Picosecond study of the near infrared absorption band of hemoglobin after photolysis of carbonmonoxyhemoglobin

Robert C. Dunn and John D. Simon
Department of Chemistry, University of California at San Diego, La Jolla, California 92093-0341 USA

ABSTRACT Picosecond absorption spectroscopy is used to examine the position and band shape of the near infrared absorption band of hemoglobin as a function of time after the photodissociation of CO from carbonmonoxyhemoglobin. For the earliest delay time probed, 35 ps, the peak of the transient spectrum is at 765 nm, red shifted by 6 nm from that characteristic of equilibrium deoxyhemoglobin. No evolution in either the peak position or band shape is observed for time delays up to 60 ns. In addition, the position and shape of the spectrum are independent of photolysis energies ranging from 15 μJ/pulse to 150 μJ/pulse, spanning conditions under which the photon/heme ratio is varied from 0.01 to 2.0. This indicates that the geometry in the heme group is unrelaxed and that equilibration of the surrounding protein structure occurs on a time scale longer than 60 ns.

INTRODUCTION

Hemoglobin, the protein responsible for carrying oxygen from the lungs to the outer tissues, has been the subject of much interest due to its unique ability to cooperatively bind ligands. Since the crystal structures for the oxy and deoxy forms were reported by Perutz (Perutz, 1970; Perutz and Ten Eyck, 1972), the basis of the cooperativity was thought to be structural, regulated by two quaternary structures referred to as the R (relaxed) and T (tense) states, respectively. This led to the further development of the two-state allosteric model of ligand binding (Monod et al., 1965) in which Hb can exist in a high ligand affinity state with fully liganded quaternary structure (R) or a low ligand affinity state with fully unliganded quaternary structure (T). This approach predicts that ligand affinity is expected to be affected only by the quaternary structure and not necessarily by the number of ligands bound.

In the past decade, much work has centered on the question of how ligand binding at the hemes and the associated tertiary structural changes can initiate the large scale movements of the α and β subunits involved in the R to T transition. There are several interesting structural changes associated with the transition from the oxy to the deoxy form including a movement of the iron (II) out of the heme plane, a doming of the porphyrin ring, a tilting of the proximal histidine accompanied by a movement of the F-helix to which it is bound, and a quaternary structural change in the relation between the α and β subunits (Perutz, 1970; Perutz and Ten Eyck, 1972). The basis for the communication between the four binding sites (Warshel, 1977) and hence the dynamics of the above structural changes is still poorly understood and a number of transient spectroscopic techniques have been employed to help unravel the events after photodissociation for a large variety of ligands (for a review see Hochstrasser and Johnson, 1988).

Considering the picosecond to millisecond time range, five relaxational processes have been identified from detailed transient absorption experiments on the photodissociation of CO from carbonmonoxyhemoglobin. After dissociation, geminate recombination and a competing tertiary structural change occur at ~50–100 ns (Shank et al., 1976; Greene et al., 1978). A second tertiary change occurs between ~0.5 and 1 μs after ligand loss (Hofrichter et al., 1983). The transition from the R to T quaternary structure takes place at ~20 μs (Hofrichter et al., 1983) and, finally, bimolecular binding of the CO ligand to the R and T forms occurs at ~200 μs and ~10 ms, respectively (Ackers and Johnson, 1981). Interestingly, unlike that reported for the oxygenated species (Chernoff et al., 1980), no picosecond geminate recombination is observed in carbonmonoxyhemoglobin (Shank et al., 1976; Greene at al., 1978; Chernoff et al., 1980) which makes it a useful model for studying the long time relaxational processes of hemoglobin following photodissociation of its ligands.

Time-resolved resonance Raman work has concentrated on the iron-histidine stretching mode as this vibrational mode is believed to be sensitive to the tilting of the proximal histidine (Friedman et al., 1982, 1983). It has been shown that after photodissociation, the fre-

Dr. Simon is a Camille and Henry Dreyfus Teacher-Scholar 1990–1995.
Address correspondence to Dr. Simon.

