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Electronic Relaxation and Oxygen Recombination Processes in Photodissociated Oxyhemoglobin after Picosecond Flash Photolysis

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Oxyhemoglobin photolysis has been investigated with picosecond laser techniques. Transient light absorption changes observed within 500–600 nm reveal two processes following photodissociation: electronic relaxation up to 400 ps after dissociation and a partial religation during 3 ns. The kinetics of oxygen geminate recombination at pH 7 and 22°C has a monoexponential decay with a lifetime of 1.5 ns ± 0.1 ns.

INTRODUCTION

A number of nano-[1,2] and picosecond[3,4] photolysis investigations have been used to describe the dynamics of hemoglobin structural adjustments by following the binding kinetics of the sixth axial ligand (CO or O_2). Recent experiments indicate that hemoglobin,[5] like a large number of other protein studied,[6] has rapid structural fluctuations that permit the penetration of oxygen molecules into the protein matrix. These studies generate a new

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interest in describing the details of the pathway by which the ligand reaches the heme.[5,7] CO geminate recombination in hemoglobin, at room temperature,[1] has been shown to proceed via a random walk mechanism[8] in the protein phase. Previous efforts to observe the same effects with O_2 as the ligand, at low[9,10] and ambient[4] temperatures, have been equivocal.

In the present work we show that relaxation processes in hemoglobin—causedby ligand detachment—perturb the optical observation of the ligand recombination kinetics during the first 400 ps. We also demonstrate that picosecond photodissociation experiments provide information on oxygen recombination (after photodissociation) only if the kinetic evolution is followed at a wavelength where the absorption change due to these relaxation processes is low. We have determined such a wavelength and have attempted to approach the dynamics of the picosecond oxygen binding in hemoglobin.

METHODS

Hemoglobin was extracted by the technique of Perutz,[11] from fresh human adult blood. Oxyhemoglobin samples were used at room temperature (22°C ± 0.5°C), 50 mM potassium phosphate buffer (pH 7). The concentration used in the experiments was adjusted to give an O.D. = 1 at 532 nm and a path length of 1 mm. The hemoglobin sample was periodically exchanged with fresh solution to prevent possible denaturation. Photodissociation was made with solutions saturated with O_2 at atmospheric pressure and also under 30 mm of oxygen.

Photodissociation of HbO_2 was produced by a 30 ps pulse of the second harmonic (532 nm) from a YAG Quantel laser.

The wavelength of the interrogating beam was selected by a monochromator (Huet, focal length 1.25, 1200 grooves/mm); the interrogating beam continuum[12] was produced by focusing the 40 ps residual fundamental pulse (1064 nm) on a cell filled with D_2O (Figure 1). The resolution of the interrogating beam was 0.5 nm and the beam aperture was f/200. Under these conditions the modification of the pulse shape is negligible. A variable optical delay was used to obtain the time dependence of the absorption change.

A reference beam (I_2), by passing the sample, was isolated by a glass beam splitter (2 cm thickness). The remaining fraction (I_1), the interrogating beam, passed through the sample volume at a small angle (< 40

