PICOSECOND TRANSIENT ABSORPTION STUDY OF PHOTODISSOCIATED CARBOXY HEMOGLOBIN AND MYOGLOBIN

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ABSTRACT The optical transient absorption spectra at 30 ps and 6.5 ns after photolysis are compared for both carboxy hemoglobin (HbCO) and carboxy myoglobin (MbCO). Both 355- and 532-nm excitation pulses were used. In all cases the shapes of the optical difference spectra thus generated are stationary over the complete timescale studied. The photolysis spectra for MbCO are not significantly different from the equilibrium difference spectra generated on the same picosecond spectrometer when measured to an accuracy of ±0.5 nm. In addition, spectral parameters for deligated HbCO generated on the same spectrometer but detected by two different techniques, either by a Vidicon detector or point by point with photomultiplier tubes, are reported; the results are different from some of the previously reported picosecond experiments.

INTRODUCTION

In recent years there has been considerable interest in the time dependence of the optical absorption spectra of heme proteins after photodeligation. This interest is sparked by the possibility of correlating the various structural changes with the observed spectroscopic evolution and extracting kinetic parameters for these processes. Processes which occur on the nanosecond and subnanosecond timescale are of particular interest because spectroscopic changes would be expected to reflect protein structural changes that are coupled to the movement of the iron out of the heme plane after deligation (1).

While there has been a good deal of work on the transient optical spectra of the photolysis products of these proteins in the nanosecond (2–8), picosecond (9–15), and femtosecond (16, 17) timescales, there are relatively few experiments that carefully monitor the evolution of these spectra between the picosecond and nanosecond time regimes. The spectra reported for these two timescales are different (3, 4, 9, 12), which, if correct, implies that structural changes must be occurring on the timescale 10 ps to 5 ns. For example, at 10 ns after photolysis the difference spectra for carboxy myoglobin (MbCO) are identical with those observed for an equilibrium mixture of the ligated-deligated pair (4). In contrast, most of the picosecond and subpicosecond spectra thus far obtained for MbCO show transient spectra that are somewhat different from the equilibrium difference spectra (13). The same is true for the corresponding carboxy hemoglobin (HbCO) difference spectra (3, 9, 13). Presumably if there are changes occurring at the heme during the period from a few picoseconds to 10 ns they should produce changes in the optical absorption spectrum. The purpose of the present study is to discover whether such changes are observable.

MATERIALS AND METHODS

Commercial ferric human Hb and sperm whale Mb (Sigma Chemical Co., St. Louis, MO) were further purified on a Sephadex g100 or g25 column, buffered to pH 7.4 with 0.02 M Tris, degassed, converted to the deoxy form with the addition of Na dithionite, and saturated with carbon monoxide. The concentrations were adjusted with CO-saturated buffer to give optical densities of 0.8–1.2 at the Soret maximum in a 1-mm sealed quartz cell. Samples prepared in this manner were stable indefinitely when stored at or below room temperature.

The laser system used in these experiments consisted of an actively and passively mode-locked pulsed Nd-YAG laser, generating a train of pulses 30 ps in width. The selected pulse was amplified in three stages to give a maximum output energy of ~20–30 mJ at 1.064 μm. Second and third harmonic generation of the amplified infrared pulse was used to obtain the pump beams used in these experiments. The residual 1.064-μm beam in the amplified pulse was used to generate a broad band continuum by tight focusing into a cell containing D_2O. The continuum was separated into two parallel beams of nearly equal intensity with the use of a 50% broadband beam splitter. The two principal reflections from the beam splitter served as the probe and reference signals in our optical detection scheme. The two beams were focused on separate regions of the sample cell, collimated, then imaged on the slits of a spectrometer. The pump beam (532 or 355 nm) traversed a variable delay line arranged so that delays between the pump and probe of up to 7 ns were obtainable. The pump beam was overlapped with the probe beam at the sample at an angle of ~15°. Overlapping of the pump and probe beams was not difficult because the pump beam was only lightly focused at the sample

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cell to provide a beam waist of ~1.5 times that of the probe. Residual scattered pump beam was removed with the appropriate cutoff filter at the entrance slit of the spectrometer.

