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Spectral evidence for sub-picosecond iron displacement after ligand detachment from hemoproteins by femtosecond light pulses

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We have measured spectral and kinetic differences in protoheme, sperm whale or horse heart myoglobin and human hemoglobin following photodissociation induced by optical pulses of 80 fs duration. Full ligation was performed with oxygen or carbon monoxide. Femtosecond kinetics and transient difference spectra revealed the appearance of a deoxy species with $\tau \cong 250-300$ fs. The transient deoxy species in myoglobin and hemoglobin evidenced a 3–4 nm red shift of their ΔA spectra compared with the equilibrium ΔA spectrum. This shift was not observed after photodissociation of the carbon monoxide liganded protoheme. We proposed that the 250 fs time constant corresponding to the appearance of the deoxy-like species is related to the displacement of the ferrous iron out of the heme plane. Consequently, the small red shift of the ΔA spectra observed in photodissociated hemoproteins may be tentatively attributed to changes in the vibrational modes of either the proximal histidine-Fe^{2+} bond and/or of the N_4 porph-Fe-N_ϵ His (F_8) bent.

Key words: hemoproteins/photodissociation/femtosecond spectroscopy/molecular dynamics

Introduction

Picosecond (ps) spectroscopy is a valuable method for the analysis of the initial events following photodissociation of ligands in hemoproteins (Shank *et al.*, 1976; Greene *et al.*, 1978; Noe *et al.*, 1978; Cornelius *et al.*, 1981). We have recently extended its range into the femtosecond (fs) time scale (Martin *et al.*, 1982a,b, 1983). From kinetics and spectral recordings, a deoxy-like species with a 250 fs time constant has been resolved. The large difference between the quantum yields of carbon monoxide (CO) and dioxygen (O_2) liganded hemoproteins was found to have its origin at least in part in relaxation processes during the first picosecond. The present article describes kinetics and spectroscopic experiments of photodissociated protoheme and hemoproteins with a better time resolution (50 fs) associated with a 5 Å wavelength accuracy. Monitoring the early events after photodissociation we are trying to find out whether the displacement of the iron from the heme plane really triggers tertiary structural changes in the protein as proposed by Perutz (Perutz, 1980; Perutz *et al.*, 1982) and Hoard (1971) for the origin of the cooperativity of ligand binding in hemoglobin (Hb). From both theoretical (Hoard, 1971) and experimental (Perutz, 1979) studies, it has been shown that qualitative information on the axial Fe-N_ϵ His (F_8) bonding interaction may be extracted from the visible and Soret spectra. In the stable deoxy spectrum, shortening the Fe-N_ϵ bond either by Fe spin transitions (Perutz, 1979; Scheidt and Reed; 1981) or steric factors led to significant red shift in the visible and Soret bands. With this in mind we have analysed the ΔA transient spectra in photodissociated hemoproteins and ferrous protoheme solutions.