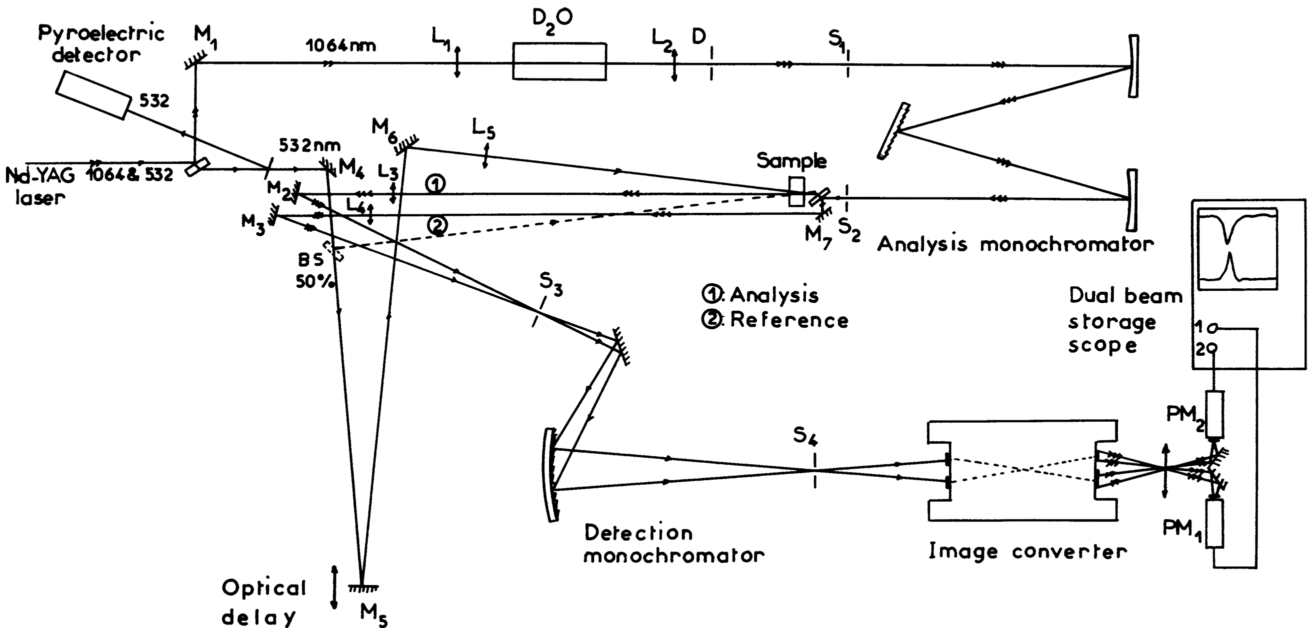

FIGURE 1 Experimental arrangement BS beam splitter, M_i mirror, L_i lens, S_i monochromator slit, F KG3 filter, G and H gratings, PM_i photomultiplier, M_5 optical delay

mrad) to the photodissociation pulse. The reference and interrogating beams were focused on the same point of the entrance slit of a 0.5 m monochromator (home built) working in the first order with a 1800 groves/mm grating from Jobin-Yvon. This experimental arrangement eliminates all parasitic light, particularly residual light due to the photodissociation beam, from the interrogating beam. The two beams emerge from the monochromator at different angles and fall upon two different areas of the photocathode of an image intensifier tube set 50 cm beyond the exit slit of the monochromator. Each spot on the image intensifier emits light at 425 nm with a lifetime of $\simeq$ 400 microseconds. Separate photomultiplier tubes were used to detect the intensity of each spot. The difference between the logarithms of the intensity ratio, I_1/I_2, with and without photodissociation, gives the variation of absorbance in the sample as a function of the time delay as determined by the setting of the optical delay line. The time delay was variable up to 6 ns.

The photodissociating and interrogating beams overlapped in the sample volume; the photodissociating beam diameter was 1 mm while the interrogating beam diameter was 0.8 mm. The intensity of the interrogating beam was adjusted low enough to avoid photodissociation yet high enough to permit the measurement of the absorbance to within an accuracy of $\pm$ 0.02 for an optical density of 1.

The effect of the photodissociating pulse (532 nm) energy on the dissociation rate was investigated by using a fraction of this pulse, delayed by 53 ps, to interrogate the sample after the incidence of varying energies of the main pulse. A saturation effect of the photodissociating beam appeared above 0.3 J/cm^2; all studies reported here were performed with beam energies of 0.4–0.6 J/cm^2. To check if successive pulses could generate local thermal gradients and produce irreversible protein damage, the same region of the sample was excited twice by pulses delayed 4.5 ns with respect to each other. Exactly the same absorption changes were found at the end of only one pulse monitored at 532 nm or after the two pulses (4.5 ns) demonstrating that the first photodissociating laser pulse did not cause hemoglobin degradation. However, each interrogating volume of the sample was exposed to only one laser shot, and examined in a 5 ns time range.

Although full dissociation of HbO_2 was produced by the energy of the laser pulse, the measured O.D. variations at some wavelengths indicated that photodissociation was incomplete (Figure 2, spectrum A). This phenomenon is a general problem for all picosecond spectroscopic investi-

