Femtosecond photolysis of CO-ligated protoheme and hemoproteins: Appearance of deoxy species with a 350-fsec time constant

(femtosecond spectroscopy/CO-heme photolysis kinetics/Excited states of hemoglobin, myoglobin, and protoheme/molecular dynamics)

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ABSTRACT Photolysis of HbCO, MbCO, and CO-protoheme has been investigated by measuring transient differential spectra and kinetics of induced absorption after excitation with a 250-fsec laser pulse at 307 nm. Probing was performed by a part of a continuous pulse between 395 and 445 nm. Photodissociation of the three liganded species occurred within the pulse duration. By contrast, the formation of deoxy species appeared with a mean (±SD) response time of 350 ± 50 fsec. This time constant was identical for the three species and independent of the presence or absence of the protein structure. Our results suggest the formation of a transient high-spin in plane iron (II) species which relaxes in 350 fsec to a high-spin stable state with concerted kinetics of CO departure and iron displacement. The spin transition is suspected to occur via liganded excited states which relax in part to non-reactive states with a 3.2-psec time constant.

Picosecond spectroscopy has revealed that the dissociation of ligand from the heme in Mb and Hb occurs within 4 psec and that the nonliganded species (Mb or Hb) were formed with a time constant of 11 psec (1, 2). This has been confirmed recently by Cornelius et al. (3) who observed the appearance of Mb species after photodissociation of MbO2 with a 12-psec time constant; their results on MbCO showed a "simultaneous development of both bleaching and absorption intensities." From results of studies with 0.5-psec optical pulses at 615 nm in a pump probe experiment, Shank et al. (4) deduced that the photolysis of CO from HbCO should occur in less than 0.5 psec.

More recently, Greene et al. (5) and Chernoff et al. (6), using 8-psec excitation pulses at 353 and 530 nm, obtained transient absorption spectra in the Soret and visible regions at 10 and 680 psec which indicated that deoxy species formed in a time shorter than their pulse duration. Terner et al. (7) used picosecond resonance Raman methods and found formation of HbCO photoproducts with spectra similar to those of deoxy Hb and corresponding to a high-spin at least partially out-of-plane Fe(II) state. These events occurred within 30 psec, their pulse duration.

Up to now, the kinetics of photodissociation of ligand from heme in hemoproteins has not been precisely resolved. Recent theoretical approaches dealing with the energies involved in ligand reactions to and from the heme and their relationship with the protein matrix surrounding the heme (8, 9) have led to tentative models describing the probable pathway(s) through energy barriers of the ligand in the heme pocket. It is of primary importance for such theoretical approaches to be supported by accurate quantitative measurements of the very early steps of the chemical events leading to the formation or dissociation of the ligand–heme complex.

In the present work, we have limited ourselves to the investigation of the kinetics of formation of the nonliganded species after dissociation of CO from MbCO and HbCO. In order to appreciate the possible influence of polypeptidic environment on the heme reactivity in these hemoproteins, we have also studied the kinetics of CO dissociation from protoporphyrin and of deoxyprotoporphyrin formation. These experiments were carried out with a newly developed spectroscopic technique operating with laser pulses of 250 fsec duration to excite the hemes at 307 nm.

MATERIAL AND METHODS

Preparation of the Hemoproteins and Protoheme Solutions. Purified human adult hemoglobin was prepared from fresh human blood by DEAE-Sephadex chromatography (10). The experimental solution was 0.1 mM on the basis of heme; it was diluted in 0.1 M K phosphate buffer at pH 7 or 5. The solution was first deoxygenated under moist argon before equilibration with CO at 1 atm (1 atm = 1.013 × 10^5 pascals). MetMb (horse heart type III, Sigma) was converted to MbFe^2+ by addition of a 5 molar excess of freshly prepared Na dithionite (Merk) under strict anaerobic conditions and then equilibrated with pure CO. This stock solution was chromatographed on an ion exchange resin column to remove dithionite and then diluted to 0.1 mM heme in the same buffer as HbCO. The purity of HbCO and MbCO was checked by isoelectric focusing which revealed a single band for both proteins. Protoporphyrin was prepared from hemin [Fe^3+] (protoporphyrin) (bovine type I, Sigma) as follows. A stock solution (2 mM) was prepared in 1 M NaOH solution and then diluted in polyethylene glycol (PEG, Merck) to a final concentration of 0.1 mM. A 5 molar excess of Na dithionite was added under anaerobic conditions and a deoxy Hb spectrum (Cary 219, Varian) was recorded.

