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Different dissociation pathways and observation of an excited deoxy state in picosecond photolysis of oxy- and carboxymyoglobin

(picosecond spectroscopy/myoglobin/hemoglobin/picosecond dynamics)

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ABSTRACT **Picosecond transient absorption spectra of Mb, MbCO, and MbO_2 have been studied at time delays of up to 10 ns after excitation at 353 nm. Particular attention has been paid to the rapid spectral changes that occur in the Soret region during the first 50 ps in MbCO and MbO_2. In MbCO both the bleaching of the Soret peak (feature I) and the appearance of new deoxy-like absorption (feature II) occur instantaneously, whereas in MbO_2 feature II is delayed with respect to feature I. A short-lived (≈12 ps) feature near 455 nm (feature III) was much more intense in MbO_2 than in MbCO and was also identified in the transient spectrum of Mb. No evidence of subnanosecond geminate recombination was found in either MbCO or MbO_2. These observations are consistent with a scheme in which MbO_2 photodissociates through an excited state of Mb, whereas MbCO under the same conditions produces ground state Mb directly. The results and conclusions are compared with those of previous picosecond studies on these molecules and related hemoglobin derivatives.**

Picosecond transient absorption spectroscopy is a valuable method for the study of heme proteins and their reactions with O_2 and CO. The unique feature of this technique is its ability to probe changes occurring on a time scale in which any large-scale movements of the protein are unlikely. In the case of hemoglobin, this permits the observation of the primary processes that initiate the protein conformational changes associated with cooperativity. Understanding these primary processes requires a detailed knowledge of the electronic states of this system and the couplings and interactions between them. To this end, a great deal of experimental and theoretical work has been devoted to establishing an accurate mapping of the states of various hemoglobin derivatives (1). We recently reported the results of a series of picosecond transient measurements on HbCO and HbO_2, in which we showed that relaxation pathways derived from spin-orbit coupling arguments could be used to explain the observed spectral changes (2). That work showed how such considerations could account for the difference in the behavior of these two molecules: HbO_2 exhibited a fast transient (<90 ps) and subnanosecond geminate recombination, whereas in HbCO both of these effects were absent.

In order to examine further the scheme proposed in these hemoglobin studies, we have used our picosecond transient absorption spectrometer to study a closely related set of myoglobin derivatives: MbCO, MbO_2, and unliganded Mb. Myoglobin has a similar electronic structure to hemoglobin but small spectral shifts result from the differences in protein environment. Because of its resemblance to a single hemoglobin subunit, myoglobin provides a natural model for the tetrameric protein, and it has been the object of previous kinetic studies in both picosecond (3, 4) and longer time regimes (5, 6). The spectral and temporal information provided by our picosecond spectrometer makes possible a more detailed investigation of the primary photolytic processes in these molecules. We report here the observation and interpretation of spectra taken at time delays of up to 10 ns after excitation at 353 nm, with emphasis on the rapid spectral changes that occur during the first 50 ps.

MATERIALS AND METHODS

Lyophilized sperm whale myoglobin (type II) was purchased from Sigma and used without purification. Reduction and oxygenation of the largely ferric form was accomplished with sodium dithionite on a Sephadex G-25 column (7). The thawed MbO_2 was diluted with 0.025 M Tris·HCl buffer, pH 8.6 at 25°C, to produce samples with a Soret peak absorbance of ≈0.9 at 418 nm in a 1-mm quartz cell. For each of two independent preparations and runs, data collection was accomplished in a single day, the continuous wave (CW) spectra showing no evidence of MbO_2 degradation over this time. MbCO was prepared by passing CO over a rotating solution of freshly prepared MbO_2 for 1 hr. The concentration was adjusted by addition of the pH 8.6 Tris buffer saturated with CO to yield MbCO samples with absorbances of 0.81 and 1.2 at the Soret maximum (423 nm) as measured in a 1-mm quartz cell. Deoxymyoglobin (70 μM) was prepared by adding a few grains of sodium dithionite to a 1-mm optical cell containing a freshly prepared MbO_2 sample. Human oxyhemoglobin, prepared by a standard procedure (8), was similarly treated to yield 70 μM deoxyhemoglobin. Despite the dithionite absorbance in the near UV, blanks made in parallel with the deoxy samples were found to have no transient absorption signal upon excitation with 353-nm pulses. All samples were characterized in terms of purity and concentration by comparing CW spectra (Cary 14) with published spectra (9).