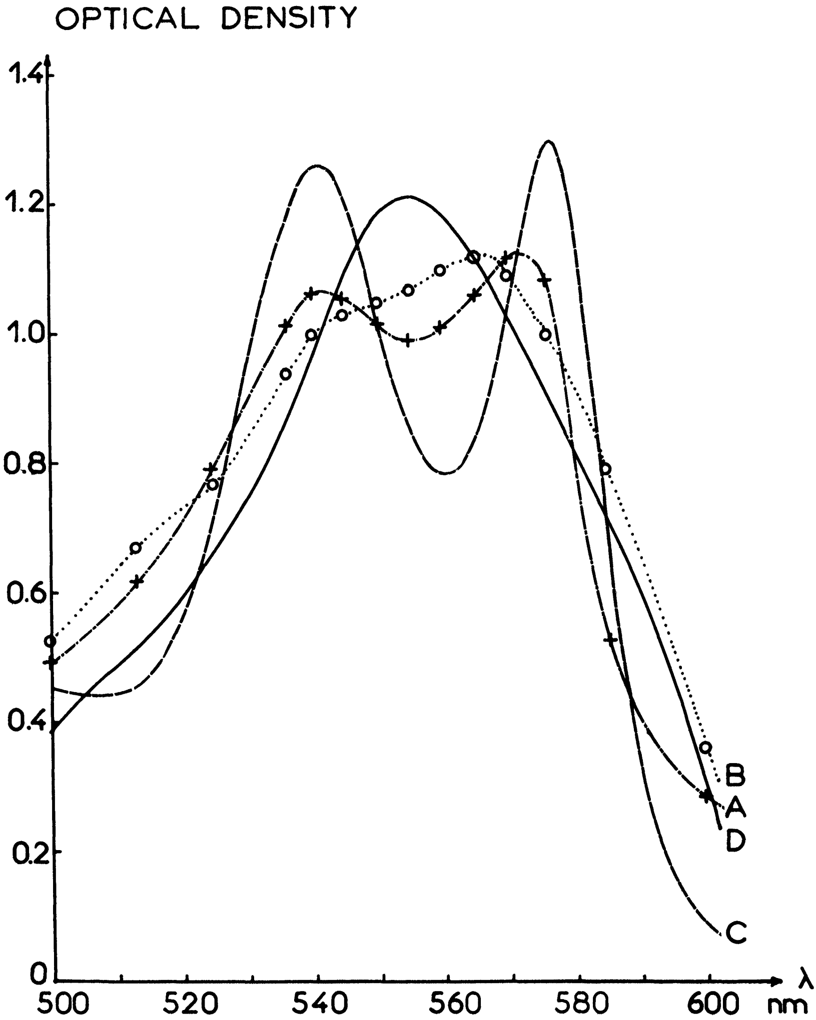

FIGURE 2 HbO_2 spectra: C, Hb stable (D), experimental transient Hb (A) and calculated (B) obtained with O.D.$^{1mm}_{532nm}$ of 1 for HbO_2 sample; the optical density was obtained with 0 delay time. Points (500,512,525,536,545,550,555,560,565,570,576,585,600nm) indicate the investigated wavelengths.

gations which use the continuum light produced by the residual 1064 nm beam. Indeed, in this case the real widths of each interrogating pulse extracted from the continuum are not identical and do not necessarily match the 30 picoseconds photodissociating pulse at 532 nm; generally these pulse widths are slightly longer. But the temporal behavior of the fundamental pulse cannot give an interrogating pulsewidth longer than 100 ps. Since the photodissociation and interrogating pulses do not have a perfect time overlap, the Δ(O.D.) at zero delay time is always lower than the Δ(O.D.) that we should obtain; thus a more or less pronounced spectral distortion is observed. In this work, only kinetic studies after picosecond photolysis have been analyzed; temporal measurements are not affected by this difficulty. Each value of the O.D. at a given time was averaged over 5 laser pulses.

RESULTS

Initial photodissociation

O.D. changes following laser excitation were monitored at 532 nm at which wavelength the interrogating and photodissociating pulses are perfectly identical. The HbO_2 photodissociation rates were analysed 53 ps after the maximum intensity of the photodissociating pulse which corresponds to the end of laser irradiation.

The amplitude of the optical density variations was directly proportional to the laser energy at low excitation energies. At high intensities the amplitude of these variations was independent of the laser energy (Figure 3). At this high energy level the photodissociation of the residual oxyhemoglobin becomes difficult because most of excitation photons are absorbed by deoxy Hb. The optical density observed under conditions of light saturation must be corrected for the influence of the interrogated pulse width and of the fraction of residual oxyhemoglobin. In the 500–600 nm range the corrected spectrum (Figure 2b), is similar although not identical, to those observed for stable deoxyhemoglobin and for unligated hemoglobin originating from photodissociated HbCO.[1]

Transient state evolution

The data reported here were all obtained under conditions of light saturation. The time dependence of the transient state was constructed from