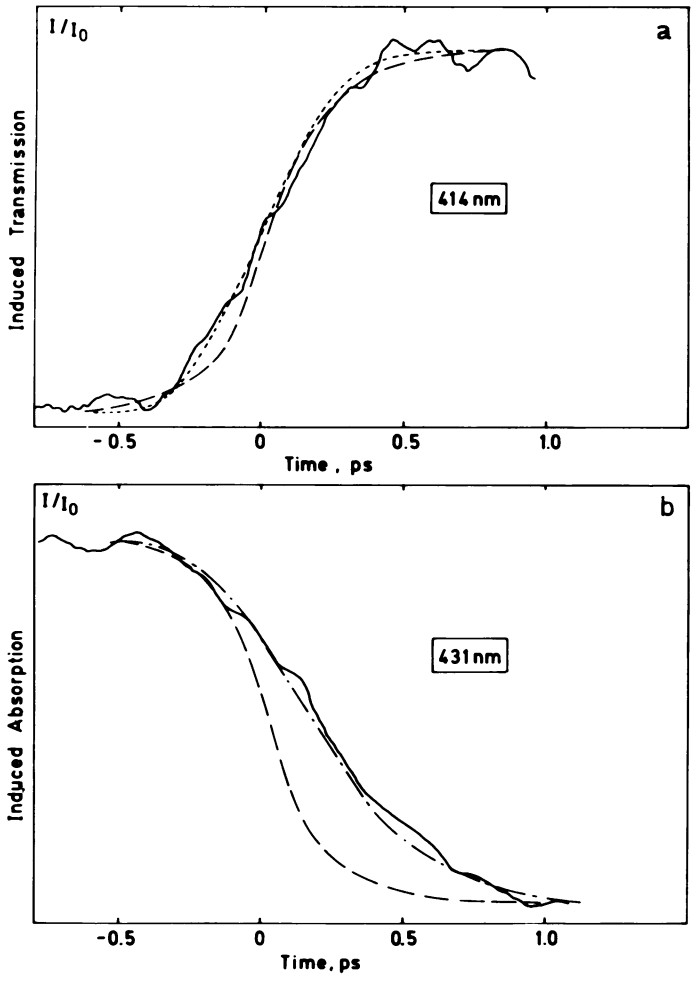

Fig. 1. Normalized kinetic results obtained after photodissociation of HbO_2 solution at 309 nm with 100 fs pulses. **Upper**: heavy lines induced transmission (I/I_0 at 414 nm showing a rise time limited by the pulse duration as indicated by the dotted line (....) which represents the integral of the cross-correlation as determined in a separate experiment. The (- - -) line is the best fit of the experimental trace (heavy curve) assuming an exponential pulse of duration 105 fs and an instantaneous bleaching. **Lower**: induced absorption at the maximum positive change in the stable difference spectrum (431 nm) corresponding to the appearance of deoxy Hb. The heavy line is experimental. The (- - -) and (-.-.-) curves correspond respectively to the computed instaneous response from the upper panel and to the best fit with a time-constant τ_{Fe} = 250 fs. This time constant cannot be due to the group velocity dispersion which shows up as a pure delay and is taken into account in the zero position. The abscissa is 2 ps full scale. Identical results were obtained using 580 nm excitation pulses.

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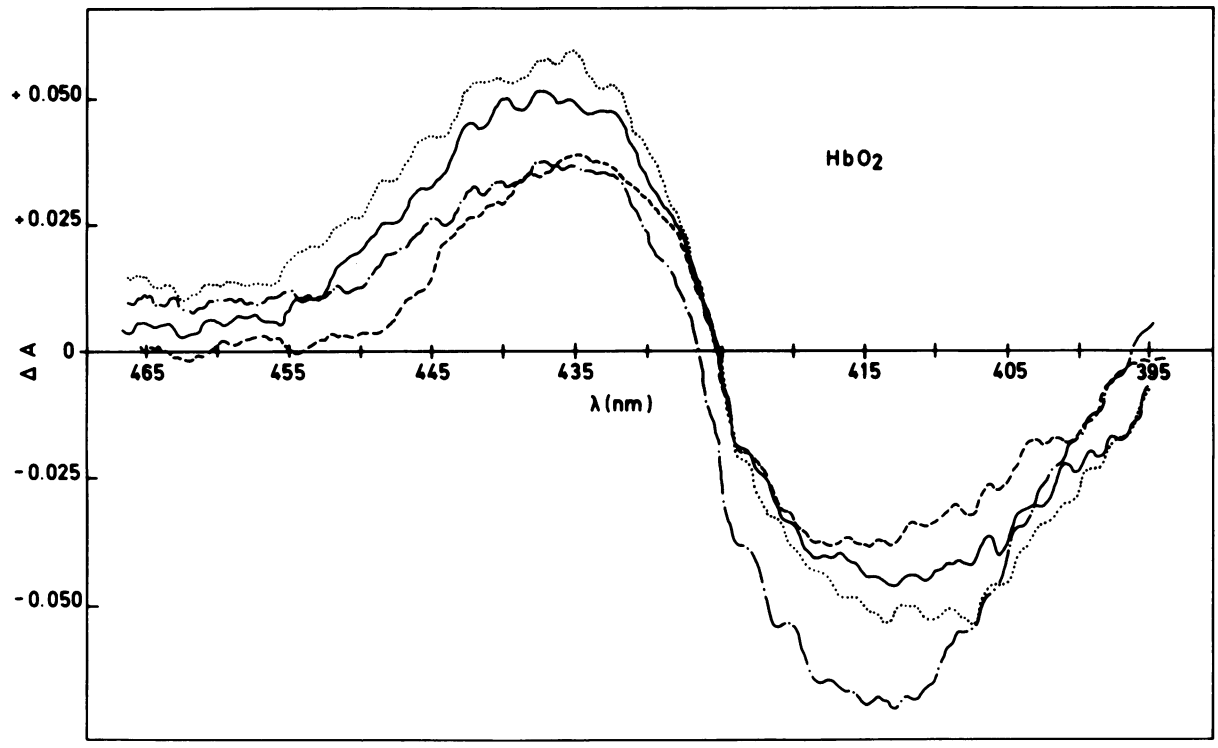

