# KINETICS AND TEMPERATURE DEPENDENCE OF CARBOXYMYOGLOBIN LIGAND PHOTODISSOCIATION

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ABSTRACT We have observed the rate of oxymyoglobin ($MbO_2$) photodissociation at room temperature and carboxymyoglobin (MbCO) photodissociation as a function of temperature (260–10 K) by means of picosecond spectroscopy. The Mb + $O_2$ and Mb + CO photodissociated states have also been characterized. Based on the picosecond experimental data, we postulate that the photodissociation of ligated myoglobin is a nonactivitated process, and the mechanism involves either a small enthalpy barrier or none at all.

## INTRODUCTION

The dissociation of oxy- and carboxymyoglobin, $MbO_2$ and MbCO,[1] by light excitation provides a convenient means for studying the naturally occurring ligand dissociation process. Previous studies (1, 2) have shown at least two intermediate states in the ligand photodissociation process of sperm whale myoglobin. The first one is characterized by a $\leq$3-ps lifetime and is primarily responsible for the observed differences in $MbO_2$ and MbCO quantum yields. The second exhibits a dissociative lifetime of 6–8 ps and is independent of ligand ($O_2$ or $CO_2$) and wavelength of excitation (355 or 530 nm). A schematic representation of the photodissociation process in MbCO is shown in Fig. 1. The initial absorption of the photon is fast ($k_1 \gtrsim 10^{14}$ $s^{-1}$). Relaxation to the intermediate state Mb · · · CO results in a lifetime of $\lesssim 10^{-12}$ s ($1/k_2 + 1/k_3 + 1/k_4 \lesssim 10^{-12}$ s). The ratio of $k_3/k_{-1}$ determines the quantum yield, which for MbCO is ~1, so that $k_1 \ll k_3$. The last step in the dissociation process from the intermediate Mb · · · CO to the dissociated state Mb + L occurs with a rate $\sim 10^{11}$ $s^{-1}$ ($1/k_5 + 1/k_6 \sim 10^{-11}$ s), and it is the temperature dependence of this step that is discussed here. Recombination is indicated by $k_7$ and occurs over milliseconds ($k_7 \sim 10^3$ $s^{-1}$). No other kinetic processes were observed during the first 300 ps of the reaction. These results are consistent with the previously published reports of Noe et al. (3), who found a 12-ps rise time in carboxyhemoglobin ligand photodissociation, which was not reported by Green et al. (4). We do not, at the present time, make any direct comparison between the myoglobin and hemoglobin photodissociation kinetics because of the structural differences between these two species.

Having previously identified the intermediate states for MbCO and $MbO_2$ photodissociation, in this report we present data that characterize the kinetics of the formation of the long-lived dissociation product Mb + L. We also show that the kinetics of this process, MbL → Mb + L, are temperature independent from 260 to 10 K, and identify Mb + L as essentially identical to deoxymyoglobin. During the time of our experiments ($\lesssim$ 1 ns), the ligand remains within the heme pocket.

## SAMPLE PREPARATION AND HANDLING

Ferrous sperm whale myoglobin samples (type II, Sigma Chemical Co., St. Louis, MO) were prepared by the method of Bauer and Pacyna (5). The sodium dithionite (Mannox Brand, Holden & Hardman Ltd., Miles Platting, England) used in reduction of the iron was removed by passing the protein through an exclusion column (P-2 Bio Rad Laboratories, Richmond, CA) equilibrated with 25-mM pH 7.4 Tris/bistris buffer. MbCO was prepared by stirring $MbO_2$ under a CO atmosphere for 1 h. Low temperature samples were prepared by the same procedure, except that they were washed from the column with distilled $H_2O$ and mixed with three parts by volume glycerol.

Low temperature experiments were performed in glasses prepared by cooling the 75% glycerol sample at a rate of ~0.5 K per minute in the range of 190 to 150 K where cracking of the glass takes place when the cooling rate is rapid. If cracks formed they could be annealed by raising the temperature. At temperature below 140 K, the glass was found to remain essentially without cracks, independent of the cooling or warming rates. Temperature regulation was provided by a Helitran Cryotip controller (Air Products and Chemicals, Inc., Allentown, PA) and the heme concentration was adjuted to ~1.2 OD in the Soret band.