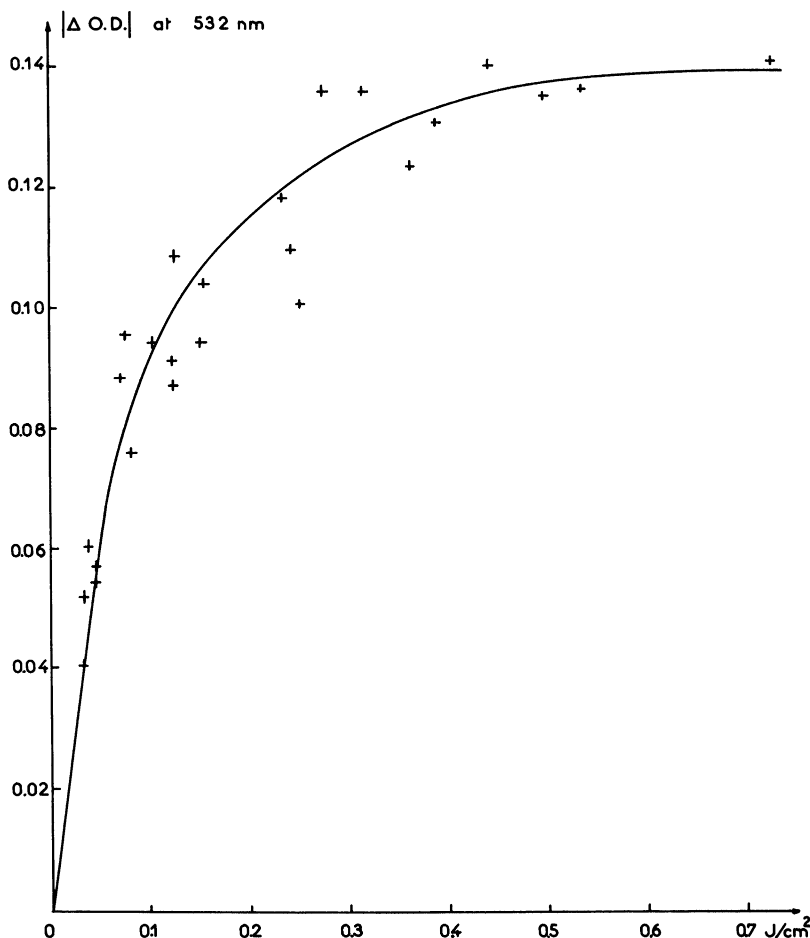

FIGURE 3 HbO_2 optical density changes as a function of laser energy. Photodissociation and measurement were made at 532nm; with a pathlength of 1 mm.

the variation of the sample O.D. measured after different delay times, Δt, from the excitation laser pulse. This treatment was made at different wavelengths of the continuum interrogating light where the Δ(O.D.) between HbO_2 and de-ligated Hb shows maximum amplitude (542,560,576 nm). It was found that for each wavelength the O.D. evolution during the first 400 ps was different. During a fraction of this time, some transient effects

