BRIEF COMMUNICATION

PHOTODISSOCIATION OF CO AND O_2 FROM α AND β HEMOGLOBIN CHAINS STUDIED BY USING PICOSECOND ABSORPTION SPECTROSCOPY

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ABSTRACT The picosecond photodissociation of the CO and O_2 forms of α and β chains of hemoglobin were studied by following $\pi\pi^*$ Soret absorption changes using a Nd^{3+} phosphate-glass laser, 531-nm pump pulse, 8 ps full width half maximum, and a pump-probe double-beam absorption apparatus. Three intermediates were observed within the first 50 ps after photon absorption. The most notable differences between the two monomers are the extent and rate of geminate recombination with the two ligands. We attribute this result to differences between the tertiary protein structure of the α and β forms of Hb, both distal and proximal.

INTRODUCTION

The picosecond photodissociation and subsequent geminate recombination of various axial ligands from Hb has become an area of intense research activity over the past ten years. Raman work has concentrated on the examination of resonantly enhanced modes which are sensitive to the position of the iron as it relaxes out of the plane of the porphyrin in the deoxy-complex after photodissociation. Fe-imidazole modes (1, 2) and modes associated with the porphyrin core hole size (3, 4) have been studied by pumping the Soret and Q-bands, respectively. Picosecond absorption measurements are of the pump-probe type and are typically done by following changes in λ_{max}, and ϵ_{max}, and shape of the $\pi\pi^*$ Soret band localized in the porphyrin plane using a Q-band pump as a function of delay time. We have studied the photodissociation of the CO and O_2 forms of a number of natural and synthetic complexes using this approach in the 1–50-ps range (5, 6), and found that the effects of proximal imidazole strain on the heme are manifest in the dynamics and mechanism of dissociation. We have isolated and kinetically analyzed a total of four or five photointermediates as they sequentially evolve during the first 50 ps and have used a model invoking excited electronic states of the heme to analyze this data in terms of predissociative six-coordinate and postdissociative deoxy five-coordinate octahedral states. Since the most interesting aspect of the present work is the difference in geminate recombination rates observed between the α and β monomers, other models emphasizing recombination within the heme pocket should be mentioned as they have a bearing on the interpretation of our results. In particular Jortner and Ulstrup (7) and Redi et al. (8) have used non-adiabatic perturbation approaches to account for the reaction rate of CO ($S = 0$) + Hb ($S = 2$) $\rightarrow$ HbCO ($S = 0$) at $T \leq 100°K$. Generally, non-adiabatic theory supports differences in recombination rates as they are dependent on the spin states of the reacting partners. Recently, however, Frauenfelder and Wolynes (9) discussed experimental results at temperatures $<200°K$ showing O_2 and CO to recombine with deoxy Hb with nearly equal rates within the heme pocket, contrary to predictions based on spin orbit considerations. They reexamined conditions under which transition state and non-adiabatic theories fail, finding that friction, the presence of intermediate states, and heme pocket geometry could effectively render non-adiabatic transitions adiabatic. In addition to these considerations, the effects of quaternary constraint on the early events in the picosecond photodissociation are not clear and this in itself prompted our interest in studying the α and β Hb monomers which are free from such restrictions.

EXPERIMENTAL SECTION

A description of the double-beam phosphate glass Nd^{3+} laser apparatus used in these experiments can be found in previous work (5). The pump-probe absorption experiments were done at a rate of 2 per min using a SHG 531 nm, 8 ps full width half maximum (fwhm), TEM_{00}, ~1 mJ pulse to pump to Q band of the parent six-coordinate CO or O_2 complex in a 1-mm optical path. Absorbance vs. wavelength spectra, $\Delta A(\lambda, t)$, in delay range settings between -12 and 50 ps are referenced to the maxima of the probe and pump pulses. Negative time frames refer to experiments where the probe pulse maximum reaches the sample cell before the pump pulse maximum. A minimum of 12 experiments were signal averaged in order to obtain the absorbance change $\Delta A(\lambda, t)$ at a particular delay setting. The spectra of the intermediates were obtained by addition of the ground state spectrum to the ΔA spectrum: $A(\lambda, t) =$

