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Quantum Yield of Thirty Picosecond Oxyhemoglobin Photodissociation. Effect of Protein Fluctuations on the Quantum Yield Values

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We have measured the absolute quantum yield of the oxyhemoglobin photodissociation with a 30 ps laser pulse excitation. We report that oxyhemoglobin dissociates with the same yield as carboxymyoglobin under photostationary conditions. Based on the picosecond and nanosecond experimental data, we show that oxygen geminate recombination is responsible for the low quantum yield values of oxyhemoglobin photodissociation measured with longer photolysis flashes. Kinetic data suggest that two geminate regimes which successively appear in oxygen recombination can be resolved. The first can be entirely attributed to the hemoglobin-iron chemical reactivity and, the second to the action of protein fluctuations.

INTRODUCTION

Since the historical observations that the heme-ligands are photodissociable,[13,24,6,1] ligand detachment by light excitation has played a central role in investigations of the ligand binding mechanisms in hemoproteins. Continuous or microsecond flash photolysis studies[1,18,4,5,21] on various liganded hemoproteins have shown that the photodissociation efficiency of these compounds appeared to be considerably dependent on the nature of the iron-ligand and the apoprotein organization. For oligomeric hemoproteins, their photosensitivity was found to vary with external factors such as temperature

and ionic strength. It was concluded[22] that the quantum yield (Q.Y.) of photodissociation of liganded heme-proteins were correlated with the electronic configuration of the heme itself. But this configuration was supposedly modulated by the nature of the ligand and the conformation of the apoprotein moiety. This hypothesis was held for several years.

Recently, Duddel *et al.*[8,9] and Alpert *et al.*[2] have independently shown that the variation in the measured quantum yield for identical heme ligand complexes in different protein environments could be explained by the fast rate of geminate recombination of photodissociated ligand. According to the latter point of view, the effective photochemical dissociation quantum yield of liganded hemoproteins must be measured before the geminate recombination processes. Under these conditions, nanosecond quantum yield measurements on carboxyhemoglobin (HbCO) and its isolated α and β subunits were given quantum yield values identical to those of carboxymyoglobin (MbCO).[2] On a longer time scale, the quantum yield of HbCO dissociation was found to decrease.[9] So, the notion that the value of the quantum yield of photodissociation of liganded heme-proteins was dependent on photolytic pulse width was proposed.

The present study represents an extension of previously reported experiments on the oxygen geminate recombination in hemoglobin.[23] In this case we have focused our efforts on the direct determination of the Q.Y. of the oxyhemoglobin (HbO_2) picosecond photodissociation. This work clearly shows that with a 30 ps pulse, the HbO_2 also dissociated with an initial quantum yield value very close to unity. Consequently, the experiments demonstrate that the protein factor does not affect the action of the light in the initial dissociation of the ligand from the heme in hemoproteins. Picosecond and long time quantum yield analysis suggest that different values of the quantum yield are obtained because of apoprotein fluctuations which increase the time of the interaction between the iron-ligand partners.

MATERIAL AND METHODS

Hemoglobin was extracted from fresh human blood according to the method of Perutz.[19] Protein solutions were adjusted to pH 7 in 50 mM

potassium phosphate buffer. Hemoglobin concentration of the samples was adjusted to give an O.D. = 1.36 in a 1 mm path length cell at the excitation wavelength 532 nm. Hemoglobin samples were periodically exchanged to prevent photochemical denaturation. Photodissociation was performed at 22°C + 0.5°C, using a 30 ps pulse of the second harmonic (532 nm) from a YAG Quantel laser described elsewhere.[11] The interrogating beam continuum was produced by focusing the 30 ps residual fundamental pulse (1064 nm) on a cell filled with D_2O. Oxyhemoglobin photolysis has been investigated by the same method of Valat *et al.*[23].

