TIME-RESOLVED CIRCULAR DICHROISM AND ABSORPTION STUDIES OF THE PHOTOLYSIS REACTION OF (CARBONMONOXY)MYOGLOBIN

STEVEN J. MILDER,* SOFIE C. BJORLING,* IRWIN D. KUNTZ,‡ AND DAVID S. KLIGER*
*Department of Chemistry, University of California at Santa Cruz, Santa Cruz, California 95064, and
‡School of Pharmacy, University of California at San Francisco, San Francisco, California 94143

ABSTRACT Time-resolved circular dichroism (TRCD) and absorption spectroscopy are used to follow the photolysis reaction of (carbonmonoxy)myoglobin (MbCO). Following the spectral changes associated with the initial loss of CO, a subtle change is observed in the visible absorption spectrum of the Mb product on a time scale of a few hundred nanoseconds. No changes are seen in the CD spectrum of Mb in the visible and near-UV regions subsequent to the loss of CO. The data suggest the existence of an intermediate found after ligand loss from MbCO that is similar in structure to the final Mb product.

INTRODUCTION

For both myoglobin (Mb) and hemoglobin (Hb) the CO adduct is photolabile, and the high spin deligated (deoxy) form is produced upon photolysis in 350 fs (Martin et al., 1983). When HbCO is photolyzed in room temperature fluid solution, the initial Hb goes through a series of intermediates until it reaches the stable T-state deoxyHb form in ~ 20 μs. The intermediates have been followed and characterized by a number of techniques including resonance Raman (Spiro and Turner, 1983; Friedman, 1985) and unpolarized absorption spectroscopy (Reynolds and Rentzepis, 1982; Hofrichter et al., 1983). However, attempts to find similar transients in the room temperature photolysis of MbCO have met with little success. The Fe-his stretching, followed by resonance Raman spectroscopy, changes from that of MbCO to that of deoxyMb within 20 ps (Findsen et al., 1985). Time-resolved absorption spectra in the Soret region of the spectrum on the picosecond time scale suggest the irradiated MbCO may not lead immediately to the same spectrum as deoxyMb (Cornelius et al., 1981; Hutchinson and Noe, 1984). However, within a few nanoseconds, the Soret spectrum of deoxyMb is obtained (Henry et al, 1983). In a time-resolved absorption study of the MbCO photolysis reaction, a small change in the transient absorption signal was interpreted as a limited amount of geminate recombination (4%) with a lifetime of 180 ns at room temperature (Henry et al., 1983).

We recently developed a technique for measuring circular dichroism (CD) changes with nanosecond time resolution (Lewis et al., 1985). To test this technique we initially looked at CD changes at single wavelengths in the photolysis reactions of MbCO and HbCO and were able to obtain nanosecond time resolution after removing linear birefringence artifacts (Einterz et al., 1985). Here, the new time-resolved circular dichroism (TRCD) technique is applied in a detailed optical study of the photolysis reaction of MbCO.

METHODS

Time-resolved optical density (TROD) measurements were carried out using an apparatus modified slightly from one described elsewhere (Milder and Kliger, 1985; Lewis et al., 1987*b*). Excitation was with either a Quanta-Ray DCR-1 (with a 7 ns pulse) or DCR-2 (4 ns pulse) Nd:YAG laser (Spectra-Physics, Mountain View, CA) frequency doubled to produce light at 532 nm. Laser energies of 5 to 20 mJ/pulse and beam diameters of 2 to 7 mm were used. The probe beam was produced by xenon flashlamps with pulse durations of 1 to 50 μs (Lewis et al., 1987*a*) for nanosecond and microsecond measurements and by a 50 W quartz-halogen lamp for millisecond measurements. For kinetic measurements, detection was with either an EMI (Thorn EMI Gencom, Inc., Plainview, NY) D279 or 9876QP photomultiplier (with rise times < 2 ns). The photomultiplier signal was digitized and analyzed by either a 100 MHz Biomation 6500 transient recorder (Gould Inc., Santa Clara, CA) linked to a Z-80 microcomputer, or by a 500 MHz 7912AD data aquisition system (Tektronix Inc., Beaverton, OR). Spectral measurements were made by use of an optical multichannel analyzer (OMA) detection system described elsewhere (Lewis et al., 1987*b*). The OMA (PAR 1420, Princeton Applied Research, Princeton, NJ) typically operated with a 10 ns gate (using a PAR 1302 pulser) in TROD measurements. The light was dispersed with a Jarrell-Ash Monospec-27 polychromator (Aries, Inc., Concord, MA) using either 150 or 300 groove/mm gratings yielding resolutions of either 0.6 or 0.3 nm/pixel. The gate of the OMA was opened at successive times after laser excitation to produce transient spectra at specified times after the initiation of the photoreaction. To avoid artifacts, the probe pulse and OMA gate were jointly delayed in time relative to the laser rather than having the OMA gate delayed relative to the flashlamp time profile. TRCD measurements were made using a pump/probe apparatus similar to that described above, except the probe beam optics were modified as described in detail by Lewis et al. (1985). Transient linear birefringence due to photoselection by the linearly polarized laser can cause a large artifact in TRCD measurements (Einterz et al., 1985). This was avoided by orienting the

