Picosecond Resonance Raman Evidence for Unrelaxed Heme in the (Carbonmonoxy)myoglobin Photoproduct†

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ABSTRACT: An actively and passively mode-locked Nd:YAG laser, producing 30-ps pulses of 1-mJ energy at 532 nm, has been used to photolyze (carbonmonoxy)myoglobin (MbCO) and generate its resonance Raman spectrum, which was recorded with a vidicon multichannel analyzer. The photoproduct spectrum was obtained by subtraction of the MbCO spectrum, obtained at lower incident power levels. Comparison with the spectrum of deoxyMb, obtained with the same apparatus, revealed frequency downshifts of $\sim$4 cm^{-1}, for bands at 1604, 1554, and 1542 cm^{-1}, which are identified with porphyrin skeletal modes ν_{10}, ν_{19}, and ν_{11}. These frequencies are known to correlate inversely with the core size of the porphyrin ring, and the shifts imply a larger core size for the photoproduct than for deoxyMb. Similar shifts have been observed for the (carbonmonoxy)hemoglobin (HbCO) photoproduct; in that case, the shifts persist for longer than 20 ns, whereas they are absent in the MbCO photoproduct spectrum within 7 ns of photolysis. The unrelaxed state of the heme group region is therefore suggested to be maintained by protein forces, which relax more rapidly for Mb than Hb. This may reflect a tighter coupling in Hb of the out-of-plane movement of the Fe atom with the proximal histidine-containing F helix.

We report resonance Raman (RR) spectroscopic evidence that the heme group of myoglobin (Mb) is unrelaxed when examined within 30 ps of (carbonmonoxy)myoglobin (MbCO) photolysis. Frequency downshifts, relative to deoxyMb, are observed for porphyrin skeletal modes which are known to correlate inversely with core size (Choi et al., 1982). Similar shifts have earlier been reported for the (carbonmonoxy)-hemoglobin (HbCO) photoproduct (Terner et al., 1980, 1981). These shifts persisted beyond 20 ns, whereas for the MbCO photoproduct, the shifts are absent in spectra recorded with 7-ns photolysis pulses. It is therefore inferred that the heme relaxation being monitored by the core-size marker frequencies is coupled to protein motions which are faster in Mb than in Hb.

Figure 1 shows resonance Raman spectra of MbCO and deoxyMb, obtained with 532-nm 30-ps pulses, generated with a passively and actively mode-locked Nd:YAG laser, with three stages of amplification. The maximum pulse energy was 1 mJ, and the repetition rate was 20 Hz. The laser beam was focused into a solution of MbCO (0.5 mM) flowing through a capillary tube in a recirculating sample cell. Raman photons were collected at 90° and focused onto the slit of a 0.75-m spectrograph equipped with a vidicon multichannel analyzer (PAR OMA II). The spectrum of essentially unphotolyzed MbCO was obtained at low pulse energies, while at higher energy the pulses produced appreciable photolysis (estimated to be $\sim$50%) of the MbCO molecules. The photoproduct spectrum was then obtained by subtracting the MbCO spectrum from that of the photolysis mixture, using the isolated 1630-cm^{-1} MbCO band as the intensity standard [a similar procedure has been followed for studies of HbCO (Terner et al., 1980, 1981) and HbO_2 (Terner et al., 1982) photolysis]. In Figure 1, the resulting photoproduct spectrum is compared with that of deoxyMb, obtained with the same apparatus. A clear frequency downshift of 4 cm^{-1} is seen for the 1604-cm^{-1} band, which is due to a porphyrin skeletal mode, ν_{10} (Terner et al., 1981). Likewise, a downshift of $\sim$4 cm^{-1} is seen for the composite band, centered at 1550 cm^{-1}, which has overlapping contributions from skeletal modes ν_{19} (1554 cm^{-1}) and ν_{11} (1542 cm^{-1}) (Terner et al., 1981). These skeletal mode frequencies are known to correlate inversely with porphyrin core size for a variety of heme complexes (Choi et al., 1982). The downshifts imply a heme core expanded by 0.008–0.01 Å in the MbCO photoproduct relative to deoxyMb (Choi et al., 1982).

Similar downshifts of these modes have earlier been observed for the HbCO photoproduct, in spectra obtained with 30-ps (Terner et al., 1980, 1981) and also 7-ns (Lyons & Friedman, 1980) and 20-ns (Terner et al., 1980, 1981) laser pulses. For MbCO, however, the 7-ns photoproduct spectrum is identical with that of deoxyMb [the ν_4 skeletal mode and the Fe–histidine stretching mode have likewise been reported to be unshifted (Friedman et al., 1982; Findsen et al., 1985)], as shown in Figure 2. These spectra were obtained with a Q-switched YAG laser (Quanta Ray DCR 1A) and a vidicon-equipped triple spectrograph (Spex Triplemate). The sampling arrangement was essentially the same as for Figure 1, but tighter focusing of the laser pulses produced essentially complete photolysis, eliminating the need for spectral subtraction. The grating employed was of higher resolution, and the ν_{19} and ν_{11} peak frequencies are clearly distinguished. The spectra are identical for deoxyMb and photolyzed MbCO to within $\pm$1 cm^{-1}. The width of the 1604-cm^{-1} line is 13 cm^{-1} for Mb, MbCO, and the MbCO photoproduct using our spectrometer. The line width of the 1604-cm^{-1} band is ca. 11 cm^{-1} when observed with an instrument band-pass of 2 cm^{-1}. We can therefore be certain that the dominant part of our

