could occur.

One possibility is that in hemoglobin it could be more difficult for CO to leave the heme pocket than in myoglobin and the presence of CO could inhibit any structural relaxation (Hofrichter et al., 1983, 1985). In this scenario CO would have to escape from myoglobin on a time scale shorter than ~1 ns so that the heme would not be prevented from relaxing to its deoxy conformation by the distal pocket. In hemoglobin, CO would remain in the pocket, preventing any relaxation until 10^{-8}–10^{-7} s. On that time scale, in some molecules geminate recombination would occur (Figures 5 and 6) and in others CO would escape, allowing partial relaxation. Additional protein constraints must be hypothesized that prevent full relaxation at the longer times. For this case, the correspondence between the conformational relaxation and the geminate recombination results from the presence of CO in the heme pocket.

A second possibility to account for the observed correspondence would have the conformational change controlling the recombination rate (Hofrichter et al., 1985; Friedman, 1985; Rousseau & Friedman, 1987). In myoglobin the conformational relaxation would occur very quickly, reaching a state of the heme with a significant barrier for recombination. Consequently, even though CO may be present within the pocket, recombination would not readily occur because of the high barrier. In hemoglobin the conformational relaxation would be postulated to be slower, so the recombination would proceed (in the 10^{-8}–10^{-7} s region) until the relaxation is complete. For this possibility, the presence of CO would not influence the structural relaxation rate and would presumably diffuse away from the heme on the 10^{-7}–10^{-6} s time scale.

To account for the correspondence between the recombination rate and the conformational change, the above two models implicitly assume that there is a homogeneous population of molecules. However, in low-temperature studies on myoglobin there is substantial evidence that the population is heterogeneous (Frauenfelder, 1983). For myoglobin at room temperature a rapid interconversion among the heterogeneous substates occurs. Thus, a third possible explanation could involve an inhomogeneous population of CO-bound molecules in hemoglobin. Upon photodissociation an inhomogeneous distribution of photodissociated molecules could be generated, resulting in a composite line in the 760-nm region. Unlike myoglobin, it must be postulated that in hemoglobin the substates within this inhomogeneous population do not interconvert at room temperature over the observed time scale. Consequently, for hemoglobin, those molecules with fast rebinding kinetics would display a wavelength near 765 nm or even longer while those with slow rebinding kinetics would have the transition near the deoxyhemoglobin value (~759 nm). As a consequence, when recombination occurs, the center of the inhomogeneous line shifts from longer to shorter wavelengths. For myoglobin, rapid structural relaxation and rapid interconversion between substates are assumed in this model so that photodissociation yields a near homogeneous distribution with a 760-nm transition at the same wavelength as that in deoxymyoglobin. Recombination to this state is presumed to be very slow.

The three models proposed above to account for the data reported here are qualitatively different. In the first, the conformational change can only occur when CO diffuses out of the pocket. In the second, the conformational change determines the rebinding rate of the heme. In the third, the absorbance wavelength is a characteristic of each substate with its unique rebinding kinetics. It is clearly important to distinguish between these three models as they have strong implications concerning the structure/function relationship in hemoglobin and myoglobin. Indeed, for hemoglobin the distinction between these models is especially important in order to determine the molecular basis for cooperativity. One would hope that the resonance Raman data would be able to clarify these models. However, the Raman data are not internally consistent, making any quantitative determinations extremely

difficult (Rousseau & Friedman, 1987). The present data show a correspondence between recombination and relaxation and thereby underscore the need to resolve the uncertainties in other data so as to understand these important mechanisms.

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REFERENCES

Ansari, A., Berendzen, J., Bowne, S. F., Frauenfelder, H., Iben, I. E. T., Sauke, T. B., Shyamsunder, E., & Young, R. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5000–5004.

Ansari, A., Dilorio, E. E., Dlott, D. D., Frauenfelder, H., Iben, I. E. T., Langer, P., Roder, H., Sauke, T. B., & Shyamsunder, E. (1986) *Biochemistry 25*, 3139–3146.

Argade, P. V., Sassaroli, M., Rousseau, D. L., Inubushi, T., Ikeda-Saito, M., & Lapidot, A. (1984) *J. Am. Chem. Soc. 106*, 6593–6596.

Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., & Gunsalus, I. C. (1975) *Biochemistry 14*, 5355–5373.

Baldwin, J., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Chance, B., Fischetti, R., & Powers, L. (1983) *Biochemistry 22*, 3820–3829.

Dasgupta, S., Spiro, T. G., Johnson, C. K., Dalickas, G. A., & Hochstrasser, R. M. (1985) *Biochemistry 24*, 5295–5297.

Doster, W., Beece, D., Bowne, S. F., Dilorio, E. E., Eisenstein, L., Frauenfelder, H., Reinisch, L., Shyamsunder, E., Winterhalter, K. H., & Yue, K. T. (1982) *Biochemistry 21*, 4831–4839.

Eaton, W. A., & Hofrichter, J. (1981) *Methods Enzymol. 76*, 175–261.

Eaton, W. A., Hanson, L. K., Stephens, P. J., Sutherland, J. C., & Dunn, J. B. R. (1978) *J. Am. Chem. Soc. 100*, 4991–5003.

Fiamingo, F. G., & Alben, J. O. (1985) *Biochemistry 24*, 7964–7970.

Findsen, E. W., Friedman, J. M., Ondrias, M. R., & Simon, S. R. (1985a) *Science (Washington, D.C.) 229*, 661–665.

Findsen, E. W., Scott, T. W., Chance, M. R., Friedman, J. M., & Ondrias, M. R. (1985b) *J. Am. Chem. Soc. 107*, 3355–3357.

Frauenfelder, H. (1983) in *Structure and Dynamics: Nucleic Acids and Proteins* (Clementi, E., & Sarma, R. H., Eds.) pp 369–376, Adenine, Guilderland, NY.

Friedman, J. M. (1985) *Science (Washington, D.C.) 228*, 1273–1280.

Friedman, J. M., Rousseau, D. L., Ondrias, M. R., & Stepnoski, R. A. (1982) *Science (Washington, D.C.) 218*, 1244–1246.

Henry, E. R., Hofrichter, J., Sommer, J. H., & Eaton, W. A. (1983a) in *Photochemistry and Photobiology* (Zewail, A., Ed.) pp 791–810, Harwood Academic, New York.

Henry, E. R., Sommer, J. H., Hofrichter, J., & Eaton, W. A. (1983b) *J. Mol. Biol. 166*, 443–451.

Hofrichter, J., Sommer, J. H., Henry, E. R., & Eaton, W. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2235–2239.

Hofrichter, J., Henry, E. R., Sommer, J. H., Deutsch, R., Ikeda-Saito, M., Yonetani, T., & Eaton, W. A. (1985) *Biochemistry 24*, 2667–2679.

Iizuka, T., Yamamoto, H., Kotani, M., & Yonetani, T. (1974) *Biochim. Biophys. Acta 371*, 126–139.

Makinen, M. W., & Churg, A. K. (1983) in *Iron Porphyrins* (Lever, A. B. P., & Gray, H. B., Eds.) Part I, Chapter 3, pp 141–235, Addison-Wesley, Reading, MA.

Ondrias, M. R., Friedman, J. M., & Rousseau, D. L. (1983a) *Science (Washington, D.C.) 220*, 615–617.

Ondrias, M. R., Rousseau, D. L., & Simon, S. R. (1983b) *J. Biol. Chem. 258*, 5638–5642.

Phillips, S. E. V. (1980) *J. Mol. Biol. 142*, 531–554.

Powers, L., Sessler, J. L., Woolery, G. L., & Chance, B. (1984) *Biochemistry 23*, 5519–5523.

Rousseau, D. L. (1981) *J. Raman Spectrosc. 10*, 94–99.

Rousseau, D. L., & Ondrias, M. R. (1985) *Biophys. J. 47*, 537–545.

Rousseau, D. L., & Argade, P. V. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1310–1314.

Rousseau, D. L., & Friedman, J. M. (1987) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Wiley, New York (in press).

Sassaroli, M., Dasgupta, S., & Rousseau, D. L. (1986) *J. Biol. Chem. 261*, 13704–13713.

Scott, T. W., & Friedman, J. M. (1984) *J. Am. Chem. Soc. 106*, 5677–5687.

Takano, T. (1977) *J. Mol. Biol. 110*, 537–568.