linear polarization of the pump laser to an appropriate angle (Einterz et al., 1985) and following this with a Soleil-Babinet compensator (Optics for Research, Caldwell, NJ) to circularly polarize the laser beam.

Due to the nature of the TRCD technique, the TRCD probe beam is about 10^{-4} less intense than that used in TROD measurements. Because of this low intensity and the small size of the CD signals, it was necessary to use many more averages for TRCD measurements than for TROD measurements to obtain adequate signals in the kinetic mode. For the spectral experiments, a 500-ns pulse from a PAR 1304 pulse amplifier triggered by a Dynascan pulse delay generator was used to gate the OMA (PAR 1420) for TRCD measurements while 10-ns gatewidths were used for TROD measurements.

Steady state absorption spectra were taken on a 9420 spectrophotometer (IBM Instruments Inc., Danbury, CT). Concentrations of myoglobin species were determined by using literature extinction coefficients (Rothgab and Gurd, 1978). Standard CD spectra were taken on either Jasco J-20 or J-500 spectropolarimeters. The signal sizes were standardized using (+)-10-camphorsulfonic acid, whose spectral characteristics are ϵ(285 nm) = 34.5 $M^{-1}cm^{-1}$ and $\Delta\epsilon$(290.5 nm) = +2.36 $M^{-1}cm^{-1}$ (Hennesey and Johnson, 1982).

MATERIALS

Myoglobin (Mb) was obtained from Sigma Chemical Co., St Louis, MO and in most cases was used as received. For a few experiments the Mb was purified by dialysis followed by chromatography using a CMC-52 column with a pH (6 to 7) and ionic strength (0.01 to 0.1 M) gradient. No optical differences were observed between the purified and unpurified samples. In all experiments 0.1 M, pH 7 phosphate buffer was used as solvent. After dissolving the metMb, the solution was filtered through glass wool. For experiments in spectral regions above 380 nm, the Mb solutions were made 0.01 M in sodium dithionite (Aldrich Chemical Co., Inc., Milwaukee, WI). For spectral studies below 380 nm only a small excess of sodium dithionite was added to a well deoxygenated (Ar or CO purged) solution of the metMb. In this case complete reduction was determined by observing the change in the visible absorption spectrum. During data collection for either transient OD or CD measurements the sample was flowed in a closed system at the rate of ~ 2 ml/min. It was necessary to avoid contact with air, as over time this leads to spurious signals and precipitation of some of the sample.

RESULTS

TROD Spectra

Previous time-resolved absorption studies of the photolysis reaction of MbCO have primarily followed the Soret band (380 nm to 460 nm) of the heme (Henry et al., 1983), though a recent report followed the near-IR absorption of deoxyMb centered near 760 nm (Sassaroli and Rousseau, 1987). We obtained the difference spectra resulting from laser excitation of MbCO from 250 to 800 nm for times between 50 and 2,000 ns after photolysis. Of particular interest to us was whether we could observe a transient intermediate Mb state (Mb*) which preceded the formation of steady-state deoxyMb. Previous workers reported a small decay of the signal in the Soret region with a lifetime of 180 ns and ascribed this to a small amount (4%) of geminate recombination (Henry et al., 1983). We also see a small diminution of the transient absorption near 435 nm in the first few hundred nanoseconds after photolysis. However, the evolution of the transient difference spectrum of the visible Q bands during this time is not simple (Fig. 1). In particular, at 535 nm there is reproducibly a

