brief communication

Ligand dynamics in the photodissociation of carboxyhemoglobin by subpicosecond transient infrared spectroscopy

L. Rothberg,* T. M. Jedju,* and R. H. Austin‡
*AT&T Bell Laboratories, Murray Hill, New Jersey 07974; and ‡Princeton University, Princeton, New Jersey 08540

ABSTRACT Time-resolved infrared spectroscopy with 0.5-ps resolution is used to track the evolution of the CO stretching vibration after visible photoexcitation of carboxyhemoglobin in water at room temperature. Polarization measurements determine that the iron-complexed CO is oriented nearly perpendicular to the porphyrin plane. The dissociation appears to proceed via a metastable excited state with 2 ± 1 ps lifetime. The dissociated CO binds weakly in the heme pocket for at least 500 ps. This state correlates with the internally bound state observed by Frauenfelder et al. at low temperatures in myoglobin.

A problem of central importance in biophysics is that of the energetics and dynamics of protein motion. Myoglobin (Mb) and hemoglobin (Hb) have become model systems to address these issues both because of their biological importance and because a great deal is known about their structure and spectroscopy (1). One problem which has attracted much attention is to understand the reaction paths of small ligands such as O_2 and CO during the binding process and the influence of the protein structure upon those paths. The accepted crystal structure of hemoglobin sterically prohibits access of even these small molecules to the binding site on the heme group (2). Thus, an understanding of the microscopic details of ligation will require insight into the motion of the ligand and protein in solution.

Substantial progress has been made by experimental and theoretical studies of the inverse problem, ligand photodissociation from ligated Mb and Hb. Most of the previous time-resolved work has concentrated on the heme group evolution after photolysis. Some picosecond time-resolved studies suggest rapid photodissociation (3) and appearance of the nearly equilibrated delegated protein with 350 fs (4). There are, however, other indications of transients on several picosecond timescales in the electronic spectroscopy which have been interpreted as states before dissociation (5–8). This apparent dilemma has yet to be resolved. Transient Raman scattering studies with 30-ps resolution (9) essentially confirm rapid delegation but are not yet sufficiently sensitive to detect the dissociated fragment in the protein pocket or sufficiently rapid to resolve the aforementioned controversy.

Matrix isolation infrared spectroscopy (10) has been used to observe the CO group after photodissociation of carboxymyoglobin (MbCO). CO is an ideal "probe" for this work due to its strong infrared absorption and the environmental sensitivity of its stretching frequency. Below 200 K, the CO is found to be trapped at several sites inside the hydrophobic protein pocket ("B state"). At higher temperatures, CO is thought to escape the protein with high quantum yield (11). The escape and recombination kinetics of the photodissociated ligand have been studied by the Frauenfelder group (12, 13) who identify a sequence of potential barriers to CO binding (escape) that they associate with structural features of the protein. Molecular dynamics simulations (14, 15) also identify probable escape trajectories and provide estimates of the time required for the ligand to escape the protein.

In the present paper, we report subpicosecond time-resolved infrared measurements which focus on the ligand dynamics after photoexcitation of HbCO in water. These experiments are room temperature analogues of the matrix isolation experiments (10) whose purpose is to time resolve the ligand behavior under more biologically realistic conditions. We have chosen to work with Hb rather than the simpler protein Mb because, paradoxically, Mb is relatively more complicated in its IR absorption due to the presence of multiple CO stretching bands which arise from different conformations of the Fe-CO bound complex (16). We observe the disappearance of ground state CO and make measurements to determine the bonding angle it makes with the heme plane in ground state HbCO. We present evidence for a short-lived (~2 ps) excited state of HbCO before dissociation. Finally, we observe CO loosely bound in the protein pocket (termed the "B state" by Frauenfelder [10]) for the first time at room temperature.

Fresh samples of hemoglobin derived from human blood were prepared by standard procedures (17) and dialyzed against CO saturated D_2O buffer, 50 mM in phosphate pD 7.5. Samples of HbCO in D_2O were concentrated to 40 mM by dialysis against polyethyleneglycol. A drop of this solution was held between CaF_2 disks separated by a 100-μm Teflon spacer. Sample absorbances at the pump frequency (590 nm) were 0.5–1.