0006-3495/91/10/884/06 $2.00

quency of $\nu_{Fe\text{-}His}$ is shifted to a higher value than that of equilibrium $Hb(CO)_4$ (Friedman et al., 1982, 1983). This elevated state remains constant for several nanoseconds after which it evolves toward the equilibrium deoxy value on the microsecond time scale (Scott and Friedman, 1984). It is thought that the tilting of the His weakens the Fe-His bond which is manifested by a shifting of the frequency of the $\nu_{Fe\text{-}His}$ band, providing a convenient marker for examining the dynamics of the restructuring of the histidine residue (Scott and Friedman, 1984). There are, however, disagreements concerning this interpretation of the observed spectral shifts. It has been pointed out that the tilt of the His in Mb and Hb is 11° and 7°, respectively; yet the frequency of this band is lower in Hb suggesting that the Fe-His bond is weaker in Hb, in disagreement with the above suggestion that the tilt angle determines the stretching frequency (Fermi and Perutz, 1981). One proposal which addresses this discrepancy invokes that a rotation of the imidizole ring occurs in conjunction with the tilting motion (Bangcharoenpaurpong et al., 1984). Another proposed mechanism to account for the $\nu_{Fe\text{-}His}$ shift invokes that it is simply a result of the movement of the iron out of the heme plane which pushes on the histidine from one side while this motion is resisted by the protein backbone (Spiro et al., 1990). This would lead to an initial compression of the bond, raising the stretching frequency from the equilibrium value; this compressed structure would relax as the protein backbone moves and would be accompanied by the expected change in $\nu_{Fe\text{-}His}$.

The resonance Raman spectrum in the 1,400–1,650 cm^{-1} region has also received considerable attention due to the sensitivity of this region to the porphyrin core size (Terner et al., 1981; Dasgupta and Spiro, 1986). Probing with 30 ps (Terner et al., 1981) and 7 ns (Dasgupta and Spiro, 1986) pulses, frequencies in this region for the initially formed photoproduct are observed to be downshifted from that of the equilibrium deoxy frequencies, indicating an expanded prophyrin core. These shifts are observed to relax to equilibrium within 0.3 μs (Stein et al., 1982). This expanded core is thought to result from the expansion in the Fe(II) in going from the low spin oxy to the high spin deoxy state and restraints of the protein backbone on the movement of the Fe(II) out of the heme plane (Dasgupta and Spiro, 1986). These results indicate that additional markers sensitive to the tertiary relaxation in the vicinity of the heme are needed to elucidate the structural relaxation dynamics of the protein.

Along these lines, there is a weak absorption band in the near infrared at ~ 760 nm which has been used by several workers to study the tertiary structural changes taking place at the heme (Iizuka et al., 1974; Campbell et al., 1987; Chavez et al., 1990). Present in only five-coordinate hemes, this absorption band, commonly referred to as band III, has been assigned to a charge-transfer band between the prophyrin π-system and the iron ($a_{2u}(\pi) \rightarrow d_{yz}$) (Eaton et al., 1978). The position of the charge-transfer band is sensitive to the relative orientation of the iron and the His which gives insight into the nature of the protein dynamics in the vicinity of the heme. Low temperature studies by Iizuka et al. (1971) report that band III is red shifted by 11 nm in the frozen photoproduct as compared with the equilibrium deoxy value of 759 nm (Iizuka et al., 1974). In 1987, the time-resolved behavior of this band at room-temperature was reported by Sassaroli and Rousseau (Sassaroli and Rousseau, 1987). These workers reported that band III was red-shifted by 6 nm from the deoxy value at 10 ns (the time resolution of the experiment) after photodissociation. However, between 10 ns and 100 μs, the band shifted to approximately that characteristic of equilibrated deoxyhemoglobin. In an interesting comparison, these authors found that in the case of the photolysis of carbonmonoxymyoglobin, band III had fully relaxed by 10 ns. These observations agree with other resonance Raman and transient absorption work which show a much faster relaxation for myoglobin. In fact, in the case of myoglobin, recent results from our lab indicate that band III relaxes to that characteristic of equilibrium Mb within 35 picoseconds after the photolysis of MbCO (Xie and Simon, 1991). This comparison strongly suggests the conclusion that band III lends insight into the dynamics of tertiary structural changes in the vicinity of the heme ring. In comparing the results of Sassaroli and Rousseau to the low temperature matrix work, the transient spectrum at 10 ns is blue shifted 5 nm from that observed in the matrix. This result indicates that there may be subnanosecond relaxation processes which affect the position of band III. The present study addresses this question by examining the evolution of band III from 35 ps to 60 ns after the photodissociation of carbonmonoxyhemoglobin.