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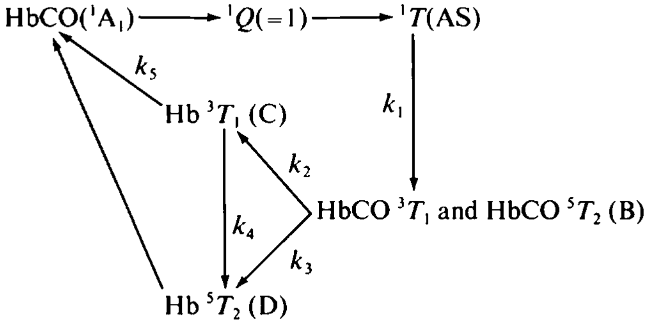
$\Delta A(\lambda, t) + A(\lambda, \text{ground})$. Six-coordinate ground-state spectra were obtained by using a tungsten lamp as the light source for the I_0/I ratio. Human HbO_2 was prepared by the methods of Antonini and Brunori (10). The HbO_2 was then treated with *p*-hydroxymercuribenzoate (11), and the chains were separated by chromatography on a diethylaminoethyl cellulose column. The sulfhydryl groups were reactivated by addition of dodecanethiol (12), and the chains were checked for purity by electrophoresis (13) and through spectral comparisons. The CO forms of the chains were prepared from the O_2 derivatives by gently blowing CO over the sample for ~1 h as it was being stirred. A small amount of CO-saturated sodium dithionite solution was added to remove any remaining oxygen. All experiments were performed at 5°C to minimize protein degradation. Ground state absorption spectra were taken before and after each set of experiments and compared with reference spectra to assure sample quality. Sample concentrations were adjusted in order to obtain an absorbance of ~1.0 at the six-coordinate Soret maximum, ~50 μM. With a pump spot size of 1 mm we estimate the sample receives a pulse of not more than 350 $\mu J/mm^2$, conditions that should minimize nonlinear artifacts. In addition, these low sample concentrations are necessary to maintain monomers for both α and β forms (14).

RESULTS AND DISCUSSION

The results of this study are consistent with an excited electronic state model of the HbCO photodissociation that we proposed for our previous work on synthetic and natural heme complexes (5, 6), namely;



The photodissociation pathway of the O_2 complex is clearly different from that of the CO forms as experiment and theory show and this will be discussed shortly. Support of this mechanism in this study comes from our ability to isolate three key photointermediates, B, C, and D from the experimental $\Delta A(\lambda, t)$ spectra at specific delays using the procedure outlined in the experimental section. Figs. 1–2 show experimental results for HbβCO: the ΔA spectra and intermediates B through D. In past work we have been able to isolate a very early intermediate AS, which we also believe to be present in the photodissociation of the α and β complexes. This early intermediate has been seen by Martin and co-workers (15) in the photodissociation of Hb and Mb compounds as early as 350 fs. However, because of noisy conditions in the early delays and the fact that we are subtracting two similar large absorbances, the existence of AS is questionable in the experiments reported here.

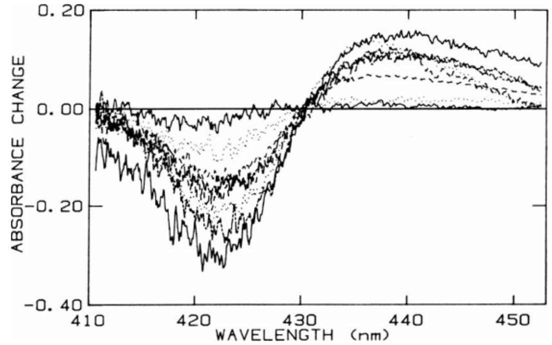


FIGURE 1 Experimental spectra for HbβCO. Delays range from −10 to 40 ps; −10, +8 (*solid*); −4, +12 (*dotted*); 0, +20 (*dashed*); +4, +40 (*broken*).

Assignment of these intermediates to specific octahedral states is clearly tentative and based on possibilities arising because of radiationless transitions among certain "bottle neck" states that were first discussed in terms of extended Hückel calculations of Eaton et al. (16) and picosecond absorption studies of Cornelius et al. (17). In order to obtain estimates of lifetimes and rate constants associated with the observed intermediates, convolution of the probe and pump pulses while they overlap in the sample with a mechanistic model of the dissociation is necessary. This kinetic modeling procedure is discussed in detail in earlier work (5) and it involves the use of a Runge-Kutta-Nystron integration procedure. It is based on the fact that the actual concentration of photoproducts will be proportional to the product of the pump-probe Gaussian (assumed) profiles as they pass through the sample. In effect the probe pulse "sees" different molecules in various stages of excitation as a function of delay. The experimental data used in this modeling procedure include λ_{max}, ϵ_{max}, and the fwhm for the intermediates as well as six-coordinate ground state. Initial estimates for the rate constants are also necessary. The rate constants are adjusted until the best fit is achieved between the computer-modeled spectra and the experimental ΔA data. There are a number of limitations to this procedure which influence the accuracy of the curve fit (5). Nevertheless, this modeling can be viewed as a worthwhile means to achieve a better understanding of how the tertiary structure affects the photodissociative pathway.