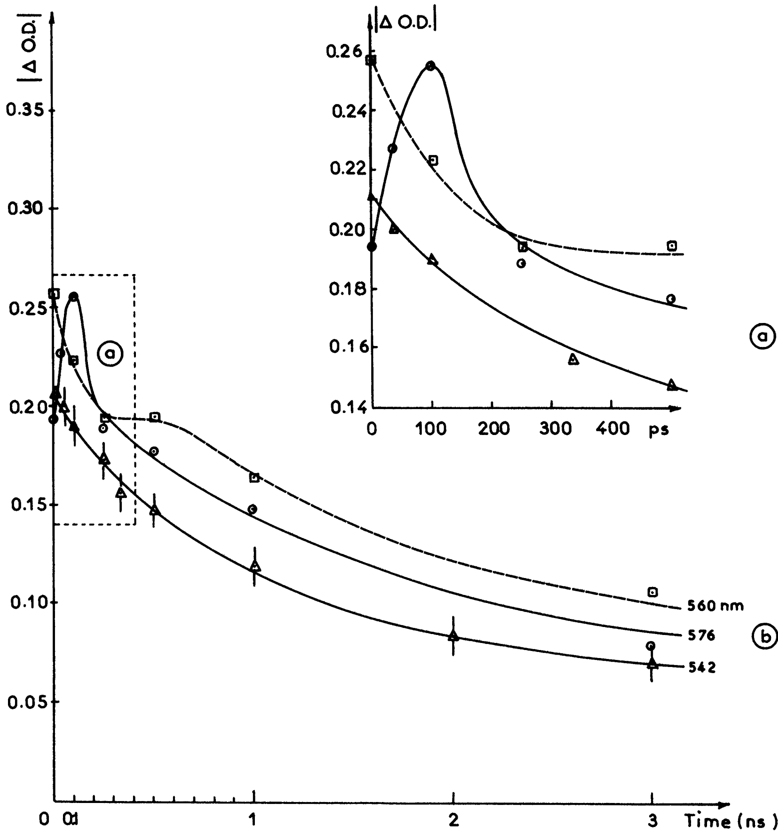

FIGURE 4 Absorption variation as a function of time after photolysis for a HbO_2 concentration corresponding to an O.D.$^{1mm}_{532nm}$ of 1. Measurement with the absolute value of Δ O.D. were made at 576 (○), 560 (□), 542 (Δ), Δ| O.D.| error bar = ∓0.01. Each point was averaged over 5 laser pulses. a/ Main preponderant electronic relaxation process recombination during the first 400 picoseconds; b/ relaxation evolution and partial oxygen recombination during the first three nanoseconds after flash photolysis.

progress in a direction opposite to that expected for a recombination process. The O.D. monitored at 576 nm first increased during 100 ps and then decreased. The O.D. monitored at 560 nm, decreased during the first 250 ps and then remained almost constant over a second period of 150 ps. Similar measurements at 542 nm showed absorption changes with a monotonously decreasing amplitude. These changes are reproduced in Figure 4. If these evolutions reflected only a single process, we should have

$$\left(\frac{\Delta O.D.\ (t)}{\Delta O.D.\ (t = 0)} \right)_{\lambda i} = K(t)$$

totally wavelength independent. On the basis of our results, we must assume that these O.D. evolutions are complex and partially due to electronic relaxation processes produced by the oxygen de-ligation. At 542 nm the corrected photode-ligated Hb and stable Hb absorbances are closely similar. This data and the spectral evolution (at this wavelength) confirm our interpretation.

Oxygen religation

After 450 ps the O.D. evolution becomes parallel for all wavelengths monitored and progress in the direction expected for a recombination process (Figure 4). The results show that 65% of hemoglobin has recombined with oxygen 3 ns after photodissociation.

Partial recombination can occur in the first few hundred picoseconds after photodissociation although we cannot observe this oxygen binding step because of spectral modifications in this time range. However, to attempt to measure the recombination of photode-ligated oxygen as a function of time after the laser pulse, kinetic analysis on the total absorption changes at 542 nm was performed. We attempted to fit the data using combinations of the terms :

$$\exp(-t/\tau) \text{ and } \exp[(-t/\tau)^{1/2}]$$

The best fitting of the data was obtained for:

$$\Delta O.D.(t) = A_1 (1 - e^{-t/\tau_1}) + A_2 (1 - e^{-t/\tau_2}) \quad (1)$$

where the ΔO.D. origin is the deoxygenated hemoglobin absorbance produced just at the end of the photolytic laser pulse. By the least square method, the fit gave:

$$A_1 = 0.018 \pm 0.005 \qquad A_2 = 0.132 \pm 0.004$$

$$\tau_1 = 190 \pm 70 \text{ ps} \qquad \tau_2 = 1.5 \pm 0.1 \text{ ns}$$

The extent of the O.D. change due to the electronic process is about 14% of those due to the total oxygen recombination during the 4 first nanoseconds. Assuming that the relaxation disappears with the same time course at all wavelengths, the oxygen recombination kinetic law is the second exponential in Eq. (1). With this hypothesis, the kinetic properties of the oxygen binding can be investigated from 250 ps after the laser flash photolysis. Oxygen recombination in the time range of 250 ps to 3 ns has not been reported previously, and this region was further investigated at different wavelengths. It was found that the rate constant ($1/\tau_2$) of HbO_2 formation at 542 nm was equally applicable at other wavelengths. Thus, the exponential model for picosecond O_2 recombination kinetics is supported by the experimental results.

Investigations with 30 mm partial oxygen pressure gave the same data. Thus we must conclude that this recombination process, after photodissociation, is produced by the O_2 excaped from its original heme. Also the rate constant observed can be attributed to this geminate effect.

DISCUSSION

Spectral differences between stable deoxyhemoglobin and the photoproduct from HbO_2 have already been reported.[3] However, the transient spectrum observed at the end of the laser photodissociation pulse depends upon the conditions of the interrogating pulse irradiation (Figure 2). The interrogating laser pulse width affects the degree of absorbance change observed under photolytic light saturation conditions. This observation may account for the apparent discrepancies in the results from various studies.