Fig. 2. Transient difference spectra of deoxy Hb-HbO_2 at 0.4 ps (-.-), + 2 ps (....), + 5 ps (—) and + 10 ps (- - -) after excitation with 100 fs pulses at 580 nm. Spectra were recorded with a 2-D optical-multichannel analyzer (OSA, B.M., Spectronik). These show the initial bleaching of the Soret liganded band simultaneously with the appearance of an absorption band in the 450–470 nm region (0.4 ps curve). Note that the latter is most sensitive to the group velocity dispersion in the continuum as indicated by the displacement of the isosbestic point. The increase in absorbance between 0.4 and 2 ps in the Soret deliganded region (435 nm) illustrates the 250 fs time constant (Figure 1, lower panel) in the appearance of the deoxy species. Note that the peak of the positive ΔA spectra from 2 ps to 10 ps lies at 435 nm, i.e., shifted by 4 nm compared with the stable difference spectrum. The 2 ps–10 ps ΔA spectra reveal the relaxation of the excited state to the ground state indicated by the simultaneous recovery of the bleaching (414 nm) and of the induced absorption (455–470 nm).

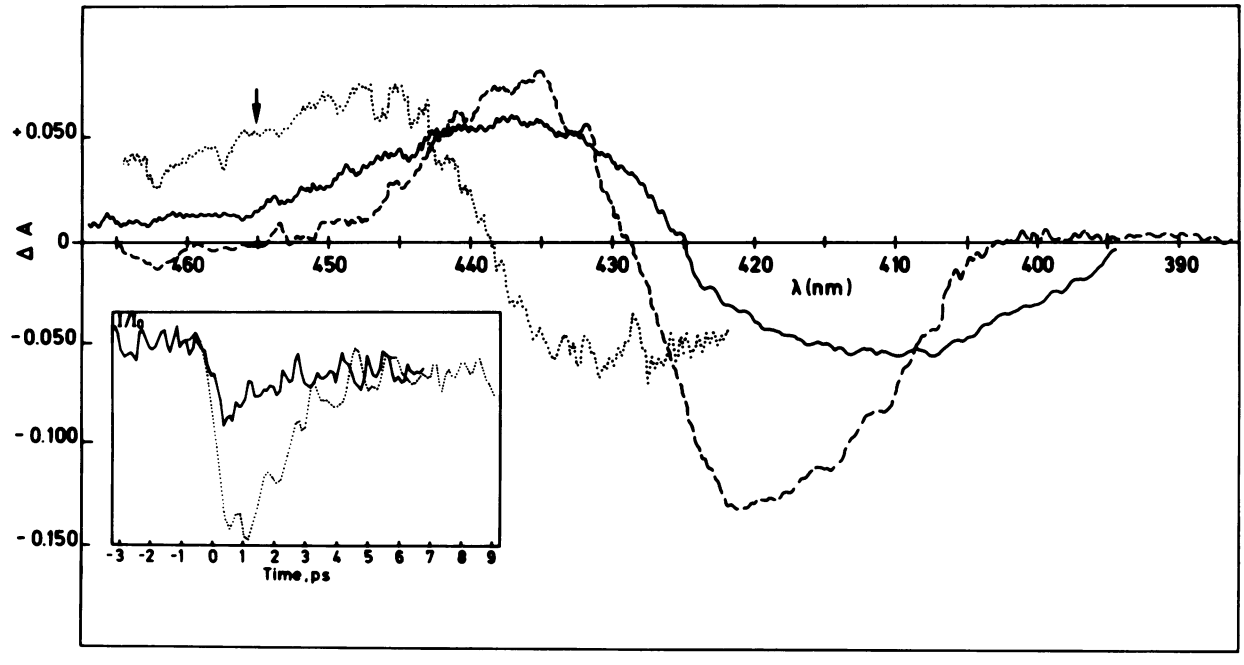