The broad band spectra reported here were obtained with a 300-groove/mm grating and an extended output coupler to disperse both the probe and the reference beams on to different regions of the target of a PAR model 1250 Vidicon detector (Princeton Applied Research, Princeton, NJ). All spectra were measured at room temperature. Data acquisition and firing of the laser were controlled with an LSI11/23 microcomputer. In this manner, the Vidicon was scanned synchronously with the firing of the laser, and the data was processed on a shot-by-shot basis. It is well known that the response of Vidicon detectors is somewhat nonlinear, and particularly so when the illuminating source is a short light pulse (18). In the experiments reported here light levels of less than 2,000 counts/channel per shot were determined to be necessary to avoid any overt nonlinear response. In addition, we found that optimum conditions required at least six scans of each track between shots. The spectra shown here are typically the average of four or five runs of 200 laser shots per run. In a normal sequence a background was collected with the laser blocked, a ratio of the probe and reference beam collected with the pump beam blocked, and the pumped transient spectra collected. In this manner long-term fluctuations in the background were accounted for. The spectrometer operated in this manner had a spectral resolution of 0.25 nm. The spectra shown in Figs. 1–3 are typical of the many sets of data obtained for the various photolyzed species. The shot-to-shot and background fluctuations resulted in nonsystematic deviations of up to 5% of the total full-scale absorptions. In addition these fluctuations would be expected to introduce a random uncertainty of ±0.5 nm to isosbestic point values.

In addition to this method, some point-by-point spectra, free from any Vidicon artifacts that might occur, are reported here. The optical pumping scheme was the same as just described. The probe and reference beams were resolved into single frequency components using a 600-groove/mm grating and 0.25-mm output slits. The two outputs were each directed to photomultiplier tubes and the resultant signals were then simultaneously sampled and digitized. In addition, the pump beam was also monitored such that signals could be normalized to account for shot-to-shot power fluctuations in the laser. A single data point reported here is typically the average of 50 laser shots.

RESULTS

Photolysis of MbCO

Fig. 1 shows the optical transient absorption spectrum generated by the photolysis of MbCO by a 532-nm pulse at time delays of 0 and 6.5 ns after photodeligation as obtained with a Vidicon detector. The spectra are independent of time delay within the limits of the resolution of our spectrometer. As mentioned in the previous section these are typical spectra. The wing at 450 nm for the 6.5-ns data is a "random" difference which amounts to ~3% of the total OD scale but appears exaggerated in the plotting process. In other sets of spectra the optical densities for the two time delays agree to within 0.2%. A similar situation is observed when the MbCO is photolyzed with 355 nm light. In addition, the spectra generated by both the 532 and 355 nm photolyzing beams appear identical to each other. The critical parameters that characterize the spectra reported here are listed in Table I. As one can see, the values for the minima and maxima of absorption as well as the isosbestic point are somewhat different from those obtained for a Mb-MbCO equilibrium difference spectrum using a conventional spectrophotometer. The difference indicates a red shift of the spectra similar to that reported by other workers (12) for picosecond photolysis of MbCO. However, the equilibrium difference spectrum obtained by separately inserting Mb and MbCO samples into the picosecond continuum as the illuminating source and using the Vidicon head as a detector gave difference spectra identical to those observed by transient photolysis (Fig. 2 and Table I). Furthermore when the difference spectra were recorded using photomultipliers (PMTs) the parameters were identical to those of the equilibrium difference spectra. This indicates that the Mb-MbCO transient difference spectra observed here are not significantly different from the equilibrium difference spectrum within the resolution of our spectrometer.