EXPERIMENTAL

Human hemoglobin (Sigma Chemical Co., St. Louis, MO) was prepared in a neutral tris-bis buffer at concentrations ranging from 0.1 to 0.8 mM. The sample was sealed in a rotating sample cell of 2 mm pathlength.

The laser system consisted of a mode-locked, Q-switched, cavity-dumped Nd:YAG. This system, described in detail elsewhere (Xie and Simon, 1989), was used in two different configurations to collect the transient spectra reported.

In one configuration, the YAG oscillator was used to

synchronously pump two dye lasers. One dye laser was operated using rhodamine-6G, providing photolysis pulses at 570 nm (35 ps FWHM, ≤ 25 μJ/pulse). The second dye laser was operated without any intracavity tuning elements, using a mixture of LDS dyes to enable covering the desired spectral range. These broad band probe pulses were passed through a computer-controlled double subtractive monochrometer, focussed on the sample and detected by a PMT. The signal from the PMT was amplified, processed by a track-and-hold circuit (Stanford Research) and detected by a lock-in amplifier (EG&G 5209). The output of the lock in was digitized by an IMB-PC/AT computer. The photolysis beam was chopped at 500 Hz, half the repetition rate of the laser. The time resolution of this experiment was 35 ps.

To perform the power dependent studies, in addition to pumping the probe dye laser, the YAG oscillator was electro-optically cavity dumped at the peak of the Q-switched envelope; the output pulses were doubled to 532 nm (80 ps FWHM, 150 μJ/pulse) and used for photolysis. In this configuration, the time resolution of the experiment was 80 ps, limited by the duration of the photolysis pulses.

RESULTS

Fig. 1 shows the transient spectrum obtained at 35 ps and 60 ns after photolysis. Spectra were recorded at various other time delays between these two limiting values (data not shown). Several spectra were also collected at varying sample concentrations (0.1–0.8 mM) and pump energies (15–150 μJ) to examine the effect of partial photolysis on the dynamics of band III. We find that at 35 ps the band maximum is located at 765 nm, red-shifted by ~6 nm from that characteristic of equilibrated deoxyhemoglobin, $\lambda_{max} = 759$ nm. Under all experimental conditions studied no evolution of the band maximum nor any changes in the band shape were observed.

DISCUSSION

As mentioned earlier, Eaton et al. (Eaton et al., 1978) have assigned band III to a charge transfer between the porphyrin π-system and the d_{yz} orbital of the iron ($a_{2u}(\pi) \rightarrow d_{yz}$). Furthermore, photolysis of $HbCO_4$ at low temperature reveal that band III in photogenerated Hb has an absorption maximum of ~770 nm which, upon heating, relaxes to the equilibrium deoxy value of ~759

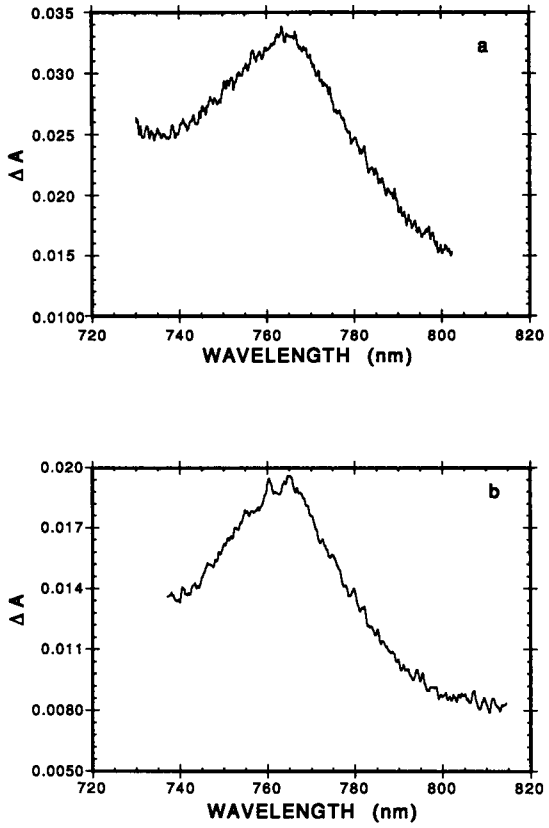

FIGURE 1 Transient absorption spectra of band III observed (*a*) 35 ps and (*b*) 60 ns after photodissociation of $Hb(CO)_4$.