Fig. 3. Transient difference spectra recorded 2 ps after excitation of HbO_2 (heavy line), HbCO (heavy dotted line) and deoxy Hb (light dotted line) with 100 fs pulses at 580 nm. Note the induced absorbance in the 450–470 nm region in deoxy Hb and HbO_2 which is absent in photodissociated HbCO. The inset shows the relative amplitude of this excited state at 455 nm (see the arrow) which is twice as large in deoxy Hb compared with HbO_2. Note the similar evolution in time for the two kinetics.

Table I. Wavelengths of the positive maximum difference spectra at equilibrium and 2 ps after photoexcitation in various liganded hemoproteins

(nm)	Protoheme CO	MbCO	MbO_2	HbCO	HbO_2
$\lambda(\Delta A$ max) stable	425.5	438	437	431	431
$\lambda(\Delta A$ max) + 2 ps	425.0	441	440.5	435.5	435
$\Delta\lambda$	−0.5	+3.0	+3.5	+4.5	+4.0

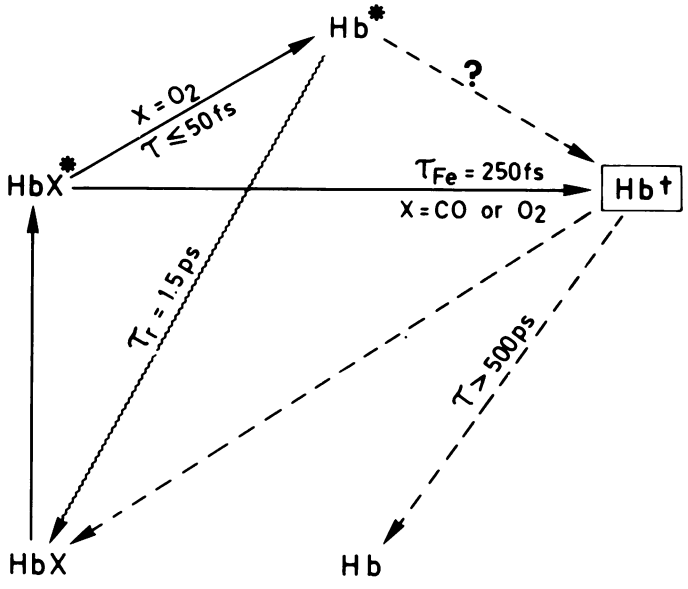

Fig. 4. Schematic model of the photolytic pathways of CO or O_2 liganded Hb after excitation with 100 fs pulses at 580 nm. Hbx* represents the photodissociation excited states below the Q band. x indicates either CO or O_2. Hb* is a transient excited deoxy species which absorbes in the 450–470 nm region. $Hb^{\dagger}$ is a vibrationally excited state (see text). The excitation of deoxy Hb is also shown.