The apparatus is a variation of one used previously to study rapid lattice deformations in *trans*-polyacetylene. The subpicosecond tunable infrared pulses are generated by "seeded" optical parametric amplification (18), and a detailed description of how these are applied to transient vibrational spectroscopy is provided elsewhere (19). An amplified synchronously pumped dye laser is used to obtain ~1-mJ pulses of 350 fs duration at 590 nm with repetition rate of 10 Hz. Part of these pulses is used to photoexcite the HbCO and the remainder is devoted to generating synchronized infrared pulses. This is done by parametric mixing of some of the 590-nm light with a white light continuum generated by self phase modulation of the intense monochromatic light in D_2O. In practice, the white light continuum must be preamplified to obtain sufficient infrared to do transmission spectroscopy through aqueous solution. The nonlinear mixing is done in a short $LiIO_3$ crystal and provides 350-fs pulses of mid-infrared radiation from 4.2 to 5.5 μm.

The infrared pulses have nanojoule energies and are focused to $\lesssim$200-μm spots on the sample. The visible beam is routed through a variable pathlength delay and is overlapped with the infrared probe on the sample. The pump pulse of ~50 μJ is focused to a spot size of 0.5–1 mm, corresponding to photon fluences of ~1.5–6 $\times$ 10^{16} photons/cm^2. Whereas the number of photons per pulse and HbCO molecules in the interaction region are comparable, the temperature rise from ambient temperature of 20°C due to absorption is only 0.2°C once the energy equilibrates with the solvent. (The temperature rise is calculated simply by dividing the absorbed energy by the heat capacity of the absorbing volume. Naturally, this does not address transient heating of the protein by the absorbed photon). The infrared pulse bandwidths are several hundred centimeters^{-1}, as determined by crystal phase matching. The transmitted pulses are therefore dispersed by a monochromator and a ~15-cm^{-1} bandwidth is monitored by an InSb detector. In this way, resolution up to the transform limit can be obtained without spoiling our time resolution and subpicosecond vibrational spectroscopy is feasible.

Fig. 1 illustrates bleaching of the bound CO stretching bands at 1,952 cm^{-1} after 590 nm excitation. The bleaching appears much faster than the apparatus resolution of 0.5 ps. The size of the bleaching is consistent with dissociation of one CO per absorbed photon as in previous measurements (11). The bleaching is roughly linear with excitation pulse energy, and damage to the sample, as evidenced by changes in the sample IR spectrum, occurs at lower intensities than saturation of the bleaching. Less than 10% bleaching recovery is observed out to a pump-probe delay of 1 ns, which is consistent with some previous measurements (20), but only barely with those of Petrich et al. (21). The bleaching spectrum around 1,952 cm^{-1}

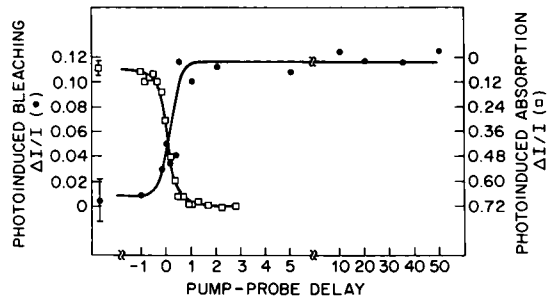

FIGURE 1 590-nm photoinduced bleaching in HbCO at 1,952 cm^{-1}. For reference, the rise of free carrier absorption in Si using the same apparatus and wavelengths is shown to illustrate the instrumental resolution of ~0.5 ps.

closely follows the infrared absorption spectrum at all delays to 1 ns. This means that there is no large effect of the photodissociation on the undissociated CO groups on the other hemes in the tetrameric Hb complex. These subunits would seem to be kinetically isolated on the picosecond time scale, as expected from the results of Eaton and co-workers (22).

We can use the polarization dependence of the bleaching to determine the relative orientation of pump and probe dipole moments. Assuming that the electronic absorption moment is isotropic in the heme plane and that the infrared absorption is along the C—O bond, the angle between the C—O bond and heme plane is given by (23)

$$\theta = \cos^{-1}[(2 - R)/(0.5 + R)]^{1/2}, \quad (1)$$

where R the ratio of bleaching for perpendicular polarized probe to bleaching for parallel polarized probe. This has been done by Hochstrasser and co-workers in elegant photoselection experiments on MbCO (23, 24) and HbCO (24) using picosecond dye lasers and a continuous narrowband infrared source tuned to the bound CO stretching absorptions. They measure a 72° angle between CO bond and the heme plane in HbCO. We obtain a polarization anisotropy of 0.2 ± 0.2 giving an angle between heme plane and CO absorption moment of 85 ± 10°. This value is somewhat higher than that of reference 24 but not inconsistent given both experiments' error bars. Numbers derived from x-ray (25), neutron (26), and resonance Raman (27) studies of HbCO lie between these two values.