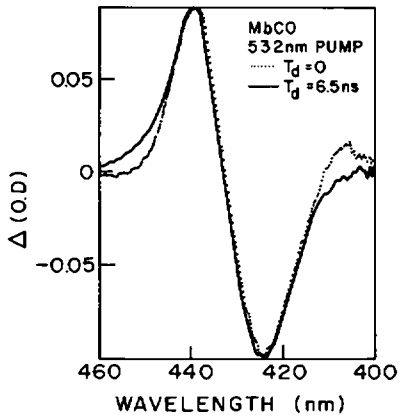

FIGURE 1 Mb-MbCO difference spectrum with 532-nm excitation at delays of 0 and 6.5 ns. The amplitudes of the spectra shown here have each been normalized to an arbitrary value and smoothed with a five-point fitting routine. Actual values of the minima and maxima of difference optical density for the two spectra agreed to within 10%. The wavelengths are accurate to within 0.25 nm.

Photolysis of HbCO

The results obtained for the photolysis of HbCO are analogous to those for MbCO. The spectra obtained with either 532- (Fig. 3) or 355-nm excitation do not change through 6.5 ns nor do the critical parameters depend on the excitation wavelength (Table I). The plots for 355-nm excitation of HbCO are identical to those for 532-nm pump and are therefore not shown here. These parameters are different in this case than those for the conventional Hb-HbCO equilibrium difference spectrum, in agreement with nanosecond studies which show that the transient Hb spectrum evolves into the equilibrium Hb spectrum with relaxations at ~50 ns, 0.5–1.0 μs, and 20 μs (3, 5, 6). The pump spectra are easily distinguishable from the equilibrium spectrum as generated on our spectrometer. The time dependence of the Hb-HbCO photolysis spectra was also obtained using the point-by-point technique described in the experimental section. There is no significant time evolution of the spectra through 6.5 ns.

TABLE I
Mb-MbCO AND Hb-HbCO DIFFERENCE SPECTRA PARAMETERS

Species	Pump	Delay	Apparent wavelength		
			Minimum	Isosbestic	Maximum
		ns	*nm*	*nm*	*nm*
Mb–MbCO	532	0/6.5	424	433.7	439
	355	0/6.5	424	433.5	439
	532*	0/6.5	423	430	437
	Continuum probe	Equilibrium	424	433.5	439
	Reference 4	10 and equilibrium	423	430	437
Hb–HbCO	532	0/6.5	418.5	427.8	435
	355	0/6.5	418.8	427.5	435
	532*	0/6.5	417	425.5	432.5
	Conventional spectrometer‡		419	425	433

*Spectra taken by point-by-point method. ‡Reference 11.

DISCUSSION

The optical transient difference spectra observed on photolysis of MbCO and HbCO with 30-ps pulses are the same as those observed with longer pulsed (10 ns) laser systems and, in the case of MbCO, by steady-state techniques. However, they differ markedly from those previously observed for the same species when picosecond or subpicosecond pulsed lasers were used for photolysis.

The first complete picosecond transient absorption spectrum was recorded by Greene and co-workers (9) for HbCO using 8-ps pulses at 532 and 353 nm for excitation and a picosecond-generated continuum. The double-beam method of recording picosecond transient spectra introduced in that study and which was used subsequently by many investigators on a variety of time scales yielded photoinduced difference spectra that appeared in a time shorter than the pulse duration, and were shifted and broadened compared with the equilibrium difference spectra. Furthermore these differences persisted for hundreds of picoseconds (9, 11, 12). It was suggested (9) that multiphoton processes may have distorted the spectra. The more recent femtosecond studies (16, 17) showed the Hb and Mb spectra appearing more slowly than the Soret bleaching, suggesting that the iron shifts out of the heme plane in a few hundred femtoseconds, a conclusion supported by molecular dynamics simulations (19). However additional transients having lifetimes of ~1 ps were observed and attributed to electronically excited forms of ligated or deligated heme protein (16, 17, 20). Obviously in experiments using 8-ps pulses these intermediates would be efficiently excited by later portions of the pulse, and nonlinear effects could become important. In contrast, in the work by Rentzepis and co-workers (10), where the transient spectra of photolyzed MbCO were recorded point-by-point with a PMT as a detector, the spectra were claimed to be identical with equilibrium difference spectra.