Results and Discussion

Figure 1 shows the changes in absorption in the Soret region of oxyhemoglobin (HbO_2) after excitation at 309 nm. Similar results were obtained when the excitation wavelength was set below the Q band region at 580 nm. These changes consisted of the bleaching of the Soret band of HbO_2 at 414 nm and the appearance of an absorption at 431 nm, close to the absorbance band of deoxy Hb. The intensity of the bleaching at 414 nm corresponding to the disappearance of the liganded species was instantaneous, i.e., limited by the pulse duration, but the induced absorption at 431 nm occurred with a time constant of 250 fs (best fit of the experimental trace, Figure 1b). These kinetics were identical to those obtained in oxymyoglobin (Martin *et al.*, 1982a) indicating their independence of the protein structure. Precise examination of the ΔA spectra in the few first picoseconds following photodissociation of O_2 (Figure 2) showed an excited species whose broadness extended up to 470 nm with a maximum absorbance peak near 455 nm (ΔA spectrum of HbO_2 in Figure 3). The appearance of this species coincided within 50 fs with the bleaching at 414 nm, which was instantaneous. This species relaxed with a time-constant τ_r = 1.5 ps at least partly to the ground state, as indicated by the simultaneous attenuation of the bleaching at 414 nm.

It has been mentioned above that an induced absorption at 431 nm occurred with a time constant of 250 fs. The spectral