The deoxy solution was equilibrated with CO and kept in ice water until used. We observed that the deoxy spectrum of protoporphyrin was very dependent upon the PEG/NaOH ratio (vol/vol), the highest value of ε(450) = 138.6 M^−1 cm^−1 being obtained with a ratio of 94:6 or more. Changes in absorbance of deoxy protoporphyrin solution and, to a lesser extent, of fully liganded protoporphyrin with the nature of the solvent have been attributed to aggregation of the protoporphyrin molecules in aqueous media (11). In the CO-protoporphyrin solutions (ε(411) = 207.3 M^-1 cm^-1), the Na dithionite was not removed before the experiment because of the rapid oxidation of the iron complex during the ion exchange chromatography (12). However, at the concentration used in this study, the absorbance of the protoporphyrin samples at 307 nm was not very different from that recorded in the MbCO or HbCO samples.

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Aliquots of the stock solutions of carboxylated protoheme, MbCO, or HbCO were transferred anaerobically into a 1-mm recirculating quartz cuvette (both openings sealed with rubber bungs) filled with CO (Hellma, France). Optical spectra of the solutions were recorded both before and at the end of each experiment. It was regularly observed that the two spectra were identical, indicating no alteration of the solutions during the run. This was obtained by moving the horizontal cuvette perpendicularly to the beam at a linear speed of 12 mm/sec, so that each pulse at 10 Hz excited a new region of the sample. All experiments were performed at room temperature.

**Time-Resolved Spectrophotometric Method.** For the initial laser source we built a passive-mode locked CW dye laser (13). The rhodamine 6G gain stream is located near the center of the resonator and is optically pumped by 4–5 W of a CW argon ion laser at 5145 Å. The second stream near one end of the resonator contains a mixture of two saturable absorbers, 3,3'-diethylxadicarbocyanine iodide and malachite green. The duration of the pulse (0.7 psec) and the simultaneously measured frequency spectrum (about 20 Å) indicate excess bandwidth due to dispersion in the cavity. Direct compression is then achieved by using a grating pair technique (14): two gratings of inverse constant, 1,300 lines/mm, with a separation of 90 cm are effectively used in first-order reflection at λ = 614 nm at an angle of incidence of 45°. Undesirable energy in the pulse wings is eliminated by removing the yellow part of the spectrum by using an aperture in the light path between the two gratings.

The resulting pulses of duration <0.3 psec are close to being transform-limited and have an energy of the order of 1 nJ. For time-resolved spectroscopy, we amplify these pulses to higher power to generate new wavelengths covering a large spectrum range. Amplifying these pulses to powers in the gigawatt range requires special considerations to avoid temporal broadening.

The specific amplifier configuration used in our experiment has three stages pumped by a Q-switched frequency doubled Nd-YAG laser generating pulses of 14 nsec in duration, 500 mJ energy at 530 nm for a 10-Hz repetition rate (15). This setup produces final pulses with a peak intensity up to 3 GW while maintaining a pulsewidth of 0.25–0.30 psec.