We also observe the appearance of two new CO stretching frequencies after photoexcitation. Fig. 2 shows a transient infrared spectrum in the CO stretching region 1 ps after photoexcitation. Aside from the bleaching, a new band appears at 2,009 cm^{-1} which we associate with CO still bound to Fe in an excited electronic state of HbCO. As seen in Fig. 3, this absorption appears in less than the

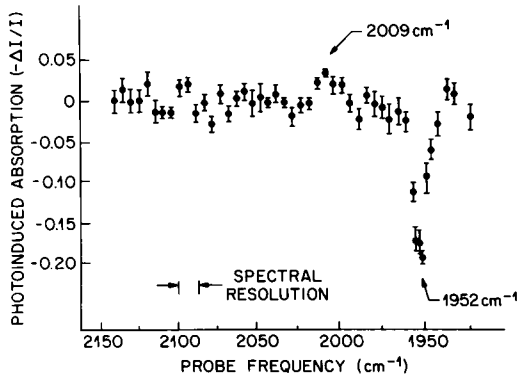

FIGURE 2 Polarization dependence of the bleaching at 1,952 cm^{-1}. Pump and probe are linearly polarized, as described in the text. The probe pulse is set at 1 ps (?) delay relative to the pump.

0.5-ps resolution of the apparatus and disappears with decay constant of 2 ± 1 ps. We attribute its disappearance to the dissociation of CO from this metastable excited state. The 2,009 cm^{-1} absorption we observe at zero delay is four to five times weaker than the bleaching. Because the CO oscillator strength is inversely correlated with stretching frequency (see, e.g., reference 10), this is consistent with high (near unity) quantum yield of this metastable excited state. Martin et al. (4) claim that dissociation occurs in ≲350 fs based on the appearance of a "deoxy species." They note, however, that their transient spectrum "differs significantly" from the stable deoxy spectrum. They also speculate on a ligated excited species with 10% yield and 3 ps lifetime which relaxes to the ground state. The species we observe is unlikely to be one with such low quantum yield. It is possible that the 3-ps transient seen by Martin et al. is associated with a spectral relaxation of the heme due to CO dissociation as discussed by Jongeward et al. (28). It is also possible that the photodissociation dynamics differ when HbCO is pumped into its Soret band (~400 nm) from those when pumping at 590 nm, though this is not the case for the transient bleaching dynamics (24).

We observe 5 ps after excitation a CO stretching absorption at 2,132 cm^{-1} as evidenced in Fig. 4 *a*. This is the first time that the "B" state (10, 19) has been seen in HbCO at room temperature. This state is one where CO is van der Waals bound inside the hydrophobic protein pocket. The relative absorption strength of this band is in rough accord with the observed low-temperature absorption strength of the "B" state (10) assuming unit yield of formation. Apparently, the CO remains bound within the protein for at least 500 ps (Fig. 4 *b*). An estimate of the lifetime of this bound state can be made roughly by analysis of the extrapolated low-temperature kinetics along the line of Frauenfelder and co-workers (12, 13).

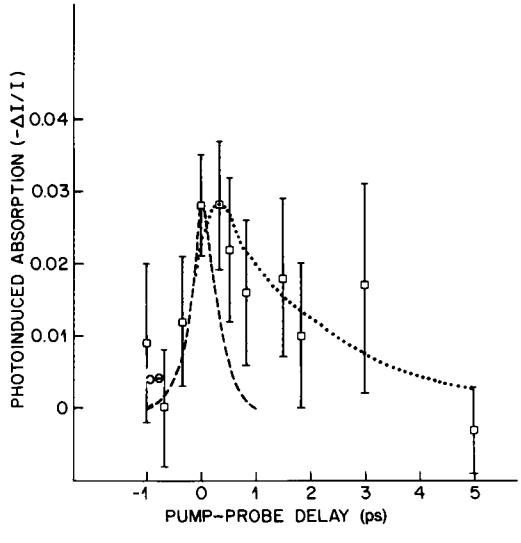

FIGURE 3 (*a*) Transient IR spectrum of HbCO in D_2O at 1 ps after photoexcitation. Error bars were determined by standard deviations from 10 (?) data sets. Calibration of the monochromator is done using H_2O vapor absorption lines in air. (*b*) Time dependence of the 2,009-cm^{-1} line after photoexcitation. Solid line is an exponential fit to the rise time of the signal, with time constant 2 ± 1 ps time constant.