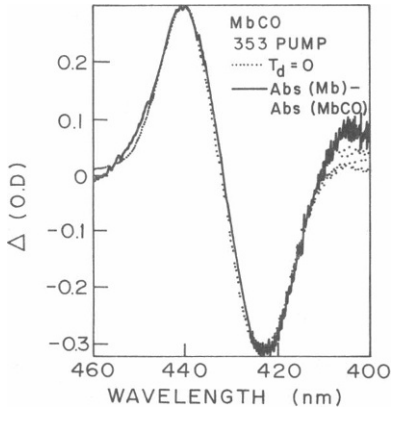

FIGURE 2 Mb-MbCO difference spectrum with 353-nm excitation at zero delay and equilibrium difference spectrum taken on the transient spectrometer with the picosecond continuum as a source. Data treated as in Fig. 1.

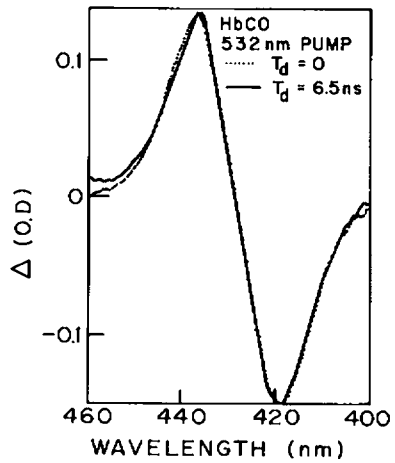

FIGURE 3 Hb-HbCO difference spectrum with 532-nm excitation at delays of 0 and 6.5 ns. Data treated as in Fig. 1.

This experimental result was questioned by Martin and co-workers (16). Transient difference spectra obtained for the photolysis of MbCO using 10-ns pulses (4) are also virtually identical with the equilibrium difference spectra.

Picosecond resonance Raman studies also have generated some unreconciled results concerning the evolution of the photolyzed protein structure during the picosecond-to-nanosecond time regime. For MbCO photolysis the Fe-histidine stretching frequency was found to reach its equilibrium value by 30 ps after photolysis (21), whereas the coremarker bands were reported to be shifted at 30 ps (22). Furthermore, the Fe-histidine bands of Hb from HbCO had the same frequencies at 30 ps and 7 ns, suggesting that photogenerated Hb undergoes no changes in heme-histidine structure during that period. The Raman and optical absorption spectra are related directly, so that the observation of a Raman shift at 30 ps (22) implies that the optical spectrum must also be shifted. However the visible band resonance was used to obtain the core marker Raman spectrum and the Soret for the present absorption studies, so that it is difficult to make a quantitative relation between the two observations. Our results do suggest that it might be worthwhile to examine the transient absorption in the visible region where the effects of any slight perturbations on the structure as indicated by the Raman shifts might be more evident.

In this work, the 30-ps pulse duration precludes observation of the fast processes observed earlier. However the higher repetition rate allows extensive averaging of the data and hence improvements in signal-to-noise ratio. Furthermore, the peak powers are at least 10 times smaller than in the 8-ps pulse experiments and less than 1/100 of the femtosecond pulse intensities. Whereas the difference spectrum for the photolysis of MbCO at 0 and 6.5 ns appears red shifted compared to the standard equilibrium difference spectrum, it is virtually identical to the equilibrium difference spectrum obtained with the same picosecond spectrometer. In addition, the transient difference spectrum of HbCO yields an isosbestic point identical to the reported (3, 5) nanosecond photolysis value when the spectrum is obtained point by point with a PMT, although it appears shifted to longer wavelength on the spectrometer when a Vidicon is used as the detector. In both MbCO and HbCO we see no evolution of the transient difference spectrum between 30 ps and 6.5 ns. These results indicate that all of the fast (picosecond) processes which occur are complete well within the 30-ps resolution of our spectrometer, and the spectra we obtain are not distinguishable from those generated with nanosecond pulses.