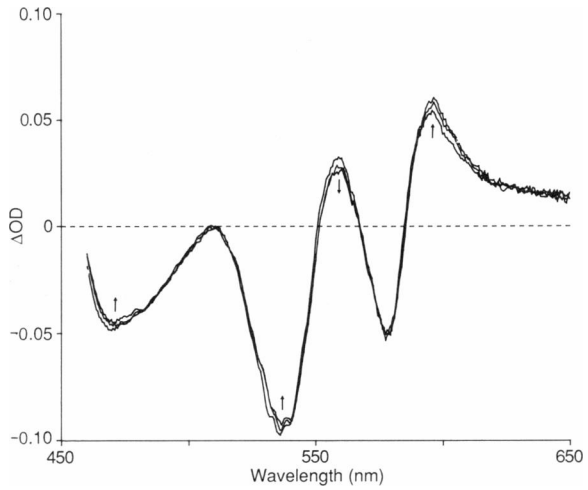

FIGURE 1 Time-resolved optical density difference spectrum of the photolysis reaction of MbCO in the 460 to 650 nm region. Each spectrum represents 128 averages. Spectra displayed were taken at 50, 500, and 2,000 ns after photolysis and changed with time as indicated by the arrows. Note the decrease in the bleaching signal near 540 nm, the decrease in the absorption signal near 560 nm and the increase in the absorption signal near 600 nm. Spectra obtained between 50 and 500 ns (not shown) show that the evolution of the changes takes place on the order of a few hundred nanoseconds.

decrease in the bleaching signal of ~5% and at 560 nm there is a decrease in the transient absorption signal of ~ 20%. However, at 600 nm there is reproducibly a small (~10%) increase in the transient absorption. Thus, the changes in the 460 to 650 nm region do not simply represent a decrease in the signal at all wavelengths during the first microsecond after the initial photolysis reaction. We attempted to measure the kinetics of these changes in the submicrosecond time regime at many different wavelengths. However, the kinetic signals were too small to measure reproducibly.

Since the absorption in the visible is only from the heme while the absorption in the 250 to 300 nm region is at least partly from the aromatic amino acids of the protein, one might expect different kinetics to be seen here. From the difference spectra obtained in the 250 to 400 nm region (not shown), it is clear that there are only subtle changes in the shape and magnitude of the difference spectra during the first microsecond after photolysis.

We also obtained spectra at different times after photolysis in the near-IR region of the spectrum. The MbCO does not absorb here so all of the signal is due to absorption of the photoproduct. For MbCO dissolved in a matrix at and below 4 K a transient form of deoxyMb, Mb* has been observed after photolysis. Mossbauer (Marcolin et al., 1979; Spartalian, et al, 1976), x-ray absorption (Chance et al., 1983), resonance Raman (Rousseau and Agarde, 1986), and near-IR absorption (Iizuka et al., 1974) all showed that this photoproduct is not the normal deoxyMb. The spectral maximum of Mb* was observed at 772 nm instead of the maximum of 758 nm observed for deoxyMb under the same conditions. After photolysis of MbCO

under the same conditions, the visible absorption was the same as that of deoxyMb. Thus, we thought that if this same Mb* state was the cause of the subtle changes in the visible difference spectra obtained in our conditions, we would see shifts in the spectra in the near-IR on a time scale of a few hundred nanoseconds. As also reported by others recently (Sassaroli and Rousseau, 1987), we observed no obvious shift in the transient absorption in this region of the spectrum of this time scale. On comparison with the spectrum of deoxyMb in this spectral region, it is clear that at our resolution the deoxyMb spectrum in the near-IR is obtained within 50 ns.

TRCD Spectra

We showed previously (Lewis et al., 1985) that we could obtain the same shape of the CD spectrum for the Q bands of MbCO as reported on a conventional instrument (Garnier, 1972). However, to obtain the magnitude of the literature values we had to multiply our CD values by 1.6. Here, we measured the CD spectra of MbCO and Mb using a conventional instrument. These spectra are shown in Fig. 2. Using (+)-10-camphorsulfonic acid as our standard, we found that the previously reported values (Bolard and Garnier, 1972; Nicola et al., 1975) for $\Delta\epsilon$ are in error by a factor of 1.6. Thus, the static spectra of MbCO and Mb we previously obtained using the TRCD technique agree quantitatively with spectra we present here measured with a standard spectropolarimeter.