Our picosecond double beam transient absorption apparatus, designed to acquire transient measurements at delays ranging up to 10 ns after excitation, has been described in detail (2). Very briefly, a single 8-ps pulse is switched out of the pulse train generated by a passively mode-locked Nd/phosphate glass laser. This pulse is amplified to 20 mJ and then used for second and third harmonic generation; in this set of experiments the UV pulse at 353 nm is used to excite the sample. The second harmonic can be delayed optically before it is focused into a cell of CCl_4, generating a weak continuum pulse of 8-ps duration. A beamsplitter selects a fraction of the continuum light as a reference beam, which traverses an unexcited region of the sample. The main continuum pulse propagates collinearly with the excitation pulse, both beams passing through a 400-μm-diam-

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Abbreviation: CW, continuous wave.

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eter pinhole located directly before the sample cell. The excitation pulse at 353 nm contains approximately 10 μJ of energy on entering the sample cell. The transmitted continuum, both "pinhole" and "reference" beams, provides two tracks of spectral data when imaged onto the slit of a low-dispersion spectrograph fitted with an optical multichannel analyzer vidicon detector.

In order to minimize the effects of shot-to-shot fluctuations in the continuum spectrum, we have employed the following data collection procedure throughout these studies: With the excitation pulse blocked, we collect a series of two-track continuum spectra. From this group we select and average five shots whose continuum spectra are similar. The reference track of each subsequent shot (with excitation present) is compared with the reference track of this average of five shots. If the spectral content differs beyond a certain tolerance, as determined by least squares analysis, the shot is rejected. In addition, we reject laser pulse trains containing satellite pulses and shots whose energy at 353 nm falls outside the typical range. We find that this data collection procedure results in reliable and reproducible spectra. All of the spectra presented here are averages of at least five laser shots and have an average noise level of $\approx$0.01 absorbance units in the region 430–500 nm, and $\approx$0.03 between 400 and 430 nm.

RESULTS

MbO_2. Excitation of MbO_2 results in the bleaching of the ground state Soret band and the production of a new absorption feature near 440 nm. The bleaching feature is fully formed within 3 ps, whereas the new absorption does not achieve its maximal amplitude until a time delay of 15 ps. This difference cannot be accounted for by the effect of group velocity dispersion on the probing continuum, which might contribute a delay of at most 3 ps over this small wavelength region. Fig. 1 illustrates the evolution of the difference spectrum immediately after the initial buildup. At 13 ps the absorption shows a tail extending into the red, which has disappeared by 40 ps. Within our experimental error, this spectral change is not accompanied by recovery of the bleaching feature or increase of the main absorption peak at 440 nm. Fig. 1 suggests that the rapidly relaxing component is centered around 455 nm, and from our data we estimate the lifetime of this peak to be 12 ± 3 ps. From 40 ps to 9.5 ns the shape of the transient difference spectrum undergoes no further change, while the amplitude decreases by at most 15%. While this is in accordance with recent studies

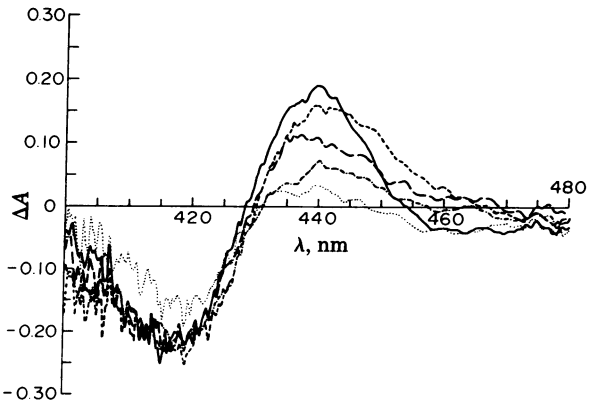

FIG. 1. MbO_2 transient difference spectra, Soret region, after ps excitation at 353 nm. These spectra show different rates of growth for the absorption and bleaching features: ·····, 0 ps; –·–, 3.3 ps; ———, 6.6 ps; ----, 13.2 ps; ——, 40.0 ps. Sample A_{418} = 0.90 in 0.025 M Tris buffer, pH 8.6 at 23°C, 1-mm cell.