The pulse train is then split into two parts. After passing through a variable delay line driven by a stepping motor, one beam is focused into a 0.75-mm-thick (potassium dihydrogen phosphate) crystal to generate pulses at 307 nm. This thin crystal ensures phase-matching of the whole pulse bandwidth without pulse broadening but gives a low efficiency for the second harmonic conversion—i.e., 10 μJ at 307 nm. The UV beam is focused on a 300-μm-diameter spot in the sample and initiates the photodissociation of the sample. The other half of the 614-nm beam is focused into a 2-cm cell filled with water and generates a white light continuum. The blue part of the spectrum is selected with colored filters and split into two beams: one beam probes collinearly the excited part of the sample and the other (reference beam) misses the cuvette. These two continuum beams are then focused on the 400-μm entrance slit of a 0.25-m spectrophotograph used in the first-order of a 600-groove-per-mm grating (ensuring, with a 400-μm output slit, a spectral resolution of 20 Å) and directed to two diodes. The two signals, corresponding to the probe and reference pulses energy at a given wavelength, are sent through an electronic chain (pulse shaping, sampling holder, and digitizer) to a computer; their ratio is determined and used in a multichannel analyzer program. Shots for which energy varied more than 50% from the mean were rejected. In one sweep, corresponding to 10 psec, 100 positions of the delay line were examined and 5 of these ratios were averaged for each position. Typically, 40 sweeps have been realized to get a good signal-to-noise ratio.

For each sample, we analyzed the signal rise time at two different wavelengths: the first one corresponded to the peak of the CO liganded species and the second corresponded to the peak of the deoxy species.

The absorption change induced by a pulse of intensity \( I_p(t) \) on a test pulse of intensity \( I_0(t) \) with \( \tau \) as the delay between the two pulses is given by (16):\[
\Delta \alpha(\tau) = \int_{-\infty}^{\infty} A(\tau - \tau') I_p(t) I_0(t + \tau') \, dt \, d\tau'.
\]

In this expression, \( A(\tau) \) is the impulse response of the molecular absorption. In case of instantaneous response, \( A(\tau) \) is a constant and the medium response is then determined by the integral of the pulse's correlation function. From other experiments (17) we know that the shape of our pulses is close to one-sided exponential with \( \Delta t \), the full width at half maximum, of the order of 230 fs.

In this case, all the curve rise times corresponding to the disappearance of the liganded species are proportional to the integral of the pulse's correlation function with \( \Delta t = 230 \) fs.

These best fits have been realized by using the expression:

\[
\frac{I(t)}{I_0(t)} = \exp[-\Delta \alpha(t)]
\]

in which \( I \) is the medium length, and \( I/I_0 \) is the probe-pulse measured transmission at delay \( \tau \) between pump and probe.

The appearance of the deoxy species could not be fitted with an instantaneous response but were well fitted with the response function

\[
A(t) = 1 - \exp(\frac{-t}{\tau_X}), \quad t \geq 0
\]

in which \( \tau_X \) is the response time of the molecular species \( X \).

An important point is to estimate the influence of group velocity dispersion. A time-broadening factor occurs when the UV and the blue pulses overlap inside the sample length but travel with different group velocities.

We have computed this broadening to be of the order of 90 fs, but we must point out that this slowing down of the response is almost the same from 400 nm to 435 nm and then is taken into account in the instantaneous response best fit and has no effect in the determination of the appearance of the deoxy species time constants. We must also consider the group velocity dispersion in the continuum pulse. The dispersion between two different wavelengths may be estimated by summing the differences in the propagation time through the different dispersive media between the continuum generation and the sample (water, lens, beam splitter, and filters). We have computed the difference to be \( \delta \tau / \delta \lambda = 40 \) fs/ncm. This will appear in two ways: (i) a broadening of 60 fs into the spectral width of the observed continuum pulse, as previously taken into account in the instantaneous response, and (ii) a pure delay of the time coincidence between pump and probe when the probe wavelength is shifted from the liganded spectrum maximum to the deligated spectrum maximum. The typical shift (15 nm) induced an observed delay at 600 fs, which is in good agreement with our estimation.