The kinetics of the initial CD changes upon photolysis of MbCO as measured with the TRCD technique are shown in Fig. 3. We used the procedure described above to eliminate linear birefringence artifacts (Einterz et al., 1985). Data were collected through the visible and near-UV regions of the spectrum. At all wavelengths the CD changed as expected for the loss of MbCO and the production of Mb (see Fig. 2). Fig. 3 shows the CD changes obtained with our highest time resolution. Both in the Soret region (417.5 and 435 nm) and in the near-UV region (270 nm) the rise time of the transient is less than

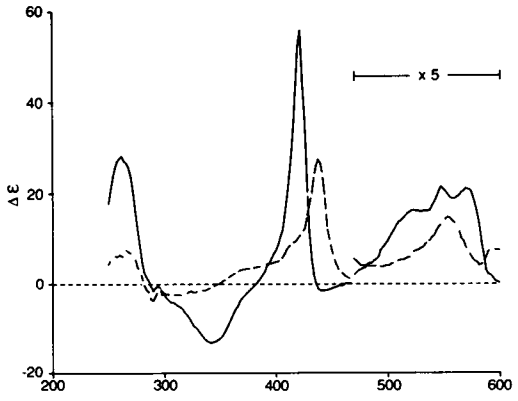

FIGURE 2 The CD spectra of MbCO and deoxyMb taken on a conventional spectropolarimeter. The displayed resolution is 2 nm.

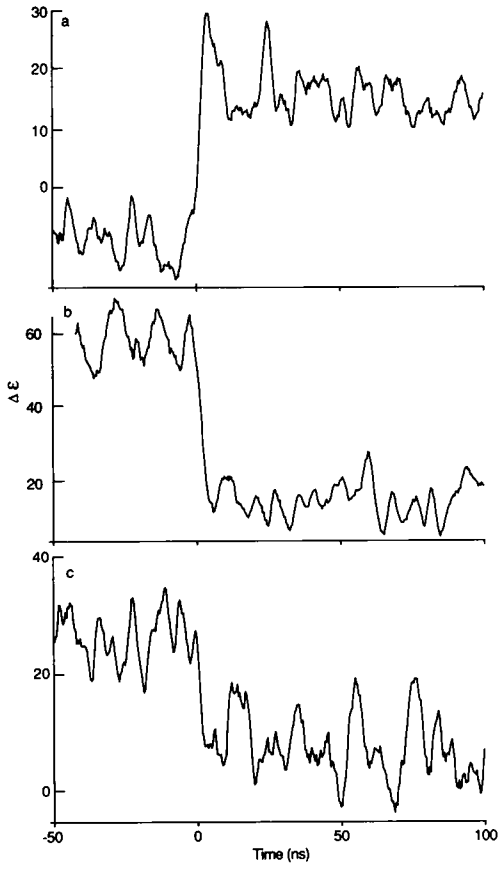

FIGURE 3 Kinetic TRCD signals obtained upon photolysis of MbCO: *a* 435 nm, 1024 averages; *b* 417.5 nm, 1,024 averages; *c* 270 nm, 6,144 averages. Upon photolysis, at all three wavelengths the CD signal changes within the time resolution of the apparatus and, within the limits of the noise, maintains a metastable value on the hundred nanosecond time scale.

the time resolution of our measurements (<5 ns). Attempts were made to obtain TRCD kinetic results in the 560 to 600 nm region, as this spectral region exhibited the largest changes in the TROD spectra on the few hundred nanosecond time scale. However, commercial myoglobin has an impurity which luminesces in this region, and prevents detection of CD changes in the first few hundred nanoseconds after excitation. Even column purified myoglobin has significant residual luminescence, presumably from a small amount of a highly emissive impurity. This made detection in this spectral region in the first 150 ns impossible, since even small amounts of luminescence can overwhelm our detection system, which is optimized to collect the low intensity probe beam.