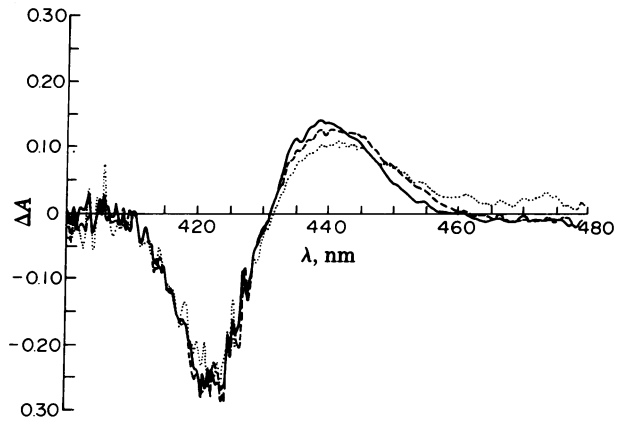

FIG. 2. MbCO transient difference spectra at 6.6 ps (·····), 13.2 ps (----), and 100 ps (——). Absorption and bleaching features develop simultaneously. Sample A_{423} = 0.81; excitation at 353 nm.

of HbO_2 recombination in the nanosecond regime (10), it indicates an absence of the significant (40%) amount of subnanosecond geminate recombination observed in HbO_2 excited with 530-nm ps pulses (2).

MbCO. Fig. 2 illustrates our finding that excitation of MbCO also leads to bleaching of the ground state Soret band and the appearance of new absorption near 440 nm. Spectra obtained at 0, 3.3, 6.6, and 13.2 ps indicate a *simultaneous* development of both bleaching and absorption intensities; these features are fully formed within the pulsewidth of the laser. During the first 100 ps after the buildup of the signal the new absorption band exhibits a spectral change similar to that in MbO_2, as depicted in Fig. 2. The difference spectrum at 6.6 ps has absorption extending to the red that disappears rapidly, and the change in shape can be attributed to a broad, weak absorption centered at 455 nm. Because this effect is much smaller here than in MbO_2, we were unable to estimate a lifetime for this band. The qualitative resemblance to MbO_2 is nevertheless clear from the figures. The shape that the MbCO transient difference spectrum assumes after the relaxation of the 455-nm component is subsequently unchanged up to 9.5 ns after excitation. The difference spectrum remains constant in amplitude, in contrast to MbO_2, which suggests that recombination of CO does not occur on this time scale.

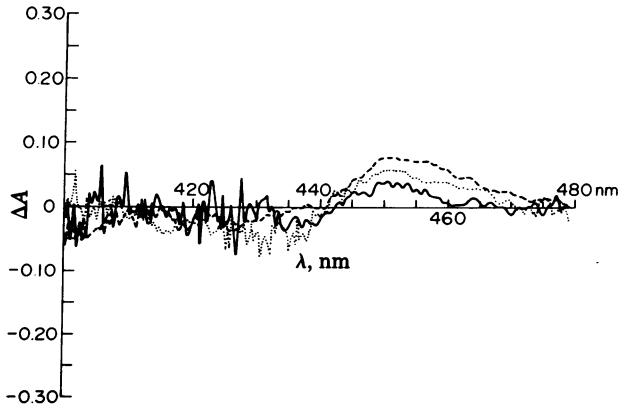

FIG. 3. Short-lived difference spectrum of deoxy Mb (·····) and difference-of-difference spectra (13 ps − 50 ps) of MbO_2 (----) and MbCO (——) illustrating a common absorption feature near 455 nm. The different amplitudes of these curves, which are normalized for ε_{353}, and their similarity in shape and lifetime (τ = 12 ± 3 ps) suggest that an excited state of Mb is formed through different pathways in the photolysis of MbO_2 and MbCO.