Results of this study are summarized in Table I, which · shows that the essential difference betweent the α and β

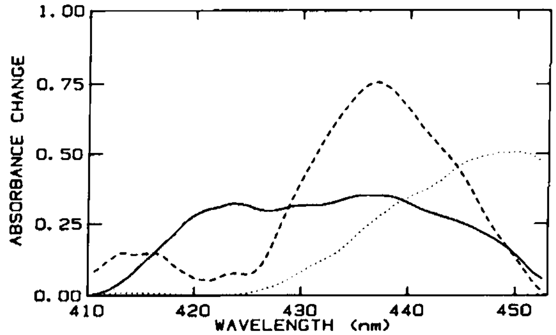


FIGURE 2 Smoothed spectra (31 point algorithm) of HbβCO intermediates: B (*solid*), C (*dotted*), and D (*dashed*).

TABLE I
RATE CONSTANTS AND LIFETIMES FOR SOME NATURAL HEME COMPLEXES AND THEIR INTERMEDIATES

Compound	k_2	k_3	k_4	k_5	τ_B $(k_2 + k_3)^{-1}$	τ_C $(k_4 + k_5)^{-1}$
					ps	*ps*
HbβO$_2$	0.20	0.10	0.20	0.25	3.3	3.7
HbβCO	0.80	0.30	0.013	0.13	0.9	7.0
HbαO$_2$	0.21	0.10	0.025	0.015	3.2	25
HbαCO	0.15	0.10	0.017	0.015	4.0	31

forms of the hemoglobin monomers occurs in the rate of geminate recombination (C $\xrightarrow{k_5}$ A) and the resultant lifetime of C. Modeled spectra for HbβCO are shown in Fig. 3. Lifetime τ_B suggests that either form photodissociates in ~4 ps. The ratio of CO to O$_2$ geminate recombination rates is 2:1 for the β monomer where the recovery is ≤30% by 50 ps. However, because the ΔA noise is high in the blue region of the Soret spectrum, ~0.05, we cannot readily distinguish between the CO and O$_2$ geminate rates for the α monomer, though the recovery is limited to ≤15% in this case, by 50 ps. They may be identical. We see that the recombination rate of β is roughly 10-fold greater than α, leading to a lifetime ratio $\tau_c(\alpha)/\tau_c(\beta)$ of five. These conclusions are supported by both the raw experimental ΔA data and the estimates of kinetic parameters found through the modeling procedure.

Our interpretation of these results is referenced to the excited octahedral energy-level diagrams for O$_2$, CO, and deoxy Hb (18, 19) and to the mechanism presented at the beginning of this section. Radiationless relaxation from the ^1Q to the singlet $^1T_1$ is expected to occur on a subpicosecond time scale, giving rise to the first intermediate, AS. Conversion from the $^1T_1$ state to other spin multiplicities in the six-coordinate manifold is expected to be the next step. For the CO forms the $^5T_2$ and $^3T_1$ are the lowest excited "bottle necks" available and the observed intermediate B is likely to be a mixture of these states. The arrival of this photoproduct can be seen in Fig. 1 by the bleaching or decrease in absorbance at 422 nm and only a slight increase in absorbance at 435 nm. What we have characterized as intermediate C has been reported by a number of other laboratories (18, 20, 21). This intermediate is manifest through the rapid changes of absorbance in the red end of the spectrum. By 8 ps the red shoulder has broadened considerably and then it rapidly sharpens up and blue shifts by 20 ps, indicating the presence of intermediate C. We attribute this species to be the five-coordinate $^3T_1$, a short-lived triplet which with time has the possibility to intersystem cross to the final electronic intermediate D, or $^5T_2$. Spectroscopically, photointermediate D is much sharper and blue shifted, distinguishing it from C. Our best indication of D being the relaxed out-of-plane five-coordinate Hb can be seen in the α forms, for both CO and O$_2$, where it has a maximum of ~432 nm, which is very close to the λ_{max} for the ground state Hbα, 431 nm. On the other hand, in the β chain for both CO and O$_2$, D as shown in Fig. 2 has a λ_{max} of ~439, which is considerably red shifted from the equilibrium five-coordinate Hbβ, λ_{max} of 431.5 nm. This suggests that the β chain geminately recombines faster and that the D species in Hbβ is a mixture of five-coordinate intermediate spin $^3T_1$ with the iron partially relaxed and fully relaxed out-of-plane deoxy $^5T_2$ iron complex.