Further experiments followed the kinetics of the CD changes in the Soret region of the spectrum over a 2 μs period. In this time regime there is no obvious change in the CD signal after the initial change during photolysis. We also obtained CD changes in the Soret band in the millisecond time regime using a CW lamp as our probe source. At the wavelengths probed (435 and 417.5 nm), the CD returned to its initial value with a first order rate,

which increased with the degree of saturation of CO in the solution. When the solution was saturated with CO at 1 atm the first order rate observed was $2 \times 10^2\ s^{-1}$. Under indentical degrees of CO saturation, TROD and TRCD signals had identical kinetics.

Previously reported TRCD spectra were obtained by measuring kinetic data at many wavelengths to determine the spectra point-by-point. This method has the advantage of having nanosecond time resolution, but is very tedious and subject to artifacts due to changes in the sample and optical elements over time. An alternative is to use a gated optical multichannel analyzer (OMA) as the probe beam detector. In this case the entire CD spectra can be obtained simultaneously at some cost to time resolution. Fig. 4 shows the CD spectra of MbCO and Mb in the 380 to 460 nm region obtained with the TRCD technique using OMA detection (with 500 ns time resolution) and compares them with the spectra obtained with a conventional CD instrument. The two sets of spectra are quite similar. The CD spectrum in the 380 to 460 nm region of the MbCO sample after photolysis is shown on Fig. 5. Here the OMA gate began 200 ns after photolysis by the laser and lasted for 500 ns. The deoxyMb CD spectrum taken on the TRCD apparatus is also shown. The spectra are similar, and any differences can probably be ascribed to noise in the TRCD spectra. The TRCD spectrum measured point-by-point in the kinetic mode (not shown) indicates that the spectrum obtained 50 ns after photolysis is also the same as that of deoxyMb.

The near-UV CD spectrum of MbCO and the spectrum

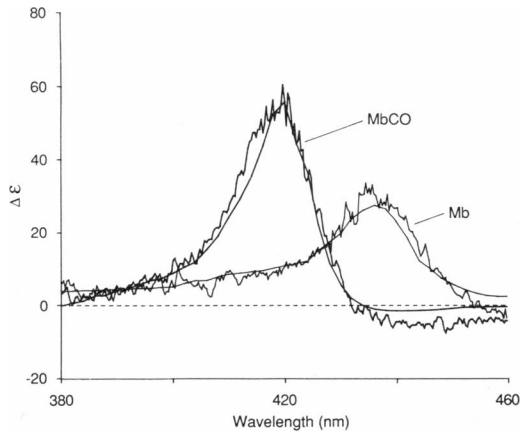

FIGURE 4 CD spectra of MbCO and deoxyMb taken on a conventional spectropolarimeter with 2 nm per point resolution (*smooth curves*). Also plotted are the CD spectra of MbCO and deoxyMb obtained using the TRCD apparatus without laser excitation (*noisy curves*). For these spectra the time gate of the OMA was 500 ns and each spectrum represents 1,024 averages. The magnitude of the conventional spectra was determined by comparison of signal sizes with (+)-(10)-camphorsulfonic acid. The magnitude of the TRCD spectra were determined directly from the signal size and the experimental parameters. The differences in the spectra obtained by the two methods is probably due to noise in the spectra obtained using the TRCD apparatus and, perhaps, imperfections in the TRCD probe beam optics.

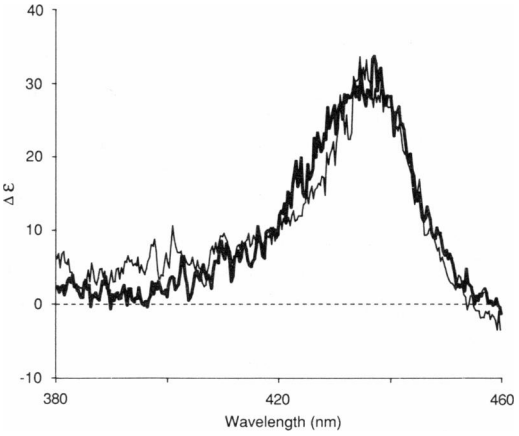

FIGURE 5 Comparison of the CD signal of deoxyMb (*thick line*) and the MbCO photolysis product (*thin line*). For both spectra the TRCD apparatus was used with a 500-ns gate. The deoxyMb spectrum represents 1,024 averages of a steady-state solution of deoxyMb. For the photolysis product of MbCO, the OMA gate began 200 ns after photolysis and the spectrum represents 1,536 averages.

of the photolysis product of MbCO 200 to 700 ns after photolysis are shown in Fig. 6. These are compared with CD spectra of MbCO and Mb taken on a conventional instrument. Again it appears that the spectrum obtained a few hundred nanoseconds after photolysis is similar to the steady-state deoxyMb spectrum.