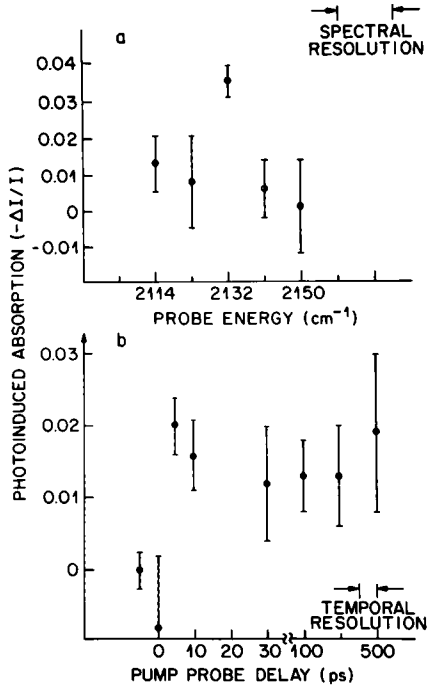

FIGURE 4 (*a*) The infrared spectrum in the nearly free CO stretching region 5 ps after excitation. These data were recorded using 2-ps laser pulses which afford higher signal/noise ratio. (*b*) The time dependence of the 2,132 cm^{-1} transient absorbance, using 2-ps light pulses.

Unfortunately, the same detailed analysis that has been done on myoglobin has not been done on hemoglobin, but crudely we can say that the lifetime of the "B" state should be $1/k_{ba} + k_{bc}$, where k_{ba} and k_{bc} are the rates for rebinding and pocket escape, respectively. The 300 K extrapolated rates for these two processes are on the order of 10^8 s^{-1}, suggesting a "B" state lifetime of ~5 ns, consistent with our measured minimum of 500 ps. An estimate based on an Arrhenius unbinding rate with attempt frequency of $\sim 10^{13}$/s gives a lower bound for the CO unbinding barrier in the B state of 5 Kcal/mol. The observation of the B state at room temperature points out the need to refine molecular dynamics calculations to include energy loss and ligand binding in the heme pocket more realistically. Transient infrared studies on longer time scales are in progress to permit a more quantitative understanding of this state.

In summary, we have explored the ligand dynamics during and after photodissociation of HbCO using subpicosecond transient infrared spectroscopy. We find no evidence for significant ligand rebinding at times < 1 ns. We argue that dissociation proceeds via a metastable excited state with 2 ± 1 ps lifetime and a CO stretching frequency of ~2,009 cm^{-1}. After this, we observe CO weakly bound in the protein pocket for >500 ps. This is the first observation of this state at a temperature > 200 K and may provide significant tests for differing models of protein reactivity.

L. Rothberg would like to thank Dr. Denis Rousseau for many helpful suggestions and stimulating discussions. R. H. Austin would like to thank Dr. Joel Friedman, Prof. Hans Frauenfelder, and Prof. Thomas Spiro for helpful discussions, and the ONR for financial support. We are most grateful to Chang Sue and Prof. Spiro for helpful discussions and the preparation of purified hemoglobin samples. We appreciate careful reviews by referees of this paper who have contributed substantially to the accuracy and clarity of the text.

Received for publication 12 April 1989 and in final form 27 July 1989.