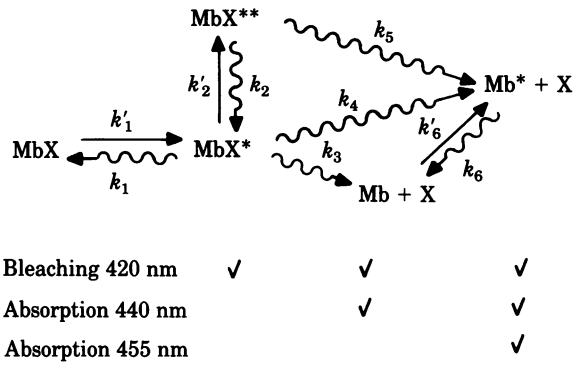

FIG. 4. Diagram defining possible photolysis and relaxation pathways in MbCO and MbO_2. MbX* and MbX** could represent bound, excited electronic states; Mb* could be an electronically excited, unliganded myoglobin. Straight arrows represent transitions involving at least one photon; wavy lines are radiationless processes, which may include intermediates not shown. Check marks beneath each species indicate which spectral features are associated with it in our experiments. Our results suggest that in our experiments the dominant pathway in MbO_2 involves k_4, k_5, or both and produces Mb*, whereas in MbCO the dissociation is through k_3 to ground state Mb.

Mb. The excitation of deoxygenated Mb produces a weak, short-lived transient difference spectrum. A bleaching feature at 433 nm and a new absorption peak at 451 nm both attain their maximal amplitudes ($\pm\Delta A = 0.05$) at a delay of 10 ps (see Fig. 3). The relative prominence of the absorption peak relative to the bleaching in this spectrum indicates that the transient state produced has a strong Soret absorption. The transient decays rapidly and cannot be observed at 20 ps.

Summary of Results. The photolysis of MbX (X = CO or O_2) can be described in terms of three spectral features listed in Fig. 4. The bleaching at 420 nm (I) results from the disappearance of ground-state MbX. The absorption at 440 nm (II) is characteristic of deoxy Mb and monitors the production of that species. The rapidly relaxing absorption at 455 nm (III) may originate from an excited state of the deoxygenated molecule, denoted Mb*. The basis for this assignment is discussed in the next section.

Fig. 4 also presents a diagram representing some possible pathways for excitation and relaxation in the MbX system. It should be emphasized that this diagram is shown here for didactic purposes and is not intended to exclude more complex or detailed descriptions. Transitions from one species to another in this diagram may involve many steps and include intermediates not shown; in some cases transitions may require absorption of at least one additional photon.

DISCUSSION

In this section we examine the above experimental findings, together with previous work on myoglobin and hemoglobin, with a view to unifying the description of the available information. As a first step, we show in Fig. 5 the photoproduct spectra for both MbO_2 and MbCO photolysis. These have been constructed by using the transient difference spectrum at relatively long times (>50 ps) and the absorption spectrum of the starting material in each case, using the fractional photoconversion as an adjustable parameter. The photoproduct spectra are similar to each other though somewhat different from the CW spectrum of deoxygenated Mb. With respect to the spectrum of Mb the photoproduct spectra are slightly broader and weaker, and they show a 3-nm shift to the red. These results suggest that the photolysis products are the same in MbCO and MbO_2, although different from stable deoxygenated Mb.

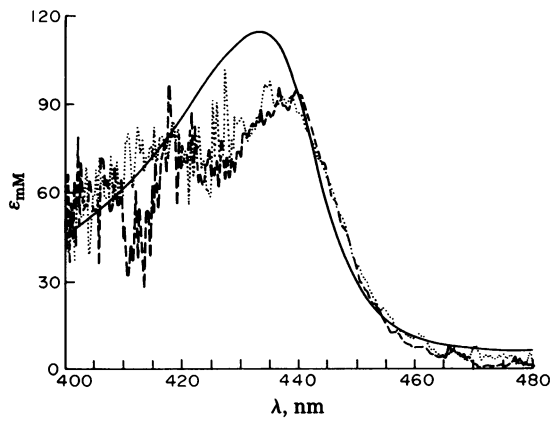

FIG. 5. Calculated photoproduct absorption spectra at $t > 50$ ps for MbCO (·····) and MbO_2 (----) compared with the CW Soret absorption band of Mb (——).