DISCUSSION

Transient optical density experiments show that there are small but reproducible changes in the transient difference spectrum within the first few hundred nanoseconds of photolysis of MbCO. However, the TRCD signals do not reflect the presence of a transient intermediate in the

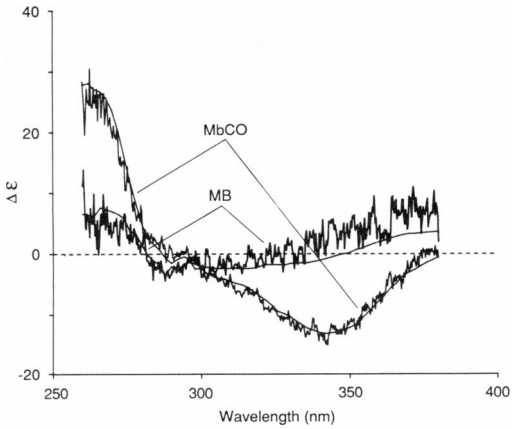

FIGURE 6 CD spectra of MbCO and deoxyMb taken on a conventional spectropolarimeter with 2 nm per point resolution (*smooth curves*). The CD spectrum of MbCO obtained using the TRCD apparatus is also displayed as well as the spectrum of the photoproduct of the photolysis of MbCO. Both spectra obtained with the TRCD apparatus used a 500 ns OMA gate and represent 1,024 averages. For the MbCO photolysis product spectrum the OMA gate began 200 ns after the laser fired.

photolysis reaction of MbCO on this timescale. The TRCD was followed in the Soret region of the spectrum, which probes the heme and its interactions with the surrounding protein matrix (Hsu and Woody, 1969). One might expect that there would be significant changes in the CD of the heme absorptions if there were protein movement around a rigid heme. The CD was also followed in the near-UV, a region whose CD is indicative of both CD from aromatic amino acids and from heme absorptions (Nicola et al., 1975). In neither spectral region was there a large change in the CD subsequent to the initial photolysis event.

Previous kinetic spectroscopic studies of the photolysis of MbCO suggested that the final deoxyMb is formed within 1 ns in room temperature fluid solution. Our transient optical density results suggest that perhaps there is an intermediate state which lives for a few hundred nanoseconds, but that it is spectroscopically very similar to deoxyMb. The CD spectrum of the intermediate is not detectably different than that of the final deoxyMb, so the intermediate must have a similar protein structure in the vicinity of the heme group. It is possible that this putative intermediate is the same as one which is seen as a kinetic intermediate in low temperature matrices. Fraunfelder and coworkers (Austin et al., 1975; Ansari et al., 1985) have shown that at low temperature the recombination of CO to Mb after photolysis of MbCO can be multiphasic, and they postulate that the CO can reside in at least a few different positions within or along the protein on its way out of and back into the heme binding site. It is reasonable that the putative intermediate could be one of the more weakly CO-bound intermediates seen in their low temperature experiments, such as the Mb_1^* intermediate they discuss. A very weakly CO-bound intermediate might not be expected to have a heme or protein structure significantly different than in the final deoxyMb, and thus might have optical density and CD spectra very similar to deoxyMb.

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REFERENCES

Ansari, A., J. Berendzen, S. F. Bowne, H. Frauenfelder, I. E. T. Iben, T. B. Sauke, E. Shyamsunder, and R. D. Young. 1985. Protein states and proteinquakes. *Proc. Natl. Acad. Sci. USA.* 82:5000–5004.

Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* 14:5355–5373.

Bolard, J., and A. Garnier. 1972. Circular dichroism studies of myoglobin and cytochrome c derivatives. *Biochim. Biophys. Acta.* 263:535–549.

Chance, B., R. Fischetti, and L. Powers. 1983. Structure and kinetics of the photoproduct of carboxymyoglobin at low temperatures: an x-ray absorption study. *Biochemistry.* 22:3820–3829.