A photodissociating gaussian pulse produced an inhomogeneous irradiation in the sample volume which perturbs the data. Thus the laser beam was defocused to a small circle having a diameter of 1.5 mm. Under these irradiation conditions, the photon distribution in any part of the irradiated surface is the same with an experimental error <7% between the periphery and the center of the circle.

The absorbed energy of the photodissociating pulse by the sample was investigated by measuring the reflected fraction of this pulse on cell (Figure 1). To check the linearity of this method, we measured samples having only neutral absorption (without any photochemical decomposition). The intensities of the two lights beams, the reflected fraction (R) was measured with a ED 100 pyroelectric detector and the transmitted (T) with a RKP 335 energy probe. Until 100 μJ, the complementary intensities between the absorbed ($1-T$) and the

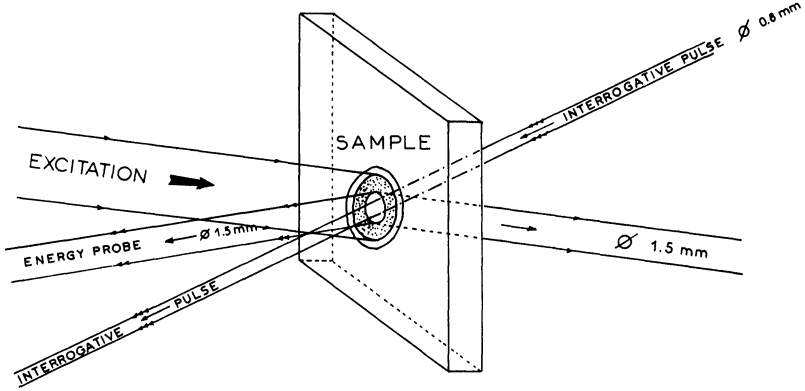

FIGURE 1 Experimental set-up for the measurement of the absorbed energy in the irradiated sample volume.

reflected (R) pulses were correct. Since the O.D. change of picosecond photodeligated and stable deoxyhemoglobin species has the maximum amplitude at 576 nm and 100 ps after the laser irradiation,[23] analysis of the effect of the energy of photodissociating pulse on the dissociation rate was performed at this wavelength and 100 ps after the photodissociating pulse. Compare to the data obtained during the 30 ps photodissociating pulse, these conditions increase about 20% the precision of the photolysis observation.

The photodissociating (532 nm) and interrogating (576 nm) beams overlapped in the sample volume. The interrogating beam diameter was 0.8 mm and the intensity of its spot was minimized to avoid photodissociation yet still high enough to measure the absorbance of the sample with an accuracy of 0.02.

RESULTS

1. Photodissociation

In this work only initial photolysis of HbO_2 after laser excitation has been measured. In Figure 2 the extent of HbO_2 photodissociation was plotted against laser energy absorbed by the sample. At low laser excitation energies, the O.D. amplitudes measured were directly proportional to the absorbed light energies. At high intensities the O.D. amplitudes became non linear, being dependent on the laser energies owing to a light saturation effect. Under light saturation conditions, previous work indicated that the transient spectrum was not the stable deoxy spectra. The measured O.D. variations at some wavelengths indicated that photodissociation was about 25% incomplete.[23] At this high photodissociation level, the picosecond optical density would have to be corrected for a fraction of deoxy hemoglobin (Hb) which would absorb some excitation photon. Care should be taken to avoid these effects in picosecond ligand photodissociation. Since the exact mechanism of the HbO_2 photodissociation is not known it is possible that for some other reasons,[20] the HbO_2 molecules present in the interrogated volume cannot be completely deligated. Under the experimental conditions employed herein there was the possibility that a fraction of HbO_2 was not photodissociated at high laser energies. We must note that with shorter pulse widths Chernoff *et al.*[7] report a dissociation of 75% of the HbO_2.