nm. This spectral shift has been interpreted in terms of structural changes which take place in the immediate vicinity of the heme following ligand loss. In the report by Sassaroli and Rousseau (Sassaroli and Rousseau, 1987), 10 ns after photolysis of HbCO at room temperature, band III is found to be red shifted by 6 nm, exhibiting an absorption maximum at 765 nm. These workers found that the absorption band relaxed to ~763 nm in 50 ns and then changed slowly out to 100 μs where it became too weak to follow (Sassaroli and Rousseau, 1987). In the present study, immediately after photolysis (35 ps), the band maximum is located at 765 nm; however, in disagreement with the earlier report by Sassaroli and Rousseau (Sassaroli and Rousseau, 1987), no movement of the band maximum is observed between 35 ps and 60 ns. The fact that we see no evolution from 35 ps to 10 ns is not surprising. Transient absorption studies on the Soret region (Greene et al., 1978; Chernoff et al., 1980) and resonance Raman work on the $\nu_{Fe\text{-}His}$ stretch (Terner et al., 1980, 1981; Friedman et al., 1982; Findsen et al., 1985) all show no structural relaxation in this time domain. In the remainder of this

section, two issues will be examined. First, possible reasons for the differences between the present data on band III and those observed in previous nanosecond studies are examined. Second, the possible involvement of relaxation processes occurring on a time scale faster than our resolution (35 ps) is discussed.

Band III is a strongly forbidden transition ($\epsilon \sim 150$) which has hindered the study of its dynamics on the picosecond time scale. Using the above described absorption apparatus, the maximum of band III can easily be resolved to within 2 nm. By using nonlinear least squares, and a basis set of 200 polynomials, the tops of the absorption spectra (~80–100% of the total signal) can be expressed analytically. Examining the derivatives of these functions results in a determination of the band maximum to better than ±0.5 nm. The accuracy of this method was determined by fitting generated spectra of varying resolution whose maximum are determined by an input parameter.

Unlike previous time-resolved studies, we have not attempted to subtract out the contribution from the Q-band in this spectral region. The maximum of band III is taken to be that wavelength with the largest total absorbance. Depending on the shape of the underlying tail of the Q-band absorption, the spectra of band III could change both in band shape and maximum. However, since the time evolution of the Q-band is not independently known, no adjustments to the experimental spectra were made.

A second major difference between the present work and previous studies is the photolysis energies used to excite the sample. Sassaroli and Rousseau (Sassaroli and Rousseau, 1987) used photolysis energies which were ~3 orders of magnitude greater than those in the present study. This difference in laser fluence could lead to different dynamics through several mechanisms. First, the spectral shift observed by these workers on the tens of nanosecond time scale may be due to the effects of complete photolysis (loss of four COs from each $Hb(CO)_4$ molecule) versus incomplete photolysis (loss of one, two, or three COs from each $Hb(CO)_4$ molecule). The observation that the transient data reported in this study were not dependent on photolysis energy suggest that this is not the case. In particular, by varying the heme concentration (0.1–0.8 mM) as well as the photolysis energy (150–15 μJ/pulse), conditions where the photon(s) to heme ratios ranged from ~2.0 to ~0.01 were produced, all of which showed no effects on band III. Thus, under conditions of complete and partial photolysis, no differences in bandshape or band positions are observed on the picosecond and nanosecond time scale. Unfortunately, we could not produce energies comparable to those used by Sassaroli and Rousseau, however, at such higher power fluences, multiphoton effects need to be considered. Secondly, at high power fluences, coherent artifacts due to the incomplete depolarization of the pump and probe pulses may lead to artificial shifts in the transient spectra. In the present study, both the pump and probe beams were passed through depolarizers. In addition, the pump beam was further passed through a rotating half-wave plate to randomize any residual linear components. When the beams were left polarized, artificial shifts in the position of the transient spectra due to coherent artifacts were seen in the present study.

