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Recombination Intermediates of Photodissociated CO Myoglobin at Ambient Temperatures Detected by Time-Resolved Resonance Raman Spectroscopy[†]

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In attempts to understand fast dynamics of protein structures, recombination of photodissociated CO of carbonylmyoglobin (COMb) has been extensively investigated.[1-10] Photodissociation of COMb takes place in 350 fs,[1] and the photoproduct appears to be the same as the equilibrium deoxyMb for all times greater than 3 ns,[2] although 85% of photodissociated CO remains in the globin 1 ns after photolysis and 50% remains after 50 ns.[3] The Fe–histidine stretching ($\nu_{\text{Fe-His}}$) frequency of the photodissociated species is reported to be the same between 10 ps and 10 ns following photolysis.[4] There is no return of CO to the heme in the 200–8000 ps range,[7] but afterward the recombination occurs in nonexponential ways.[8-10] Cryogenic IR measurements revealed differential recovery for different A_i sites,[9b] but no new intermediate states have been found in the recovery process. In X-ray crystallographic analysis at 1.5 Å resolution,[11] on the other hand, it has been noted that there is no pathway for the migration of CO between the buried binding site and solvent. Therefore, rapid rearrangements of the protein structures should be accompanied by recombination of CO. Here we report the first direct evidence from time-resolved resonance Raman (TR^3) experiments for the presence of a transient species, which is generated after CO migrates into the heme pocket but before it binds to the heme.

Sperm whale Mb was isolated from stored meat according to Yamazaki et al.,[12] and the purified preparation was adjusted to a concentration of 25 μM in 50 mM tris-HCl buffer, pH 8.0. The metMb was placed in the airtight spinning cell, and its atmosphere was replaced by CO gas. Then the protein was reduced by a small amount of dithionite. The nanosecond pump/probe TR^3 experiments ($\Delta\tau = 7$ ns) were carried out as described previously[13a] with the pump (532 nm, 4 mJ) and probe pulses (416 nm, 100 μJ), a triple polychromator (Spex 1877) equipped with an intensified photodiode array (PAR, OMA III 1421B), and a slow spinning cell (25 rpm). To measure the time profile of RR spectra, the delay time (Δt) of the probe pulse from the pump pulse was varied between −20 ns and 1 ms, and spectra for different Δt_i were measured quasi-simultaneously.[13c] Accumulation time for each time profile spectrum was 70 min, but it was 210 min for precise spectra.

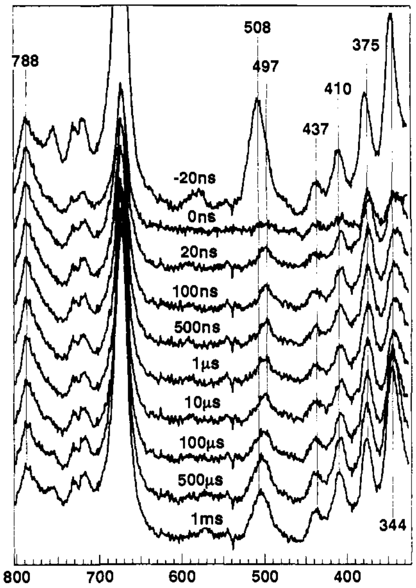

Figure 1. The TR^3 spectra of $^{12}C^{16}$OMb in the time range from Δt = −20 ns to Δt = 1 ms. Instrumental conditions: pump, 532 nm, 4 mJ, 10 Hz, and $\Delta\tau$ = 7 ns; probe, 416 nm, 100 μJ, 10 Hz and $\Delta\tau$ = 7 ns. In the quasi-simultaneous measurements for different Δt, the computer-programmed data acquisition proceeded as follows: The TR^3 spectrum for a selected Δt was measured for 8 s, and then the measurement was made for the next Δt value. After measurements were made for all Δt values, another series of measurements were repeated from the initial Δt value and the others following it. This was repeated 500 times. The total accumulation time for each spectrum was 4000 s.