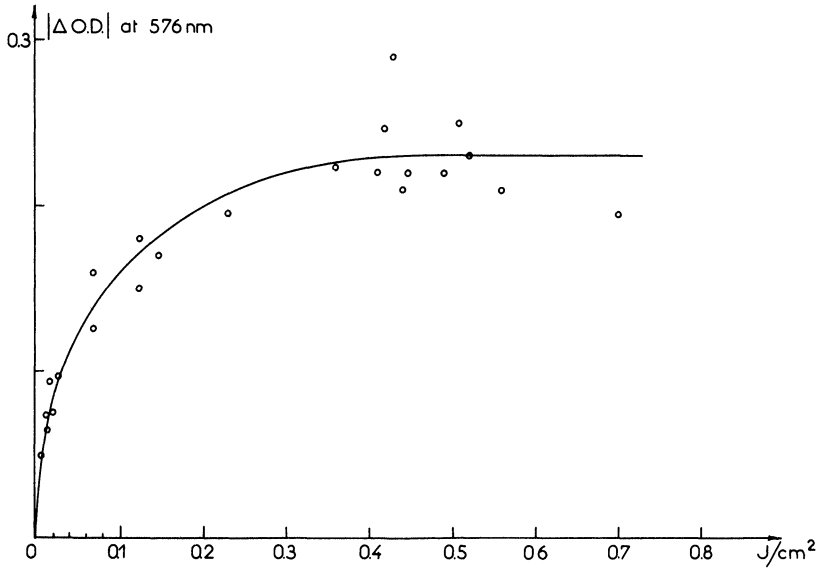

FIGURE 2 Optical absorption changes (at 576 nm) versus energy density of light excitation (at 532 nm) $O.D._{532nm}^{1mm} = 1.36$ for HbO_2 sample.

2. Quantum yield

The course of the spectral change under light saturation gave the extent of the HbO_2 photodissociation. In the worst case a fraction of 25% is not photodissociated. For this reason, each experimental photolysis yield was systematically corrected by 25% and plotted as a function of laser energies. Consequently the Q.Y. value determined by this method must be interpreted as the lowest limit for the HbO_2 photodissociation.

Preferably the Q.Y. should be measured when there is a very small perturbation induced by light. In fact, it is not possible to determine the Q.Y. for the picosecond photodissociation of HbO_2 at very low levels of excitation intensity.

We have worked between approximately 15-45% on full photodissociation, and used the initial slope of the photolysis curve extrapolated to zero level of photodissociation (Figure 3). From the slope, 15×10^{14} photons was used to photolyze 9.6×10^{14} heme molecules (in

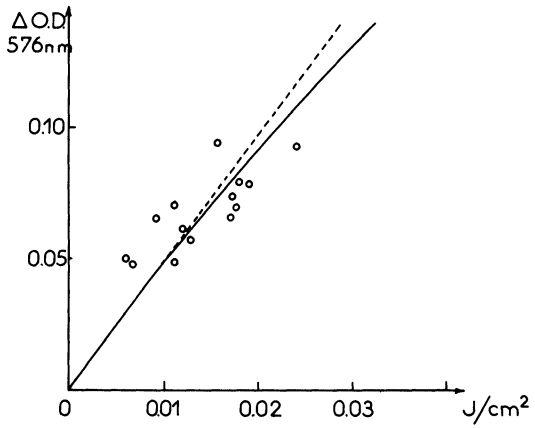

FIGURE 3 Slope determination of HbO_2 low photolysis rate.

1 mm^3) with two main sources of error: (a) ±5% in the slope determination, (b) ±10% for the exact value of the absorbed energy. From these considerations the lowest limit of the picosecond photodissociation quantum yield of HbO_2 was 0.65 ± 0.15.

However if the picosecond transient spectrum represents the full dissociation of HbO_2, the Q.Y. reaches 0.85.