One important question which remains is whether or not band III exhibits any spectral relaxation on a time scale faster than 35 ps. In principle, one might expect that upon photolysis, band III would shift from 770 nm (observed in the low temperature matrix work) to 759 nm. Observation of a λ_{max} of 765 nm at 35 ps suggests that there is either a fast relaxation process that is not detected in the present study or that the matrix distorts the protein structure of $Hb(CO)_4$, thereby causing the charge transfer absorption to be observed at 770 nm upon photolysis. In comparing the primary photoproducts absorption spectra in room temperature solution to that observed in matrices, one must be careful to consider the effects on the optical spectra. Temperature dependent steady state studies show that for both oxy and deoxyhemoglobin, all of the optical transitions in the region from 350–1,350 nm shift and narrow as the temperature is decreased (Cordone et al., 1986; Leone et al., 1987). In particular, band III blue shifts with decreasing temperature (Cordone et al., 1986). These experimental observations support the conclusion that the difference in the matrix photoproduct adsorption ($\lambda \sim 770$ nm) and that observed at 35 ps in solution ($\lambda \sim 765$ nm) does not result from temperature effects on the optical transitions. Although a definitive answer to this question requires femtosecond measurements on band III, insight into this issue can be gained from other complementary studies. With a time resolution of 200 fs, transient absorption studies on $Hb(CO)_4$ reported by Martin et al. (Martin et al., 1983) reveal a pulse width limited bleach of the oxy like spectrum and a slower 300 fs appearance of a dexoy like spectrum which these authors attribute to the emergence of a ground state deoxy heme with the iron displaced from the heme plane. In agreement with this assignment, Henry et al. have used molecular dynamics simulations to study the movement of an iron out of a heme plane (Henry et al., 1985). With no protein surrounding the heme, this motion takes <150 fs. However, including the protein, the initial displacement of the iron was smaller indicating that further movement was occurring well into the picosecond time scale. This conclusion is also supported

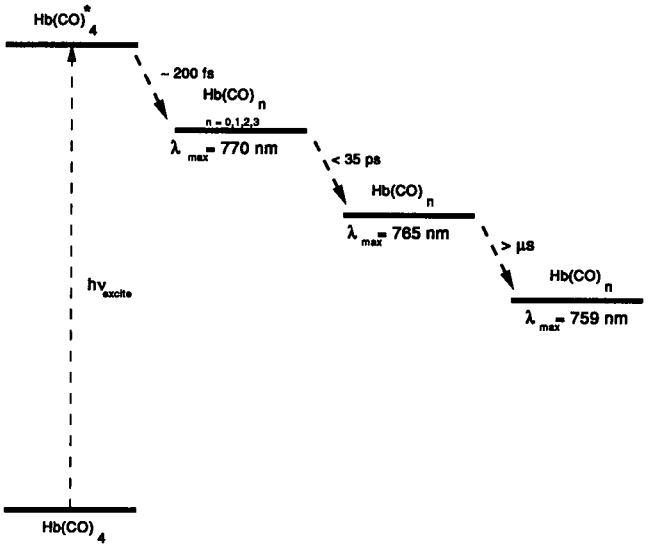

FIGURE 2 Time scales for the evolution of band III of hemoglobin after photodissociation. Immediately after ligand loss, λ_{max} = 770 nm. On the subpicosecond time scale, band III shifts to 765 nm, reflecting the motion of the iron out of the heme. No evolution in λ_{max} is observed for ~60 ns. This indicates a stable unrelaxed structure which requires microseconds to achieve equilibrium.

by recent resonance Raman work on the heme core size marker (Terner et al., 1981; Dasgupta and Spiro, 1985; Dasgupta and Spiro, 1986). Transient studies on this vibration reveal a shift in frequency which indicates an incomplete relaxation of the Fe/heme planar geometry that last for at least 20 ns. It is reasonable to associate the low-temperature band III intermediate at 770 nm with an unliganded, five-coordinated heme which is caught in a R-like structure, in which the Fe is still in the heme plane with the proximal histidine in its upright position. Thus, it is expected that on the femtosecond time scale (see Fig. 2) band III will evolve from 770 to 765 nm, the maximum of the earliest time probed in the present study.