Cornelius, P. A., A. W. Steele, D. A. Chernoff, and R. M. Hochstrasser. 1981. Different dissociation pathways and observation of an excited deoxy state in picosecond photolysis of oxy- and carboxymyoglobin. *Proc. Natl. Acad. Sci. USA.* 78:7526–7529.

Einterz, C. M., J. W. Lewis, S. J. Milder, and D. S. Kliger. 1985. Birefringence effects in transient circular dichroism measurements with applications to the photolysis of carbonmonoxyhemoglobin and carbonmonoxymyoglobin. *J. Phys. Chem.* 89:3845–3853.

Findsen, E. W., T. W. Scott, M. R. Chance, J. W. Friedman, and M. R. Ondrias. 1985. Picosecond time-resolved Raman studies of photodissociated carboxymyoglobin. *J. Am. Chem. Soc.* 107:3355–3357.

Friedman, J. M. 1985. Structure, dynamics, and reactivity in hemoglobin. *Science (Wash. DC).* 228:1273–1280.

Hennessey, J. P., Jr., W. C. Johnson, Jr. 1982. Experimental errors and their effect on analyzing circular dichroism spectra of proteins. *Anal. Biochem.* 125:177–188.

Henry, E. R., J. H. Sommer, J. Hofrichter, and W. A. Eaton. 1983. Geminate recombination of carbon monoxide to myoglobin. *J. Mol. Biol.* 166:443–451.

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.

Hsu, M.-C., and R. W. Woody. 1969. Origin of the rotational strength of heme transitions in myoglobin. *J. Am. Chem. Soc.* 91:3679–3681.

Hutchinson, J. A., and L. J. Noe. 1984. A study of the photodissociation of the CO and O_2 forms of hemoglobin and myoglobin by picosecond absorption spectroscopy. *IEEE (Inst. Electr. Electron. Eng.) Trans. Biomed. Eng.* 20:1353–1362.

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.

Lewis, J. W., R. F. Tilton, C. M. Einterz, S. J. Milder, I. D. Kuntz, and D. S. Kliger. 1985. New technique for measuring circular dichroism changes on a nanosecond timescale. Application to (carbonmonoxy)myoglobin and (carbonmonoxy) hemoglobin. *J. Phys. Chem.* 89:289–294.

Lewis, J. W., T. D. Dawes, and D. S. Kliger. 1987*a*. Flashlamp probe source for nanosecond spectroscopic studies in the ultraviolet. *Appl. Opt* 26:949–953.

Lewis, J. W., G. G. Yee, and D. S. Kliger. 1987*b*. Implementation of an optical multichannel analyzer controller for nanosecond flash photolysis measurements. *Rev. Sci. Instrum.* 58:939–944.

Marcolin, H.-E., R. Reschke, A. Trautwein. 1979. Mossbauer spectroscopic investigations of photodissociated myoglobin-CO at low temperatures. *Eur. J. Biochem.* 96:119–123.

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 350-fsec time constant. *Proc. Natl. Acad. Sci. USA.* 80:173–177.

Milder, S. J., and Kliger. 1985. Spectroscopy and photochemistry of thiouracils: implications for the mechanism of photocrosslinking in tRNA. *J. Am. Chem. Soc.* 107:7365–7373.

Nicola, N. A., E. Minasian, C. A. Appleby, and S. J. Leach. 1975. Circular dichroism studies of myoglobin and leghemoglobin. *Biochemistry.* 23:5141–5149.

Reynolds, A. H., and P. M. Rentzepis. 1982. Kinetics and temperature dependence of carboxymyoglobin ligand photodissociation. *Biophys. J.* 38:15–18.

Rothgeb, T. M., and F. R. N. Gurd. 1978. Physical methods for the study of myoglobin. *Methods Enzymol.* 52:473–493.

Rousseau, D. L., and P. V. Argade. 1986. Metastable photoproducts from carbon monoxide myoglobin. *Proc. Natl. Acad. Sci. USA.* 83:1310–1314.

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

Spartalian, K. G. Lang, and T. Yonetani. 1976. Low temperature photodissociation studies of ferrous hemoglobin and myoglobin complexes by Mossbauer spectroscopy. *Biochim. Biophys. Acta*. 428:281–290.

Spiro, T. G., and J. Turner. 1983. Heme protein structure and dynamics, studied by resonance Raman spectroscopy. *Pure Appl. Chem*. 55:145–149.