**RESULTS**

Photolysis of HbCO Solutions. Fig. 1A shows the change in transmission at 419 nm, a wavelength at which bleaching in HbCO is maximal as recorded from transient picosecond differential spectra. These kinetics were found to be limited by the pulse duration. By contrast the rate of induced absorption at 434 nm, the maximal positive peak of the transient \( \Delta A \) spectrum,
cannot be fitted to an instantaneous response (Fig. 1B). The best fit of the rate of appearance of deoxy Hb is obtained for $\tau_{Hb} = 300 \pm 40 \text{ psec} \text{ (mean} \pm \text{ SD). It has been determined that } \tau_{Hb} \text{ is independent of pH (7–8), of ionic strength (0.01–0.1 M), and of CO concentration (0.01–1 atm).}

**Photolysis of MbCO.** Similar experiments were carried out in MbCO solutions with interrogating wavelengths of 422.5 nm for bleaching and 438 nm for induced absorbance changes, the two negative and positive maxima of the transient $\Delta A$ spectrum. It is remarkable that, as for HbCO, the rate of bleaching (Fig. 2A) is instantaneous (<<$\Delta t$) whereas the rate of appearance of deoxy Mb was observed at $\tau_{Mb} = 320 \pm 35 \text{ psec}$ (best fit in Fig. 2B).

The identity of these results in HbCO and MbCO solutions prompted us to analyze protombe solutions.

**Photolysis of CO-Protopheme Solutions.** As evident from Fig. 3 the rates for bleaching and for the appearance of deoxy protombe at 410 and 420 nm, the negative and positive maxima of the transient $\Delta A$ spectrum, were similar to those observed in HbCO and MbCO solutions—i.e., instantaneous bleaching at 420 nm and $\tau_{H} = 380 \pm 30 \text{ psec}$, respectively.

Fig. 3A shows that a relaxation of the bleaching at 410 nm occurs, with a time constant of 3.2 psec (best fit of the exponential decay). Such a relaxation of the signal of the carboxylated species (not seen in deoxy species) was also observed in HbCO and MbCO (Figs. 1A and 2A) with a similar time constant (3.2 psec). However, the importance of the relaxation varies considerably between the protombe solution (≈50% decay after 20 psec) and either HbCO or MbCO solution (10% and 20% decay after 20 psec). No further decay was observed up to 100 psec. Full development of the interpretation of this relaxation will be given elsewhere.

**DISCUSSION**

**Kinetics.** Dynamics of photodissociation of ligands from heme proteins are well documented in studies using picosecond laser techniques (1–7). The best time resolution associated with an accurate optical spectrum of the initial photolyis product has been achieved by Greene et al. (5), Chernoff et al. (6), and, more recently, by Cornelius* et al. (3). They observed the formation of a deoxy-like Hb species 6 psec after 530-nm excitation. Their deliganded species appeared within their pulse duration, corresponding to the depletion of the HbCO population within the same time. Reynolds et al. (2) found that the formation of deoxy Mb was initiated in 15 psec both for MbCO and MbO$_2$ whereas the disappearance of the liganded species occurred in 5 psec. Terner et al. (7) used picosecond resonance Raman spectroscopy and found the presence of deoxy Hb in less than 30 psec after HbCO photolysis.

In the present study, we determined that the photodissocia-
within the new the of difference after is which corresponds to the absorbance of glycol after 307 nm. It can be seen that the absorbance of deoxyprotoheme, which corresponds to the absorbance of Hb, (deoxyprotoheme) relaxation can be observed in any of the three molecular species.

Fig. 3. Normalized kinetic data for CO-protoheme in 94% polyethylene glycol solution excited at 307 nm. (A) Induced transmission at the absorbance maximum of CO-protoheme (λ = 410 nm). The thin line indicates the instantaneous response. Note the well-defined relaxation of the signal whose time constant is 3.2 psec (best fit) and which corresponds to 50% of the signal at 20 psec compared to 10% and 20% in HbCO and MbCO, respectively (Figs. 1A and 2A). (B) Induced absorption at the maximum induced positive change (λ = 425 nm) (deoxyprotobium) with the best fit (thin line) for τ = 380 fs. --- and ---, as in Fig. 1. Note that in the induced absorption kinetics no relaxation can be observed in any of the three molecular species.