In a previous article,[1] Alpert et al. described a rapid CO-hemoglobin recombination process (also reported by Duddel[2]), and showed it to be a geminate reaction by analyzing that the geminate recombination[8] occurs during the time the deligated CO is diffusing in the protein matrix. Similar behavior is expected after photolysis of HbO_2. We have examined here the geminate pair recombination rate of O_2 and hemoglobin as a function of time. Careful work of HbO_2 picosecond photolysis shows the oxygen binding kinetic law as a single exponential decay (Figure 4) with a lifetime of 1.5 ns. The principal implication of this finding is that the oxygen ligand, after photolysis, contrary to the carbon monoxide, stays in the protein cavity near the heme and does not diffuse through the protein bulk before 4 ns. These differences between O_2 and CO ligation could be

interpreted in terms of electrostatic interactions between the ligands and the amino-acid residues. Further experimental work is required to determine the importance of such interactions.

Fluorescence quenching data suggest that ligand penetration is not restricted to a narrow channel.[5] The internal mobility of different parts of the protein is certainly the most important parameter determining the kinetics of ligand partition[7,13] between the protein interior and the aqueous medium. These Hb fluctuations are revealed by the random walk type diffusion of de-ligated CO molecules[8] through the protein before the rebinding to the iron.

The principal implication of this finding is that the time differences in the cage effects for CO and O_2 are not totally controlled by diffusion in the viscous protein medium. The diffusion rate inside the protein may differ for different ligands, or geminate recombination may be strongly coupled with the specific reactivity of the iron for each ligand. For O_2, the highly efficient reaction between the iron and O_2 apparently competes with ligand diffusion through the protein matrix or perhaps the O_2 recombination is due to transient trapping[14] of the de-ligated O_2 molecule on a single specific site in the protein, since refixation on the heme gives exponential kinetics.

It is also possible that the oxygen molecule is not trapped in a single site of the proteic matrix, but returns to its corresponding heme through the empty space of the heme-pocket without being trapped. With this assumption, recombination of the original partners is expected to be produced during the very first picoseconds after the photolysis. In this case, the O_2 recombination process measured 250 ps after the photolytic laser pulse ought to be a new fast re-ligation stage of hemoglobin. In these conditions, it is obvious that the rapid recombination reaction should be due to oxygen molecules in the proteic phase[5,13] located at some sites[14] in the heme pocket cavity. Whatever it may be, kinetic experiments (Figure 4) indicate that the O_2 recombination process can only be observed 250 ps after ligand removal by flash photolysis. Recently Chernoff et al.[4] have interpreted the absorbance changes in the 200 ps range of photodissociated oxyhemoglobin as being due to the geminate recombination. Our photolytic pulse has a 30 ps duration while that of Chernoff et al. is 10 ps. Perhaps the time of electronic relaxation decay depends on the laser pulse width, shorter pulses creating faster decay. However, it is more probable that the formation and disappearance of this transient electronic state are independent of the irradiation conditions. In this case, this assignment of the

picosecond spectral evolution to the geminate recombination proposed by Chernoff et al.[4] must be erroneous.

On the basis of our results we estimate the upper limit for the relaxation time to 400 ps, which must represent the rearrangements of the heme group accompanying the oxygen photolysis process. It is known that the transition from HbO_2 to Hb is followed by large structural changes in the heme[15] and its environment.[16] These changes are induced by electron transfer processes[17] between the iron and the ligand on the one hand, and the iron and the porphyrin on the other. The first phase of the picosecond absorbance (Figure 4a) change might reflect the electronic reorganization process of the unligated heme[15–18] accompanying electron transfer from the iron oxygenated form to the deoxygenated iron species.[17] Although the kinetic details of these changes in hemoglobin iron properties have not yet been investigated, their existence have been known for a long time.[19] Further detailed examination of the electronic structure of the heme after deligation at high time resolution, could identify the processes that control this relaxation. We must note, here, the pioneering work on myoglobin carried out along these lines by Eisert et al.[20] who observed an heme electronic charge redistribution within the 450 ps period following photodissociation.

In conclusion we propose that the initial changes in the absorption after photodissociation of HbO_2 is due to electronic reorganization of the heme group and that the absorption changes, observed after this reorganization, are characteristic for the geminate oxygen-hemoglobin religation.

Acknowledgments

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