DISCUSSION

1. Effect of a transient deoxyhemoglobin species on the initial quantum yield value

More than 75% or almost complete photodissociation of HbO_2 was obtained with 30 ps laser pulses having sufficient intensity. Under light saturation conditions, it is reasonable to consider that the oxygen deligation is complete and that the spectrum of photodeligated hemoglobin is different from that of stable deoxy hemoglobin.[20,23] However, at the present time, the true Hb spectrum of the picosecond photodeligated species[7] is unknown. Alternately, one may also consider that only 75% of deligated hemoglobin was obtained. In this first study, we have preferred to choose the latter position which permits us to give a lower limit of the Q.Y. Firm spectroscopic assignments for theoretical descriptions are important, but for Q.Y. determination at

low level excitation, it is obvious that the results, in both interpretations, give values of the same order. Additional experiments to find the short-lived species after photolysis of HbO_2 can only increase the picosecond Q.Y. value, which is already close to unity, by a small amount.

2. Photodissociation process

Indirect Q.Y. measurements published in the literature mentioned the possibility of different channels for photon energy dissipation. These authors[12,7] suggested that this photolysis occurred by multiphoton dissociation processes. We have examined this possibility by plotting the photodissociation yield against the intensity of laser irradiation. The pattern of the photodissociation curve shows no evidence for a multiphoton absorption effect (Figure 2). Therefore we propose that the picosecond HbO_2 photodeligation is a mono-photonic process. This is suggested by the Q.Y. value of 0.65 which we measured.

3. Photolysis pulse-width effect on the quantum yield value

When the photolysis is produced in 30 ps, the Q.Y. value of the HbO_2 photodissociation is 0.65. This value is very close to the HbCO photodissociated Q.Y. measured with 7 ns pulse-width[2] and the Q.Y. reported for MbO_2.[17] The Q.Y. of HbO_2 dissociation decreases significantly when the photolysis pulse-width increases.[1,21,9,10] The large difference between the Q.Y. values measured with picosecond and long pulse-widths can be simply explained by a O_2 geminate recombination processed with Hb. For long duration light pulses many photolyzed HbO_2 molecules recombine during the pulse, hence, it is easy to understand why the Q.Y. value will depend on the kinetics of the geminate binding and the pulse-width. From this particular aspect, it is possible to compare the fraction of the O_2 geminate recombination at different times with the values of the Q.Y. measured with corresponding pulse-widths (Table 1).

Picosecond[23] and nanosecond[16] geminate kinetics give consistent values with the Q.Y. reported previously.[21,8] The quantitative agreement between the photolysis yield and the kinetic data confirms the idea that the geminate religation of the iron-ligand pairs with rates faster than the measurement is the principal cause of the low values

TABLE 1
Kinetics and Q.Y. correlation

Time	ps	ns	μs
Q.Y. measured	0.65[a]	0.24[b]	0.05[c]
Deligated Hb fraction		0.35[d] (at 5 ns)	≃0.2[e] (at 1 μs)
Q.Y. calculated		0.65 × 0.35 = 0.23	≃0.23 × 0.2 = 0.046

[a] This work.
[b] From Duddel *et al.* (1979).
[c] From Saffran and Gibson (1977).
[d] From Valat *et al.* (1982).
[e] From Lindqvist *et al.* (1983).

of the Q.Y. for HbO_2 photodissociation (see Table 1). From our kinetic data,[23] in a 30 ps photolysis experiment less of 2% of O_2 molecules can recombine with the iron. The almost total absence of O_2 geminate recombination during the picosecond pulse and the high value of the Q.Y. stresses the importance of taking the picosecond Q.Y. value for the initial (or true) Q.Y. of the HbO_2 photodissociation.

4. Protein dynamics and quantum yield

Different factors affect the long-time quantum yield measurements.[1,18,4,5,21] Particularly, an increase on the temperature increases the Q.Y. of the photodissociation.[21,8]

In the CO geminate recombination, the rate of the escape of CO from the protein also increases with temperature.[2] This rate should depend of the ligand diffusion inside the protein. Thus, it is interesting to compare the different photodissociation yield in connection with the diffusion processes of the ligand through the protein.