## EXPERIMENTAL METHODS

In practice, there are two types of picosecond absorption systems used to study the kinetic behavior of a reaction. One is capable of monitoring the kinetics up to several hundred picoseconds at a specified wavelength; the other can display a complete difference spectrum at a single selected time making possible the identification of the intermediate states involved in a reaction.

The spectra presented in this communication were taken by the second type of picosecond spectrometer. The laser consists of $Nd^{3+}$/YAG oscillator and amplifiers that provide good pulse stability and repetition rate up to 10 Hz. The oscillator operated in $TEM_{00}$ mode is passively mode-locked (Kodak 9740 saturable dye, Eastman Kodak Co., Rochester, NY) and produces ~25-ps pulses. A single amplifier is used to boost the single pulse energy to 8–10 mJ for harmonic generation. Conversion efficiencies to the second harmonic, 532 nm, were typically 40–50%, and

[1] *Abbreviations used in this paper:* Mb, myoglobin; L, ligand.

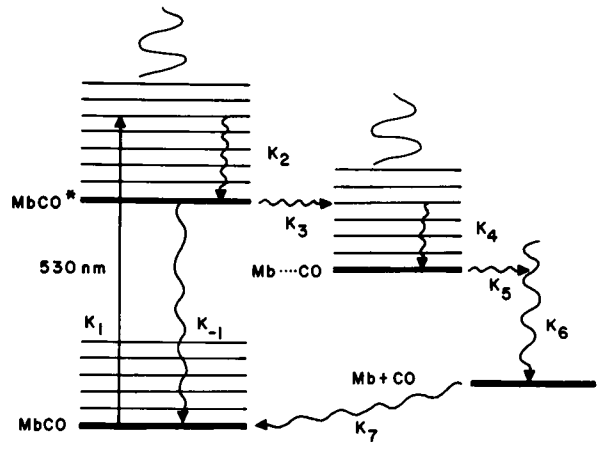


FIGURE 1 Schematic representation of the photodissociation process in MbCO. MbCO is the ligated state, MbCO* and Mb · · · CO are intermediates and Mb + CO is the dissociated state.

to the third harmonic, 355 nm, 20–25%. A second amplifier is used to boost the remaining fundamental energy to ~20 mJ for continuum generation. Data collection was done on-line by an ISIT/OMA2 system (E G & G/Princeton Applied Research, Princeton, NJ) and off-line by an Eclipse S/130 minicomputer (Data General Corp., Southboro, MA).

One of the problems associated with wavelength resolved systems is the time dispersion, or chirp, of the interrogation pulse which creates a temporal bias across the spectrum. Because red light tends to travel faster in dispersive media than blue light, kinetic differences will first appear in the short wavelength region of the spectrum and only later will such differences become evident in the longer wavelengths. We have measured the chirp in the system described above and have found a difference of ~5 ps between 420 and 440 nm. Differences between the spectra shown in Figs. 2*b* and 3 are due both to chirp and to the kinetics of the ligand photodissociation process.

## RESULTS AND DISCUSSION

The data shown in Fig. 2 show the difference spectra of MbCO and $MbO_2$ after light excitation. Fig. 2*a* shows the room temperature difference spectra between the liganded $MbO_2$ species and its product 200 ps after excitation with a 532-nm, 25-ps pulse. The corresponding MbCO difference spectrum is reproduced in Fig. 2*b*. For comparison purposes, we show the conventional static difference spectra between the ligated species and deoxymyoglobin. Only minor differences of <2-nm spectra shifts and <5% shape changes are observed. This spectral similarity indicates that the state of MbL present 200 ps after excitation is very similar, if not identical, to deoxymyoglobin. We have shown previously that this state is formed with a 6–8-ps lifetime (1). Difference spectra of these species taken at 600 ps after excitation were identical to the 200-ps difference spectra and suggest that we are observing a long-lived species with a lifetime of at least a few nanoseconds. This species we attribute to the dissociated ligand Mb + L, and its long lifetime is attributed to, and consistent with, its recombination rate to form MbL. The 6–8-ps photodissociation process observed previously is also indicated in the difference spectra of both MbCO and $MbO_2$ taken within 30 ps of excitation. However, an unambiguous assignment