The photodissociative pathway of the O$_2$ complexes is clearly different from the CO forms as experiment and theory show. Based on extended Hückel (16, 18, 20) and INDO-SCF-MO (22–24) calculations, we consider the predissociative photointermediate, B, in the O$_2$ complexes to be a mixture of a triplet $^3T_1$ state, lying just below the ^1Q

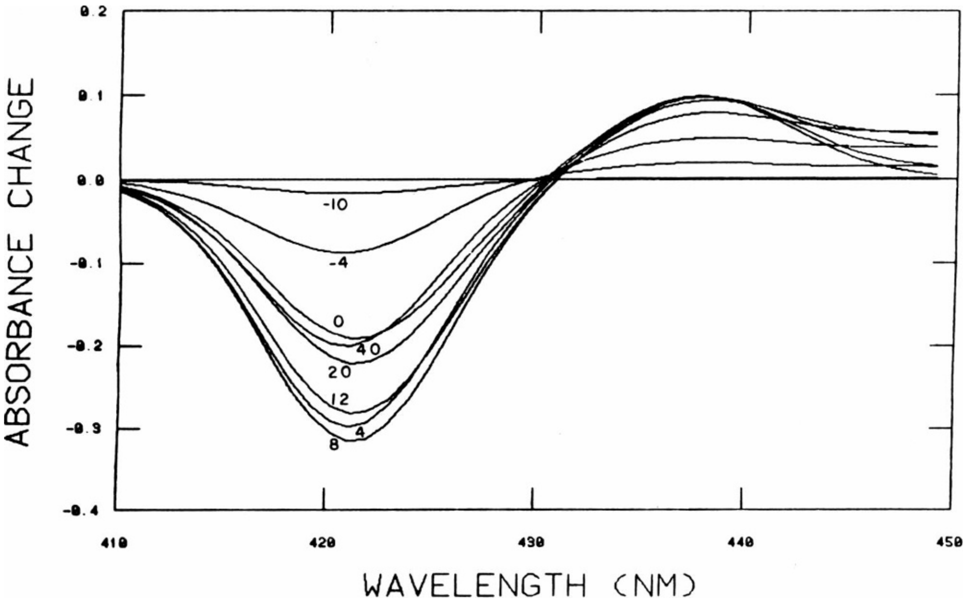


FIGURE 3 Modeled spectra for HbβCO at the delays indicated.

state, with lower lying ^{1,3}CT states. As in the CO complexes, dissociation of the O_2 complex will produce a triplet $^3T_1$ and quintet $^5T_2$ in the five-coordinate deoxy manifold, so that the C and D intermediates are considered to be the same for both complexes.

In this excited state model one may view the local deoxy geometry in terms of an iron fully relaxed from the porphyrin plane ($^5T_2$) or partially relaxed ($^3T_1$). Immediately after the dissociation, geminate recombination of CO or O_2 with these nearby states is possible, forming the parent six-coordinate heme complex depending on spin-orbit matrix elements and associated rates interconnecting these states. The favorable 2:1 geminate recombination rate ratio for O_2 and CO in the β monomer are consistent with this modified non-adiabatic model, not a proof of it. The geminate rate results for the O_2 versus CO competition in the α monomer appear, within our noise limitations, to be independent of spin-orbit considerations that would support the adiabatic model proposed by Frauenfelder and Wolneys (9). Differences in the local heme geometry between the α and β monomers may be effective in promoting the adiabatic mechanism over the non-adiabatic one. Tertiary heme geometry is certainly a key factor. Our study of synthetic complexes (6) revealed a relatively long lifetime for photointermediate C that ranged from 15 to 50 ps compared with a rate between 4 and 7 ps for the β monomer and 25 and 31 ps for the α monomer. We attributed the long-lived C intermediate in the synthetic compounds to lack of quaternary constraint. However, the results of this study show that the cause of the relative long C lifetimes is more complex, as it noticeably varies between the α and β monomers, which also lack quaternary restrictions. X-ray studies done by Perutz (25) have shown that the distal heme pocket in the β chain is smaller than that of the α chain, due to the close proximity of the histidine E7 and valine E11, and one might on this basis expect a higher rate of geminate recombination for the β chain. Although molecular dynamics trajectory simulations have been done on the dissociation of CO in the distal pocket of the α form (26), additional studies on the β form would be helpful to clarify this point. Another possibility is supported through the resonance Raman work of Friedman et al. (1, 2), which shows the importance of the tilt of the proximal histidine F8 in governing geminate recombination. In addition, Baldwin (27) found that the tilt of the proximal imidazole ring is slightly less in the β chain than in the α chain. Apparently there is less imidazole strain in the β form than the α form, favoring an intermediate degree of iron relaxation in β. This is consistent with a shorter-lived C intermediate and faster geminate recombination. Clearly, much more work remains to be done to clarify this matter.

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