Figure 1 shows the TR^3 of $^{12}C^{16}$OMb observed for Δt = −20 ns to Δt = 1 ms. Figure 2 shows the steady state RR (a) and TR^3 (at Δt = 1 μs) (b) spectra of $^{12}C^{16}$OMb and $^{13}C^{18}$OMb. The RR spectrum at Δt = −20 ns in Figure 1 is identical with the steady state spectrum in Figure 2a, which demonstrates that COMb in the repeated pump/probe illumination in the spinning cell is in the equilibrium state before pumping. The steady state RR band at 508 cm^{-1} is shifted to 497 cm^{-1} in Figure 2a, indicating that this band is associated with the Fe–CO stretching ($\nu_{\text{Fe-CO}}$) mode. This band was absent in the spectrum for Δt = 0 ns (Figure 1) and started to reappear from Δt = 100 μs. Since the spectrum at Δt = 0 ns is close to that of the equilibrium deoxy form excited at this wavelength (not shown), photodissociated Mb reached to the equilibrium state on a nanosecond time scale. Before the recovery of the 508 cm^{-1} band, a new band appeared at 497 cm^{-1} in a time range from Δt = 20 ns to 100 μs. This band is not observed in the spectrum at Δt = 0 ns, although there is a broad porphyrin band at 503 cm^{-1} for the equilibrium deoxyMb. In order to clarify the assignment of this new band, in the intermediate time region (Δt = 1 μs), that is, after appearance of the new band but before recovery of the Fe–CO stretching band, more precise TR^3 spectra with longer accumulation times were measured with

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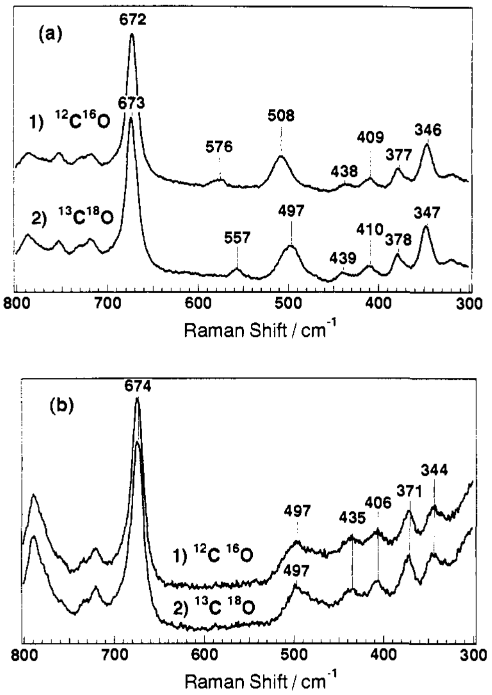

Figure 2. (a) Steady state RR spectra (probe only) of $^{12}C^{16}OMb$ and $^{13}C^{18}OMb$. Experimental conditions are the same as those in Figure 1. (b) TR^3 spectra of $^{12}C^{16}OMb$ and $^{13}C^{18}OMb$ at $\Delta t = 1$ μs. The number of repetitions was 1500, and accordingly the accumulation time was 12 000 s.

$^{12}C^{16}OMb$ and $^{13}C^{18}OMb$ and are shown in Figure2b. Unexpectedly, this new band does not show the CO-isotopic frequency shift. This result manifests that a porphyrin band, which is not present just after photodissociation, appears before the rebinding of CO.

Previously Friedman and co-workers[4] pointed out that the TR^3 spectra at Δt = 10 ps and 10 ns are alike and similar to that of the equilibrium deoxyMb. Since the present spectrum for Δt = 0 ns is an average of 0–7 ns, it is consistent with them that the spectrum for Δt = 0 ns is rather close to that of deoxyMb. The appearance of the clear 497 cm^{-1} band definitely demonstrates the presence of a transient deoxy state distinct from the equilibrium deoxyMb. The ν_{Fe-His} band was weak upon this excitation wavelength, but upon excitations at both 435 and 441 nm, the ν_{Fe-His} band seemed to have a shoulder at its high frequency side in the time range between 20 ns and 100 μs. In addition, a band at 410 cm^{-1} appeared at slightly lower frequency in the transient state. These features resemble the RR spectral characteristics of deoxyMb, but we stress that there exist distinct differences in these deoxy states as displayed by the spectra for Δt = 0 ns and 20 ns to 100 μs. This may be related to the recent note about the presence of the B_i state[14] in which CO is photodissociated from the heme but remains in the heme pocket. We are tempted to think that in the transient deoxy state found in this study CO comes back into the heme pocket but has not bound to the heme iron. Further studies using distal mutants are in progress to ascertain this proposal.

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