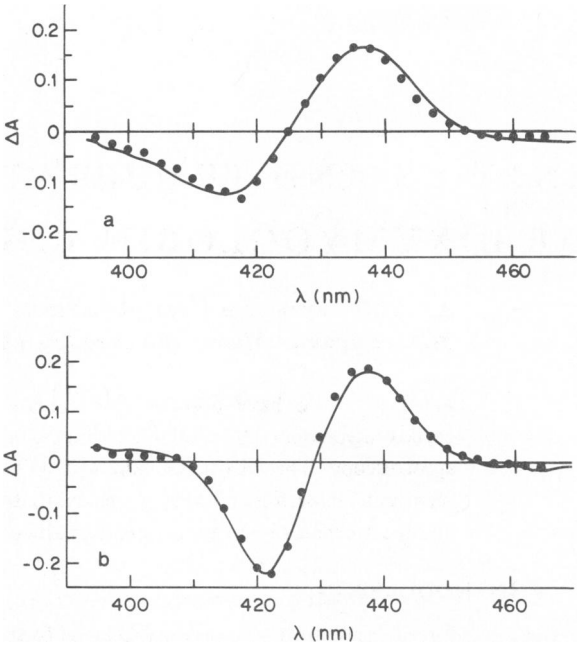


FIGURE 2 (*a*) Solid line depicts $MbO_2$ picosecond difference spectrum taken 200 ps at 280 K after excitation with a 25-ps, 530-nm pulse; dots show static difference spectrum between deoxymyoglobin and $MbO_2$. (*b*) Solid line depicts MbCO picosecond difference spectrum taken 200 ps at 280 K after excitation with a 25-ps, 530-nm pulse; dots show static difference spectrum between deoxymyoglobin and MbCO.

is difficult because of the chirp and the ~25-ps length of the excitation pulse. The photodissociation kinetics of MbCO as a function of temperature are shown in Fig. 3, monitored 25 ps after excitation at 260 and 20 K. The 25-ps time was chosen because it is most sensitive to changes in the 6–8-ps time interval. The data shown represent 20 pairs of excitation vs. no excitation spectra. The error bars are given by the standard deviation among pairs.

The spectra of MbCO at 260 and 20 K are essentially identical, with the exception of a slight narrowing in the width of the Soret band at 420 nm at 20 K. The bandwidth change at lower temperature, however, is not sufficient to suggest that a different process takes place at 20 than at 260 K. The absorbance also does not change drastically

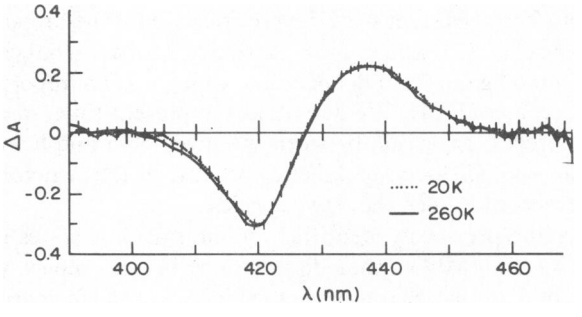


FIGURE 3 Picosecond difference spectra for MbCO in 75% glycerol 25 ps after excitation, at 260 K (—) and 20 K (---). Error bars given by standard deviations among 20 pairs of excitation/no excitation shots.

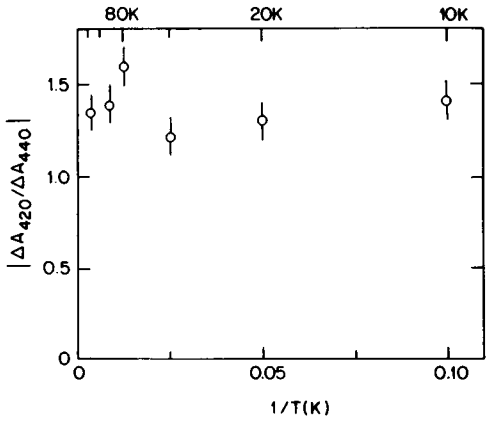


FIGURE 4 Plot of the ratio of the bleach of the Soret band absorbance maximum to the increase in absorbance at the maximum of the absorption band of the dissociated species measured 25 ps after excitation for MbCO in 75% glycerol as a function of temperature.

within the temperature range of our experiment. This is shown in Fig. 4 in the form of the ratio of the change in absorbance of the 420-nm band of the ligated species (MbL) to the 440-nm dissociated species (Mb + L) as a function of temperature. It is evident from Fig. 4 that the photodissociation rate does not change by >30% or 2–3 ps. We therefore conclude that the photodissociation process is temperature independent, and consequently that $MbO_2$ and MbCO do not dissociate via an activated process.