Absorption of CO from HbCO, MbCO, and CO-protobium occurs within the 250-fsec of our pulse duration whereas the deligated species is formed with a 350-fsec time constant. The formation of the deoxy species is demonstrated by the appearance of the new absorption maximum at the same wavelength as that observed in the stable difference spectra. This is illustrated by the transient difference spectrum of Mb and MbCO at 0 and 5 psec after 307 nm excitation (Fig. 4) compared to the fully deoxy MbCO stable difference spectrum. Similar spectra were recorded after photolysis of MbCO and CO-protobium solutions. It was also observed that, within experimental accuracy (20 Å), the isobestic points at 417, 426, and 430 nm for protobium, Hb, and Mb solutions, respectively, were close to those of the stable difference spectra.

The kinetics at the maximum of bleaching (Figs. 1A, 2A, and 3A) show a decay process more pronounced in the protobium experiments (approximately 50% of the initial signal at 20 psec) than in HbCO or MbCO where it barely amounts to a maximum of 20%. This relaxation process is detectable only in the initial absorption spectra of the liganded species. This suggests the presence of excited liganded species which relax, at least in part, to the ground state with a time constant of 3.2 psec, identical for the three molecular species. Our results may be compared to the recent work on MbCO and MbO₂ by Cornelius et al. (3). The transient spectrum of MbCO shown in Fig. 4 is similar to spectra reported by these authors 6.8 and 100 psec after excitation. As they did, we observed no geminate recombination up to 100 psec. We demonstrate here that the "simultaneous development of both bleaching and absorption intensities" means actually a rate of growth for the absorption intensity of 350 fsec.

We should also point out that our ΔΔ transient spectra, like those published by Cornelius et al. (3), differ significantly from the stable ΔΔ deoxy Mb/MbCO spectrum. The source of this difference is not clear. It may be that these transient spectra are distorted due to some degree of inhomogeneity in the overlapping of the pump and the continuum beams. This appears to be unlikely because large changes in the excitation fluence, by factors of 2 or 3, did produce similar distorted spectra. Moreover, kinetic measurements, with a narrow band selected in the

Fig. 4. (A) Stable difference spectrum of deoxy Mb and MbCO. Arrows indicate the wavelengths at which kinetic results shown in Fig. 2 were recorded. (B) Transient difference spectra of deoxy Mb and MbCO at 0 psec (——) and 5 psec (-----) after pulse excitation at 307 nm. The dashed line represents the calculated stable difference spectrum for 10% photodissociation corresponding to the ΔΔ measured at 438 nm. The transient spectrum was similar between 1 and 100 psec after excitation. Experimental conditions: MbCO 0.1 mM (λ₄₂₂ = 2.1) in 0.1 M K phosphate pH 8 buffer at 25°C; 1-mm cell; excitation, 10 μJ, 250 fsec, at 307 nm. Spectra were recorded by replacing the two photodiodes at the output of the spectrophotometer by a two-dimensional optical multichannel analyzer (OSA, B.M., Spectronik). The digital processing was as follows. After the dark current is subtracted the reference track is divided by the probe track and its logarithm is calculated. The change in absorbance (ΔΔ) is the difference in absorbance between a delay of 0 psec or 5 psec and its value at −5 psec. It has been checked that the spectra were identical before and in the absence of excitation.
continuum and two photodiodes as detectors, also revealed distorted amplitudes. Another possibility would be that about 50% of the bleaching in the Soret liganded peak corresponds to the formation of at least one excited MbCO* species with very broad and weak absorbance toward shorter wavelengths. The relaxation of the initial bleaching (20%) with a 3.2-psec time constant in part supports this hypothesis.