CONCLUSIONS

Picosecond absorption studies of the photolysis of $Hb(CO)_4$ reveal that band III has an absorption maximum of 765 ± 0.5 nm from 35 ps to 60 ns after photolysis. This value is blue shifted by 5 nm from that observed in the matrix indicating that there is relaxation in the vicinity of the heme on a time scale shorter than 35 ps. However, the band is also red shifted by 6 nm from that observed for deoxyhemoglobin. This indicates that the geometry in the heme group is unrelaxed and that equilibration of the surrounding protein structure occurs on a time scale longer than 60 ns.

The authors wish to thank Dr. Xiaoliang Xie for the many helpful discussions.

This work is supported by the National Institutes of Health through grant GM-41942.

Received for publication 13 December 1990 and in final form 24 June 1991.

REFERENCES

Ackers, G. K., and M. L. Johnson. 1981. Linked functions in allosteric proteins. Extension of the concerted (MWC) model for ligand-linked subunit assembly and its application to human hemoglobins. *J. Mol. Biol.* 147:559.

Bangcharoenpaurpong, O., K. T. Schomacker, and P. M. Champion. 1984. A resonance Raman investigation of myoglobin and hemoglobin. *J. Am. Chem. Soc.* 106:5688–5698.

Campbell, B. F., M. R. Chance and J. M. Friedman. 1987. Linkage of functional and structural heterogeneity in proteins: dynamic hole burning in carboxymyoglobin. *Science (Wash. DC).* 238:373–376.

Chavez, M. D., S. H. Courtney, M. R. Chance, D. Kiula, J. Nocek, B. M. Hoffman, J. M. Friedman, and M. R. Ondrias. 1990. Structural and functional significance of inhomogeneous line broadening of band III in hemoglobin and Fe-Mn hybrid hemoglobins. *Biochemistry.* 29:4844–4852.

Chernoff, D. A., R. M. Hochstrasser, and A. W. Steele. 1980. Geminate recombination of O_2 and hemoglobin. *Proc. Natl. Acad. Sci. USA.* 77:5606–5610.

Cordone, L., A. Cupane, M. Leone, and E. Vitrano. 1986. Optical absorption spectra of deoxy- and oxyhemoglobin in the temperature range 300–20 K. *Biophys. Chem.* 24:259–275.

Dasgupta, S., and T. G. Spiro. 1985. Picosecond resonance Raman evidence for unrelaxed heme in the (carbonmonoxy)myoglobin photoproduct. *Biochemistry.* 24:5295–5297.

Dasgupta, S., and T. G. Spiro. 1986. Resonance Raman characterization of the 7-ns photoproduct of (carbonmonoxy)hemoglobin: implications for hemoglobin dynamics. *Biochemistry.* 25:5941–5948.

Eaton, W. A., L. K. Hanson, P. J. Stephens, J. C. Sutherland, and J. B. R. Dunn. 1978. Optical spectra of oxy- and deoxyhemoglobin. *J. Am. Chem. Soc.* 100:4991–5003.

Fermi, G., and M. F. Perutz. 1981. Atlas of Molecular Structures in Biology, to Hemoglobin and Myoglobin. Clarendon Press, Oxford.

Findsen, E. W., J. M. Friedman, M. R. Ondrias, and S. R. Simon. 1985. Picosecond time-resolved resonance Raman studies of hemoglobin: implications for reactivity. *Science (Wash. DC).* 299:661–665.

Friedman, J. M., D. L. Rousseau, M. R. Ondrias, and R. A. Stepnoski. 1982. Transient Raman study of hemoglobin: structural dependence of the iron-histidine linkage. *Science (Wash. DC).* 218:1244–1246.

Friedman, J. M., T. W. Scott, R. A. Stepnoski, M. Ikeda-Saito, and T. Yonetani. 1983. The iron-proximal histidine linkage and protein control of oxygen binding in hemoglobin. *J. Biol. Chem.* 258:10564–10572.

Greene, B. I., R. M. Hochstrasser, R. B. Weisman, and W. A. Eaton. 1978. Spectroscopic studies of oxy- and carbonmonoxyhemoglobin

afer pulsed optical excitation. *Proc. Natl. Acad. Sci. USA.* 75:5255–5259.

Henry, E. R., M. Levitt, and W. A. Eaton. 1985. Molecular Dynamics simulation of photodissociation of carbon monoxide from hemoglobin. *Proc. Natl. Acad. Sci. USA.* 82:2034–2038.