If a barrier exists and photodissociation occurs by quantum mechanical tunneling, then the barrier must be very high and the tunneling distance remain very nearly the same from 10 to 260 K. If we assume that it is an electron that tunnels through a square barrier with an attempt factor of $\sim 10^{15}\ s^{-1}$, then we arrive at a minimum barrier height of ~20 kJ/mol. We believe this case is unlikely because it is expected that the tunneling distance, and hence the dissociation lifetime, will increase at the lower temperatures where the populated states are practically at the minimum of the electronic potential curve.

If the photodissociation rate is governed by the Arrhenius law, and the barrier is enthalpic, then the activation enthalpy must be <0.03 kJ/mol as any larger barrier would influence the kinetics to a greater extent than is observed. If the barrier were entirely entropic, then the ratio of the number of accessible states of the intermediate Mb · · · L to the number in the transition state between Mb · · · L and Mb + L must be $\sim 10^4$. This corresponds to an entropy barrier of ~0.08 kJ/mol K.

There is still the possibility that the excitation pulse causes the population of a highly excitated vibronic level of MbL that decays directly to the Mb · · · L and Mb + L states, rather than relaxing to the lower vibronic level and then crossing over to the intermediate states (6, 7). Under these conditions, temperature effects will be minimal. Even if a barrier existed, it would not necessarily cause dramatic changes in the rate, and photodissociation would appear as a nonactivated process. Although this mechanism is plausible and can be tested by excitation tuning with a picosecond dye laser, we believe that it is unlikely because of the high rate of vibrational relaxation in excited electronic states observed consistently for metal porphyrins and other large molecules in condensed media (8–10). To some extent this is substantiated because our results show (1) that the photodissociation rates are the same for 355 and 530 nm excitation.

## APPENDIX

Assuming the photodissociation process via an activated mechanism, the maximum activation energy is calculated using the Arrhenius equation and the maximum difference in the experimentally determined lifetimes of ~7 and ≲10 ps for 260 and 10 K, respectively.

$$k = Ae^{-H/T}, \quad H = T \log (A/k)$$

for

$$k = 1.4 \times 10^{11}\ s^{-1} \text{ at } 260\ K$$

and

$$k = 1.0 \times 10^{11}\ s^{-1} \text{ at } 10\ K. \tag{A1}$$

We calculate $H \lesssim 3.5\ K \simeq 0.03$ kJ/mol.

If there is tunneling (11, 12), we assume that the Gamow expression is applicable:

$$k \sim A \exp\left[-\frac{ad}{\hbar}\sqrt{m(H-W)}\right] \sim 10^{11} s^{-1}, \tag{A2}$$

where $A$ is a frequency factor ($\sim 10^{15}\ s^{-1}$) for an electron, $d$ the tunneling distance, $m$ electron mass, $H$ the barrier height, and $W$ the incident particle energy.

Assuming the maximum possible difference in dissociation lifetimes consistent with our experimental results, assuming small differences in rates and $W \ll H$, then

$$\frac{\Delta k}{k} \sim \frac{3}{7} \sim \frac{ad}{\hbar}\sqrt{mH}\frac{\Delta W}{2H}. \tag{A3}$$

Assuming that the transition most probably occurs at energies near the temperature of the system, then $\Delta W = 250$ K and from Eqs. A1 and A2, we obtain $H \gtrsim 2{,}500K \sim 20$ kJ/mol; $d \sim 3$ nm. If the barrier is purely entropic, then the ratio between the number of accessible states in the pre-dissociative state to the number in the transition state may be obtained from Eq. A1 by setting $H = 0$ and using $A = \nu e^S$ where $e^{-S} \sim A/k \sim 10^4$ and $\nu \sim 10^{15}\ s^{-1}$.

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