From the figures it can be seen that all three of the molecules studied show a common spectral feature, denoted III in Fig. 4. To isolate this rapidly relaxing peak in the difference spectra of MbO_2 and MbCO, we subtracted the 50-ps spectrum from the 13-ps spectrum in each case. These curves are plotted in Fig. 3 on the same scale as the induced transient in Mb, with all three curves normalized for equal ground state absorbance at 353 nm. Because these curves show approximately the same peak position and lifetime, we have assumed that all three represent the *same* state, Mb*.

The difference in amplitude among the curves in Fig. 3 is significant and must be incorporated into any model used to explain the data. In MbO_2 feature III is larger than when Mb is pumped directly, suggesting that the photolysis results in a very efficient production of Mb*. In the terms of Fig. 4 this would entail a mechanism involving either k_4 or k_5, and it would imply that the MbO_2 photoproduct rapidly reaches the excited state Mb*. In MbCO feature III is weaker than it is in the direct pumping of Mb, which could be explained by a pathway through k_3 and k'_6. In this scheme the photoproduct appears in its ground state and is subsequently pumped to Mb* by the absorption of a second photon from the excitation pulse.

The electronic structure of these molecules has been described in detail in the earlier hemoglobin work (1). In MbCO a lowest excited state is dissociative and can provide a simple pathway (k_3) for the photolytic production of ground-state Mb. In MbO_2 the presence of low-lying charge-transfer states complicates the kinetic picture, because these states could be weakly dissociative or even nondissociative. Therefore a major dissociative pathway in MbO_2 might not pass through the lowest-lying excited state. We suggest that in MbO_2 a multiple-photon mechanism operates that provides a significant amount of product through repeated excitation of a short-lived dissociative state MbX** not reached by one-photon absorption. In Fig. 4 MbX** is reached through k'_2 and dissociates through k_5 to produce a large amount of photoproduct *in an intense short pulse* even if k_2 is relatively fast.

This interpretation would account for: (*i*) the low quantum yield for dissociation of MbO_2 under lower intensity illumination (11); (*ii*) the delayed appearance of the 440-nm absorption feature mentioned above, which implies the presence of a second step in the photolysis pathway; and (*iii*) the appearance of the initial photoproduct Mb in an excited state. This scheme suggests that the short pulse quantum yield of photolysis in MbO_2 would be wavelength dependent through the step k'_2, which could account for the negligible quantum yield obtained by Shank *et al.* (12) in HbO_2, using 500-fs pulses at 615 nm.

We first compare our present results with the previous picosecond studies of myoglobins by Rentzepis and coworkers (3, 4). In their experiments the excitation characteristics were similar to ours, whereas the probing process relied principally on kinetic measurements of the ground state Soret bleaching and transient Soret absorption at 420 nm and 440 nm, respectively. For this reason their experiments did not detect feature III at 455 nm; nonetheless it is possible to compare the three sets of results with regard to features I and II. In their earlier study the photodissociation of both MbO_2 and MbCO was deduced to occur in less than 6 ps, and a reported 125 ± 50 ps decay exhibited only by MbCO apparently was preparation related (3). In their more recent report (4), Reynolds *et al.* focused more on early-time kinetics ($t < 280$ ps) in samples at 4°C that were pumped at 355 nm and at 530 nm. They found both MbCO and MbO_2 exhibited an instantaneous bleaching, corresponding to feature I, and a new absorption feature II, delayed in its appearance by 3.5–5 ps. The authors accounted for this by proposing an intermediate state (τ = 7–10 ps) in the dissociation pathway of both MbCO and MbO_2. The recent results and proposed explanations of Reynolds *et al.* (4) are consistent with our MbO_2 results reported here, but their MbCO results are at variance with ours. Thus their model accommodates neither our MbCO results nor our information regarding optically pumped Mb and feature III. Further work is required to examine quantitatively the possible effects of pulse width, pulse energy, experimental geometry, sample preparation, and sample conditions in order to explain the discrepancies between these studies. We note that Reynolds *et al.* found that, while their kinetic results were independent of excitation wavelength (530 or 355 nm), their quantum yield measurements implied that $\phi_{355,530}^{MbCO} > \phi_{355}^{MbO_2} \approx 3\phi_{530}^{MbO_2}$. Further careful determination of the wavelength dependence of the quantum yield of MbO_2 is necessary.