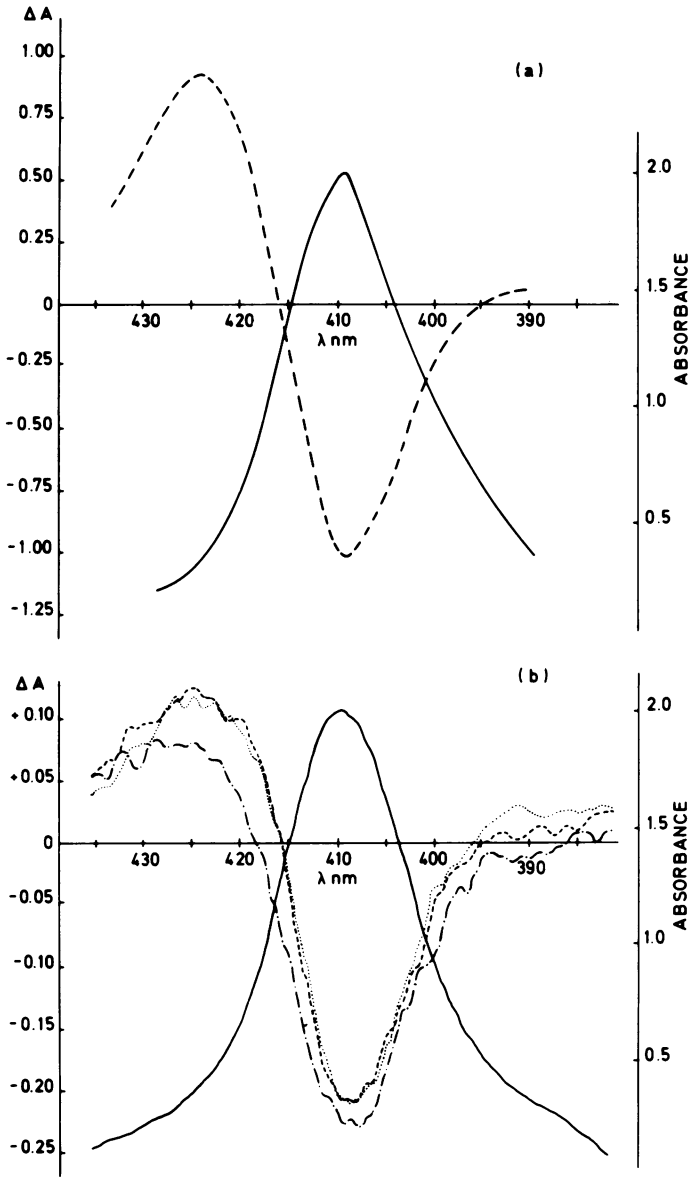

Fig. 5. Equilibrium **(a)** and transient **(b)** difference electronic spectra (deoxy-CO liganded) recorded in 95% PEG 0.1 M protoheme solutions. The middle full line curves compare the Soret liganded absorption bands recorded in **(b)** at −5 ps. Note the almost perfect superimposition of the transient spectra and of the stable ΔA spectrum. The isosbestic point at 416.3 nm is within our 5 Å wavelength accuracy identical in **(a)** and **(b)**. The scale of the ordinate in **(b)** is enlarged by a factor of 5. Symbols: _●_●_ 0.5 ps, ---- 7.5 ps, ... 50 ps after excitation respectively.

changes which accompany these kinetics are shown in Figure 2. Focusing on the ΔA spectra deoxy region (431 nm), we observed that the positive peak of the photodissociated HbO_2 lies 4 nm shifted to the red compared with the stable deoxy species. Similar results have been found in myoglobin (Mb) and Hb solutions either liganded with O_2 or CO (Table I). Identical red shifts were seen after excitation at 309 nm or 580 nm which indicated the existence of a dissociative channel below the Q band in CO and O_2 liganded heme proteins. The deoxy red shifted species in photodissociated Mb and Hb lasted up to 500 ps, the upper limit of our observation time. These intermediate states should be distinguished from the initial electronic excited state observed after photodissociation of HbO_2 in the 450–470 nm region (Figures 2 and 3). Almost

identical transients (in shape and kinetics) were found after excitation of fully deoxygenated Hb (Figure 3). However, in the latter, the change in absorbance was twice as large as in HbO_2. The short-lived electronic excited state was ~10 times less at 455 nm after photodissociation (+ 2 ps) of any of the CO liganded hemoproteins. The existence and similarity of electronic excited states in HbO_2 and deoxy Hb should stem from either steric factors like the fully developed (deoxy) or partial doming of the heme structure in HbO_2 (Shaanan, 1982) and in MbO_2 (Philipps, 1980) and/or from the presence of an equal mixture of initial spin-paired states (Case *et al.*, 1979) ($Fe^{S=1} - O_2^{S=1} \rightleftharpoons Fe^{S=0} - O_2^{S=0}$) as compared with a mainly populated low spin state in CO liganded species ($Fe^{S=0} \rightleftharpoons CO^{S=0}$).

The interpretation of our results is summarized in Figure 4. From this model, it follows that after 2–3 ps, corresponding to the disappearance of the excited species (Hb*), the only deoxy species present is an excited intermediate $Hb^{\dagger}$ whether it originates from O_2 or CO liganded proteins. The existence of short-lived Hb* species should be taken into account in the interpretation of data from picosecond absorption and resonance Raman spectroscopic studies using pulses of several picoseconds which may favor multiphoton processes and not allow these different excited species to be distinguished.

We have performed similar experiments in protoheme solutions. As previously observed (Martin *et al.*, 1983), the bleaching at 410 nm is instantaneous while the deliganded species appears at 425 nm with $\tau \cong 350 \pm 50$ fs. The initial bleaching at 410 nm is followed by a relaxation phase with a time constant of 3.2 ps which corresponds partly to the disappearance of an excited species in the 435–440 nm region. The deliganded ΔA spectrum, which lasts up to 200 ps, is within our experimental accuracy similar to the stable equilibrium spectrum in the 415–435 nm domain. Particularly, we did not observe any red shift of the maximum positive ΔA peak at 425 nm (Figure 5).