Using 30 ps fwhm pulse we have shown that only 65% of the deligated O_2 molecules recombined with the iron within 5 ns and this recombination followed a single exponential kinetic law.[23] Using laser pulses of 1.5 ns fwhm,[16] one can see the above exponential term of O_2 geminate recombination followed by a second O_2 geminate phase lasting 100 ns. This second term was found to obey $\sqrt{t}$ kinetics. It is evident than with a 30 ns fwhm pulse,[9] only the second geminate phase can be observed.

At long photolysis times (>7 ns) the ligand recombination rate (for both CO and O_2) varies with the square root of time.[15,16] In this case the kinetics of the ligand binding is influenced by several factors and especially by the diffuse displacement of the ligand inside different parts of the protein structure. This random migration of the ligand is certainly highly controlled by the structural fluctuations of the polypeptide chains which occur on a very long (>5 ns) time scale.[14] This suggestion supports the idea that the O_2 and CO molecules have approximately the same diffusion velocity inside the protein matrix. Only the chemical interaction between the iron and the ligands are dependent of the ligand nature. From this point of view the data obtained from the data at shorter photolysis times (<1.5 ns) can be interpreted. The picosecond time scale of our observation in liquid phase is too short to observe any movement of the protein, and so the ligand is inside a "rigid protein" as a cristal state. The rigid apoprotein conformation acts as a hard wall during a brief period keeping the newly freed ligand close to the heme, until with time the protein dynamics allow the ligand to penetrate into the protein matrix. During this time the ligand is sufficiently close to the heme and strongly interacts with it and can recombine with the iron. Only the chemical reactivity of the iron for the ligand determines the reaction between the partners. Exposure of HbCO and HbO_2 to picosecond laser irradiation[7,23] supports this proposition, since only HbO_2 exhibits a first-order rate constant of ligand binding during the first ns after the photolysis.

The protein mobility appears after about 5 ns and starts to govern the ligand diffusion inside the apoprotein matrix.[16] Thus, there is a competition between the iron attraction for the ligand and the fluctuations of the apoprotein which randomly displaces the ligand into the matrix. In this regime the probability of religation is inversely proportional to the iron-ligand distance. Hence for large inter-pair separation the iron-ligand interaction can be expected to vanish, and the transfer probability of the ligand from the protein to the surrounding solvent becomes important. Consequently, only a fraction of the ligand (35%) which remains inside the protein can diffuse back towards the iron.[23]

5. Conclusions

Exposure of HbO_2 to picosecond laser pulses and HbCO to nanosecond laser pulses produce ligand photodissociation quantum

yield of 0.65 and 0.8 respectively, which is close to that observed for MbCO under photostationary conditions. These findings suggest that the heme-O_2 complex which is reputed to be less photolabile than the heme-CO is in fact just as photosensitive as the latter complex. It is concluded that the protein and the ligand do not affect the heme primary Q.Y. in hemoproteins.

The data presented herein also show that the variations in the Q.Y. of HbO_2 photodissociation observed for different photolysis times can be ascribed solely to the protein fluctuations which govern the motions of the ligand inside the apoprotein. Like a large number of other proteins, structural fluctuations of hemoglobin seem to be in the nanosecond time scale (>5 ns). Thus in nanosecond photolysis experiments, the recombination of iron-ligand pairs exhibit diffusion controlled processes. In contrast, the O_2 geminate recombination after picosecond photolysis exhibits a first order kinetic reaction which certainly approaches the intrinsic chemical reactivity of the iron and the oxygen molecule. Additional experiments investigating the effect of ionic strength and protein concentration on the picosecond time scale, before the diffusional phenomenon can take place, should indicate if these factors act on the iron chemical activity. Similar studies on nanosecond time scale would assist the understanding of the protein dynamics.

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