Recently, Reynolds and Rentzepis (18) published a ΔA spectrum at 200 ps after 25-ps excitation pulses which is almost identical to a Mb/MbCO DC difference spectrum. We cannot explain the discrepancy between their spectrum on the one hand and the spectra of Hochstrasser's group and ours because no precise information on the experimental conditions (excitation fluence, heme concentration, cell pathlength, etc.) used were given by Reynolds and Rentzepis. One cannot completely eliminate the influence of the pulse duration for values more than 3- to 4-fold above the 3.2-psec relaxation time constant, associated with multiphoton photodissociation processes.

Photodissociation Process. The kinetics of formation of the deoxy species suggest the existence of spin-allowed dissociation channels. This requires the instantaneous formation of a transient high-spin liganded species, highly photodissociative via an intersystem crossing. According to the notation of Chernoff et al. (6) this intermediate product is likely the HbCO $^5T_2$ state. We must point out also that, in spite of the different experimental conditions of this study (our excitation was at 32500 cm$^{-1}$), the transient difference spectra recorded in the HbCO experiments were similar to those reported by Chernoff et al. (6) but with less red shift and no broadening compared to the stable deoxy spectrum (Fig. 4). This means that, after excitation to a high-energy state by the 307-nm pulse, the liganded species relaxed through nonradiative, nonreactive channels to the lowest electronic states that will be efficiently populated. The $^5T_2$ state appears as a likely candidate (but unfortunately not unique) because it is very dissociative toward CO in bringing an electron in the antibonding dz$^2$ orbital which would lead to an increase of the Fe—CO bond distance.

Fig. 3A shows that the CO-protopheme excited photoproduct(s) relaxes completely to the ground state with a single time constant of 3.2 ps and with no evidence of recombination during the first 100 ps after photolysis. This resulted after 20 ps, in a difference spectrum similar to that of the stable deoxy CO-protopheme spectrum. It is difficult at the present time to explain why the lack of difference between the photoprotoheme relaxation and that observed in HbCO and MbCO and the possible influence of the protein matrix on this process. The presence of a chloride ion at the 5th coordination bond in place of the N$_8$ of the proximal histidine (F8) in the protopheme as well as the presence of polyethylene glycol in the close vicinity of the heme (19) may contribute to a new electronic configuration. This could produce a direct relaxation pathway from the dissociative liganded-excited state to the ground state instead of the more complex dynamics including an intermediate non-reactive step with a life-time of more than 100 ps as observed in HbCO and MbCO.

Iron and Ligand Dynamics. It has been reported by Terner et al. (7) that partial out-of-plane displacement (20) of the high-spin Fe(II) occurred within 30 ps, their pulse duration. This result is supported by theoretical considerations on nuclear motions which allow movement of the iron atom in the range of 1 Å in the picosecond or even the subpicosecond time scale. Because our results imply the formation of high-spin Fe(II) de-ligated species with a time constant of 350 fs, it is reasonable to postulate displacement of the iron out of the heme plane within the same time scale. The fact that we did not observe any change of the electronic spectra in the spectral region of the deligated species between 1 and 100 ps sec strengthens this hypothesis.

It cannot be ruled out, however, that some out-of-plane displacement of the iron may account partially for the 350-psec dynamics. The dynamics of the CO molecules must be also examined because in the first 100 ps after photoexcitation, electronic changes in the coordination group upon ligation will modify the absorption electronic spectra. Indeed, the CO molecule interaction volume can be approximated as a sphere with a diameter of 4.4 Å (8). Starting with a bond length of =2 Å the magnitude of the displacement of the CO sphere must be at least 4 Å before the Fe—ligand interaction becomes negligible on the electronic state of the iron. Such a displacement may need a few hundred ps to take place. At this stage, reliable well-founded theoretical considerations are needed to decide which of the data interpretations is the most likely. It is tempting to speculate that the induced absorbance change indicative of a deoxy stable species could appear as long as the ligand collides into the low-energy well in close proximity to the ironporphyrin ring. Evidently much information will be gained by studying these reactions with oxygen as the ligand, at low temperatures, and with time-resolved resonance Raman spectroscopy.

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