It has been proposed (Perutz, 1979; Wang and Brinigar, 1979) that the red shift of the Soret λ_{max} observed on going from the T to the R structure of deoxy Hb be attributed to a reinforcement of the covalent Fe-N_ϵ interaction bond at the fifth coordination position of the Fe^{2+}. This has been confirmed in resonance Raman spectroscopy experiments by analysis of the shifts of the 215 cm^{-1} band assigned to the Fe-N_ϵ stretching frequency (Nagai and Kitagawa, 1980; Irwin and Atkinson, 1981; Ondrias *et al.*, 1982). The displacement of the iron out of the heme plane toward the proximal imidazole has also been suggested from picosecond resonance Raman spectroscopy of photolysed HbCO (Terner *et al.*, 1981). Our observation that the red shift occurred in the presence of the Fe^{2+}-N_ϵ bond but not in protoheme (Table I and Figure 5) suggests that the spectral changes in the 431 nm region (Figure 1b) may be attributed to the doming of the porphyrin ring following the iron displacement from the heme plane. This suggests that the $Hb^{\dagger}$ species corresponds to a vibrationally excited state, the energy of which is partially stored in the Fe^{2+}-N_ϵ bond mode (200 cm^{-1}) and in the pyrrol N_4-Fe-N_ϵ bond mode (80 cm^{-1}). This would imply a deformation of the imidazole ring of the proximal histidine restrained by the polypeptide chain until tertiary changes occurred in a nanosecond to microsecond time scale (Friedman *et al.*, 1982). In protoheme no protein restrains the direct relaxation to the deoxy ground state.

The small increase in energy stored in these different vibrational modes may be sufficient to trigger further steric changes leading to an enhancement in the free energy of the system. In tetrameric Hb, on a much longer time scale, further steps of deoxygenation and/or larger photodissociation yields might set in motion complementary contributions by the protein on the heme, as proposed by Perutz (1970).

Materials and methods

Fast kinetics and Δ spectra were recorded after photoexcitation by 100 fs pulses generated by a passively modelocked CW dye ring laser (Fork *et al.*, 1982, Martin *et al.*, 1982b). Amplification of these pulses to powers of the order of 10 gigawatts (GW) has been achieved using a four stage dye-amplifier pumped by a Q-switched frequency doubled Nd-Yag laser (Migus *et al.*, 1982). Temporal broadening due to group velocity dispersion in the dye solvent and amplifier optics was removed by a grating pair introduced at the output of the second stage (Fork *et al.*, 1982; Migus *et al.*, 1982). This set-up finally produced GW pulses at 10 Hz in the 100–120 fs time regime. Half the energy was used to generate a broad band femtosecond continuum while the other half was focused either into a 1.5 mm KDP crystal to produce up to 20 μJ, 100 fs pulses at 309 nm or into a 2 cm water-cell to generate a second continuum from which a selected spectral part was further amplified to GW power. In this latter case, we have used the tunability of the system to adjust the excitation frequency just above the presumed dissociating channel in carbonmonoxyhemoproteins (Waleh and Loew, 1982). Thus, both kinetics and transient spectra were measured after excitation with pulses at 309 nm and at 580 nm with 100 fs duration. The energy flux was adjusted to be comparable in both cases.

Purified Hb solutions were prepared from fresh human blood by DEAE-Sephadex chromatography and diluted in 0.1 M phosphate buffer pH 7 or 8 at ambient temperature. Mb (sperm whale type II or horse heart type III from Sigma) was obtained following the procedure of Rothgeb and Gurd (1978). Protoheme was prepared from hemin (Fe^{3+}.Protophorph.Cl^-) as described elsewhere (Martin *et al.*, 1983) in mixtures of 94% polyethylene glycol/6% NaOH 1 N (v/v). Dilutions of the stock solutions were made so as to obtain absorbances of the Soret liganded species of 2.2–2.4 in a quartz cuvette of 0.05 cm light path. During the experiment the cuvette was moved horizontally so that each pulse at 10 Hz excited a new region of the sample. Equilibrium spectra recorded before and after each run were identical indicating the absence of denatured material.

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