Hochstrasser, R. M., and C. K. Johnson. 1988. Biological processes studied by ultrafast laser techniques. *In* Ultrashort Laser Pulses and Applications, Topics in Applied Physics. W. Kaiser, editor. 60:357–417.

Hofrichter, J., J. H. Sommer, E. R. Henry, and W. A. Eaton. 1983. Nanosecond absorption spectroscopy of hemoglobin: elementary processes in kinetic cooperativity. *Proc. Natl. Acad. Sci. USA.* 80:2235–2239.

Iizuka, T., H. Yamamoto, M. Kotani, and T. Yonetani. 1974. Low temperature photodissociation of hemoproteins: carbon monoxide complex of myoglobin and hemoglobin. *Biochim. Biophys. Acta.* 371:126–139.

Leone, M., A. Cupane, E. Vitrano, and L. Cordone. 1987. Dynamic properties of oxy- and carbonmonoxyhemoglobin probed by optical spectroscopy in the temperature range of 300–20 K. *Biopolymers.* 26:1769–1779.

Martin, J. L., A. Migus, C. Poyart, Y. Lecarpentier, R. Astier, and A. Antonetti. 1983. Femtosecond photolysis of CO-ligated protoheme and hemoproteins: appearance of deoxy species with a 350-fsec time constant. *Proc. Natl. Acad. Sci. USA.* 80:173–177.

Monod, J., J. Wyman, and J. P. Changeux. 1965. On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* 12:88.

Perutz, M. F. 1970. Stereochemistry of cooperative effects in haemoglobin. Nature. *(Lond.).* 228:726–739.

Perutz, M. F., and L. F. Ten Eyck. 1972. Stereochemistry of cooperative effects in hemoglobin. *Cold Spring Harbor Symp. Quant. Bio.* 36:295.

Sassaroli, M., and D. L. Rousseau. 1987. Time dependence of near-infrared spectra of photodissociated hemoglobin and myoglobin. *Biochemistry.* 26:3092–3098.

Scott, T. W., and J. W. Friedman. 1984. Tertiary-structure relaxation in hemoglobin: a transient Raman study. *J. Am. Chem. Soc.* 106:5677–5687.

Shank, C. V., E. P. Ippen, and R. Bersohn. 1976. Time-resolved spectroscopy of hemoglobin and its complexes with subpicosecond optical pulses. *Science (Wash. DC).* 193:50.

Spiro, T. G., G. Smulevich, and C. Su. 1990. Probing protein structure and dynamics with resonance Raman spectroscopy: cytochrome *c* peroxidase and hemoglobin. *Biochemistry.* 29:4497–4508.

Stein, P., J. Terner, and T. G. Spiro. 1982. Hemoglobin R-state iron-imidazole frequency observed by time-resolved resonance Raman spectroscopy. *J. Phys. Chem.* 86:168–170.

Terner, J., T. G. Spiro, M. Nagumo, M. F. Nicol, and M. A. El-Sayed. 1980. Resonance Raman spectroscopy in the picosecond time scale: the carboxyhemoglobin photointermediate. *J. Am. Chem. Soc.* 102: 3238–3239.

Terner, J., J. D. Strong, T. G. Spiro, M. Nagumo, M. Nicol, and M. A. El-Sayed. 1981. Picosecond resonance Raman spectroscopic evidence for excited-state spin conversion in carbonmonoxy-hemoglobin photolysis. *Proc. Natl. Acad. Sci. USA.* 78:1313-1317.

Warshel, A. 1977. Energy-structure correlation in metalloporphyrins and the control of oxygen binding by hemoglobin. *Proc. Natl. Acad. Sci. USA.* 74:1789–1793.

Xie, X., and J. D. Simon. 1989. High energy and tunable picosecond laser pulses at 1kHz: synchronously pumping a dye laser with a mode-locked, Q-switched and cavity dumped Nd:YAG laser system. *Optics Comm.* 69:303–307.

Xie, X., and J. D. Simon. 1991. Protein conformational relaxation following photodissociation of CO from carbonmonoxymyoglobin: picosecond circular dichroism and absorption studies. *Biochemistry.* 30:3682–3692.