REFERENCES

1. Perutz, M. F. 1979. Regulation of oxygen affinity of hemoglobin. *Annu. Rev. Biochem.* 48:327–386.
2. Perutz, M. F., and F. S. Matthews. 1966. An x-ray study of axide methaemoglobin. *J. Mol. Biol.* 21:199–202.
3. Shank, C. V., E. P. Ippen, and R. Berssohn. 1976. Time-resolved spectroscopy of hemoglobin and its complexes. *Science (Wash. DC).* 193:50–51.
4. Martin, J. L., A. Migus, C. Poyart, Y. Lecarpentier, R. Astier, and A. Antonetti. 1983. Femtosecond photolysis of CO-ligated protoheme and hemoproteins. *Proc. Natl. Acad. Sci. USA.* 80:173–177.
5. Greene, B. I., R. M. Hochstrasser, R. B. Weisman, and W. A. Eaton. 1978. Spectroscopic studies of oxy- and carbonmonoxy hemoglobin. *Proc. Natl. Acad. Sci. USA.* 75:5255–5259.
6. Noe, L. J., W. G. Feisert, and P. M. Rentzepis. 1981. Kinetics of carbonmonoxy myoglobin and oxy myoglobin studied by picosecond spectroscopy. *Biophys. J.* 25:455–464.
7. Reynolds, A. W., S. D. Read, and P. M. Rentzepis. 1981. Mechanism for excited state relaxation and dissociation of oxymyoglobin and carbonmonoxymyoglobin. *Proc. Natl. Acad. Sci. USA.* 78:2292–2296.
8. Guest, C. R., and L. J. Noe. 1987. Photodissociation of CO and O_2 from α and β hemoglobin chains. *Biophys. J.* 52:885–889.
9. Findsen, E. W., T. W. Scott, M. R. Chance, J. Friedman, and M. R. Ondrias. 1985. Picosecond time-resolved Raman studies of photodissociated carboxymyoglobin. *J. Am. Chem. Soc.* 107:3355–3357.
10. Alben, J. O., D. Beece, S. F. Bowne, W. Doster, L. Eisenstein, H. Frauenfelder, D. Good, J. D. McDonald, M. C. Marden, P. P. Moh, L. Reinisch, A. H. Reynolds, E. Shyamsunder, and K. T. Yue. 1982. Infrared spectroscopy of photodissociated carboxymyoglobin at low temperatures. *Proc. Natl. Acad. Sci. USA.* 79:3744–3748.
11. Bucher, T., and J. Kaspers. 1947. Photochemical breakage of carbonmonoxyhemoglobin through UV light. *Biochim. Biophys. Acta.* 1:21–34.
12. Austin, R. H., K. Beeson, L. Eisenstein, H. Frauenfelder, I. C. Gunsalus, and V. P. Marshall. 1974. Activation energy spectrum of a biomolecule. *Phys. Rev. Lett.* 32:403–405.
13. Austin, R. H., K. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus. 1975. Dynamics of ligand binding to myoglobin. *Biochemistry.* 14:5355–5373.
14. Case, D. A., and M. Karplus. 1979. Dynamics of ligand binding to heme proteins. *J. Mol. Biol.* 132:343–368.
15. Henry, E. R., M. Levitt, and W. A. Eaton. 1985. Molecular dynamics simulation of photodissociation of CO from hemoglobin. *Proc. Natl. Acad. Sci. USA.* 82:2034–2038.
16. Flamingo, F. G., and J. O. Alben. Structures of photolyzed carboxymyoglobin. *Biochemistry.* 24:7964–7970.
17. Antonini, E., and M. Brunori. 1971. Hemoglobin and Myoglobin in Their Reactions with Ligands. Elsevier Science Publishing Co., New York.
18. Jedju, T. M., and L. Rothberg. 1988. Tunable femtosecond radiation in the mid-infrared for time-resolved absorption in semiconductors. *Appl. Optics.* 27:615–618.
19. Jedju, T. M., L. Rothberg, and A. Labrie. 1988. Subpicosecond time-resolved vibrational spectroscopy by transient infrared absorption. *Opt. Lett.* 13:961–963.
20. Chernoff, D. A., R. M. Hochstrasser, and A. W. Steele. 1980. Geminate recombination of oxygen and hemoglobin. *Proc. Natl. Acad. Sci. USA.* 77:5606–5610.
21. Petrich, J. W., C. Poyart, and J. L. Martin. 1988. Photophysics and reactivity of heme proteins. *Biochemistry.* 27:4049–4060.
22. Murray, L. P., J. Hofrichter, E. R. Henry, M. Ikeda-Saito, K. Kitagashi, T. Yonetani, and W. A. Eaton. 1988. The effect of quaternary structure on the kinetics of conformational changes and nanosecond geminate rebinding of CO to hemoglobin. *Proc. Natl. Acad. Sci. USA.* 85:2151–2155.
23. Moore, J. N., P. A. Hansen, and R. M. Hochstrasser. 1987. A new method for picosecond time-resolved infrared spectroscopy. *Chem. Phys. Lett.* 138:110–114.
24. Anfinrud, P., C. Han, P. A. Hansen, J. N. Moore, and R. M. Hochstrasser. 1988. Picosecond and femtosecond infrared spec-

troscopy with CW diode lasers. *In* Proceedings of the Sixth Conference on Ultrafast Phenomena. T. Yajima, editor. Springer-Verlag, Berlin.

25. Baldwin, J. M. 1980. The structure of human carbonmonoxy haemoglobin at 2.7 Å resolution. *J. Mol. Biol.* 136:102–128.
26. Norvell, J. C., A. C. Nunes, and B. P. Schoenborn. 1975. Neutron diffraction analysis of myoglobin structure. *Science (Wash. DC).* 190:568–570.
27. Yu, N. T., M. Tsubaki, and B. Srivastava. 1983. Resonance Raman investigation of carbon monoxide bonding in hemoglobin and myoglobin. *Biochemistry.* 21:1132–1140.
28. Jongeward, K., D. Magde, D. J. Taube, C. Marsters, T. G. Traylor, and V. S. Sharma. 1988. Picosecond and nanosecond geminate recombination of myoglobin with CO, O_2, NO and isocyanides. *J. Am. Chem. Soc.* 110:380–387.