The results of the present studies also may be compared with the previous picosecond work on HbO_2 and HbCO (1, 2). The following similarities exist: the photoproduct spectra for HbCO and HbO_2 resembled the spectrum of deoxygenated Hb but were broader and weaker. A fast transient similar to feature III in MbO_2 was seen in the HbO_2 difference spectrum. Recently we observed a rapidly relaxing transient difference spectrum, resembling feature III of Mb, in Hb pumped directly at 353 nm. The one major difference in the behavior of the Mb and Hb systems is the absence of subnanosecond recovery of the Soret bleaching in MbO_2, which was observed in HbO_2. In previous work with HbO_2 and HbCO we attributed the recovery of the Soret bleaching for HbO_2 to geminate recombination of O_2. We suggested electronic reasons why CO might not be expected to recombine so rapidly, thereby improving its probability of escape from the protein. We have shown here that there is no significant recovery of the Soret bleaching in the cases of MbO_2 and MbCO. On the other hand, the electronic structures of the hemes in Hb are not expected to be significantly different from the structure in Mb. It is obvious then that arguments based on differences of the electronic structures of Hb, O_2, and CO cannot provide a complete explanation of the photokinetic behavior of HbO_2 and HbCO. These important parallels indicate that the scheme of Fig. 4 may also describe the photokinetic behavior of Hb and its derivatives. Our earlier results therefore provide evidence for a multiple-photon dissociative pathway to an electronically excited deoxy-like photo product in HbO_2 and for the relative unimportance of this path in HbCO.

A comparison can also be drawn between our work and recent time-resolved Raman studies of HbCO (13, 14), in which a flowing sample was irradiated with 30-ps pulses and the resulting spectra were recorded for different degrees of focusing. The observed shift of an oxidation marker band was attributed to high-spin Hb, with the iron not fully relaxed out of the plane. This frequency shift persisted when the pulses were lengthened to 20 ns. This is consistent with our results on HbCO (2), in which a transient is generated within our pulsewidth and exhibits no subsequent spectral changes. However, the similarity in the peak position of feature III in MbO_2, MbCO, and Mb suggests that the iron is out of the plane at the time this peak appears (<10 ps). If the motion of the iron had not yet occurred, a shift in peak position relative to equilibrium Mb would be expected. Our work has detected quite different behavior for HbO_2, in which the dissociation apparently produces a form of Hb also as yet undetected by vibrational spectroscopy. Time resolution better than 12 ps will be needed to see these prompt species.

In conclusion, we consider first the dynamics immediately after excitation and then the results at longer delay times. The observation of feature III and its identification as an excited state of the deoxy species was made possible through careful study of transient spectra closely grouped around time zero. This feature seems to be characteristic of directly pumped Mb and was found to have an analog in Hb. It seems reasonable, then, to assign the fast-relaxing component ($\tau < 90$ ps) observed in HbO_2 to an excited state of Hb (2). It appears firmly established now that MbCO undergoes photolysis with high quantum yield and exhibits no geminate recombination phase in either the picosecond or the nanosecond time regime (15). For MbO_2 there is no clear information available regarding the nanosecond time domain dynamics. Eisert *et al.* (3) found no relaxation in the first 450 ps for MbO_2 pumped at 530 nm, and we report here an upper limit of 15% recombination in 10 ns after 353-nm excitation. Further experiments are necessary to explore the excitation wavelength and heme environment dependence of subnanosecond geminate recombination.

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