X-ray crystallographic studies on liganded and unliganded Hb in the R quaternary structure indicate that globin conformational changes associated with changes in the geometry of the heme complex upon deligation are expected to occur before the R $\rightarrow$ T quaternary transition at ~20 μs (1). These include motion of the E and F helices associated with displacement of the iron from the heme plane, doming of the porphyrin ring, and slight tilting of the heme planes (1, 23). A recent study of the nanosecond geminate rebinding kinetics of HbCO by Murray et al. (6) indicates that the difference in the overall bimolecular association rates between the R and T quaternary structures can be explained by the differences in the geminate processes, which have a relaxation time of ~40 ns. The observed scaling of the geminate yields and bimolecular rates implies that any structural changes that influence the rate of the geminate process occur in a time much shorter than 40 ns (6, 7). The present study suggests that such changes occur in less than ~30 ps. In fact, as mentioned above, spectral changes were observed on the sub-30 ps timescale (12, 16, 17, 20) and in one case interpreted as the initial iron displacement at ~300 fs (16, 17, 19). Identification of other conformational changes in this time regime will be complicated by interference from electronic and vibrational relaxation processes.

Comparison of the spectra generated on a Vidicon with those obtained using a PMT as a detector suggests that the former might lead to somewhat distorted spectra when picosecond continuum pulses are used as the probe. A marked nonlinear response of the detector in a region of strong absorption such as the heme Soret distorts the transient spectra obtained upon deligation. Another possible explanation for the occurrence of broadened spectra with ~8-ps glass laser excitation pulses, which is not seen in the present experiments, is heating of the protein molecule. Recent molecular dynamics calculations by Henry et al. (24) indicate that this could be a contributing factor in picosecond experiments. In these calculations the absorption of a photon at 532 or 355 nm is simulated by adding the photon energy to the kinetic energy of the heme, and monitoring the thermal relaxation of the heme. For Mb, a biexponential decay with components of 1–4 ps and 40 ps is observed. When the temperature vs. time profiles are convoluted with Gaussian pulses of 30 ps the temperature rise is only 30% of the maximum predicted, whereas 10-ps pulses produce 50% of the maximum (E. R. Henry, unpublished calculations). These results suggest that whereas thermal effects could contribute to the differences between spectra taken with 10 and 35 ps time resolution, those differences would not be expected to persist for the full time scale of the current experiments. In the case of the 8-ps experiments the broadened and red shifted spectra persisted to the nanosecond regime (9, 11–13), which suggests that thermal factors were not contributing in this case.

CONCLUSIONS

Our results indicate that in both MbCO and HbCO the transient difference spectra have fully evolved within 30 ps after photolysis with 532- and 355-nm pulses to those previously observed at about 10 ns. In addition, the spectra of Mb with overlapped 30-ps pump and probe pulses are identical with those obtained by steady-state methods. This

suggests that for Mb, significant changes in heme structure such as might result from direct coupling to the protein via the proximal histidine linkage and van der Waals contacts are completed within 30 ps. For HbCO photolysis, the results indicate no change is occurring in heme structure from ~30 ps to 6.5 ns. This finding, together with the results of recent nanosecond kinetic studies (6, 7), suggests that protein conformational changes within the R quaternary structure that influence the rate of ligand rebinding take place in <30 ps. Our results also suggest that nonlinear responses of vidicon detectors to picosecond continuum pulses, particularly in the region of strong absorption bands, might be responsible for the small distortions observed in the spectra reported here and earlier (11).

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