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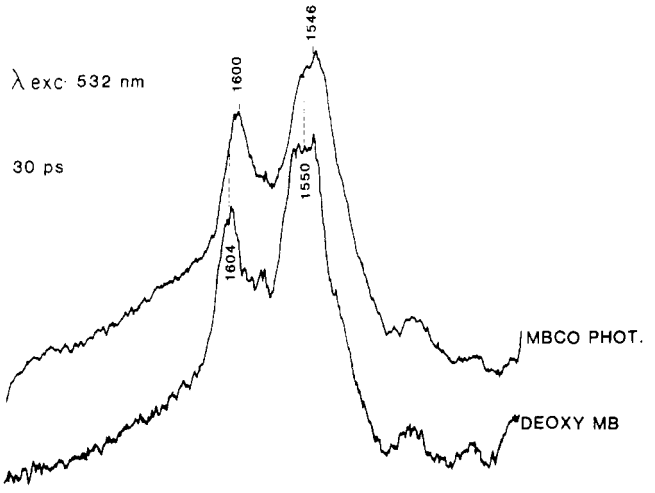

FIGURE 1: Raman spectra (bottom) of deoxyMb (Sigma, sperm whale, 0.5 mM; purified by preparative isoelectric focusing, gently stirred under N_2 with a few crystals of sodium dithionite) excited with 30-ps 532-nm YAG laser pulses (~1 mJ, 10 Hz) during 40-min spectral accumulations and (top) of MbCO photoproduct after subtraction of the MbCO contribution (see text for details). The spectra are unsmoothed.

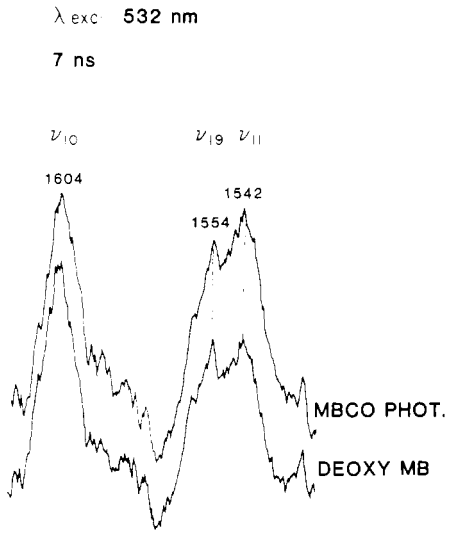

FIGURE 2: Raman spectra of deoxyMb and MbCO photoproduct obtained with 7-ns 532-nm YAG pulses (~1 mJ, 10 Hz) and 10-min accumulation. Sample conditions as for Figure 1. (See text for details.)

observed lines manifest the Raman line width and not the instrument response. The results imply, therefore, that the inhomogeneous distribution of photoproduct Raman transitions spans about the same energy for all three species. Improvements in the precision of the line-width measurements should allow more definite conclusions to be made regarding the range of structures that contribute to the Raman scattering signal.

Raman frequency shifts or line broadening can be caused by sample heating or strong-field effects. An estimate of the strength of interactions (Dick & Hochstrasser, 1984) for our pulse intensities with the heme Q band shows that strong-field effects will not cause band shifts. Sample heating occurs because the 532-nm photolyzing light is about 11 000 cm^{-1} in excess of the CO–heme binding energy. Vibrational energy redistribution is expected to be very fast (a few picoseconds or less) within the heme (Bloembergen & Zewail, 1984; Felker & Zewail, 1985). If the excess energy attains a Boltzmann distribution among the heme modes alone, the vibrational temperature can increase by ca. 200 K. An estimate incorporating thermal diffusion shows that the average heme temperature increases by about 10° at room temperature during the laser pulse if the excess energy initially populates heme modes. The resulting population of low-frequency anharmonic vibrations could cause the heme core to expand and result in a frequency shift of $\lesssim 2$ cm^{-1} (Asher & Murtaugh, 1983). The above estimate neglects relative motions of the heme and its protein environment, which may be excited initially upon heme excitation. The heme makes some 90 van der Waals contacts with the surrounding protein (Antonini & Brunori, 1971). We estimate that the population of vibrations associated with these contacts would result in vibrational heating of the heme too small to be detected in our experiment. The spectrum of deoxyMb, obtained under the same conditions, is a good experimental control, since it gives the same band frequencies as are observed with low-power continuous-wave excitation (Choi et al., 1982), and shows no evidence of heating effects.

It is unlikely that electronic excitation contributes to the picosecond transient Raman spectra, since excited-state lifetimes are expected to be much shorter than the pulse width. The initial photoproduct of heme CO has a rise time of 0.35 ps, and the resulting optical spectrum resembles that of deoxyheme (Martin et al., 1983), although a 1–2-nm red shift of the Soret band can be discerned in spectra obtained with 7-ps pulses (Cornelius & Hochstrasser, 1981; Cornelius et al., 1982; Johnson et al., 1985). It is likely that a population of electronically excited states from multiple excitations of HbO_2 with intense YAG laser pulses might account for the large downshifts and broadening of the 30-ps YAG-generated HbO_2 photoproduct spectrum (Terner et al., 1982). No such effects are seen in the present MbCO photoproduct spectrum or in the low-power HbO_2 picosecond photoproduct Raman spectrum (Nagumo et al., 1981). Multiphoton effects with 532-nm pulses are less likely for heme–CO than for heme–O_2 complexes, the former lacking the low-energy charge-transfer states of the latter (Greene et al., 1978), which can act as intermediate states in multiphoton absorption (Chernoff et al., 1980).

The possible contributions of a nonequilibrium heme pocket to the <30-ps Raman spectrum must be considered. The diffusion of water in the heme pocket following photolysis, for example, could affect the Raman spectrum. The presence of a partially occupied water site in the distal heme pocket has been detected in the deoxyMb (Takano, 1977) and MbCO (Phillips, 1980) X-ray structures. Experiments are planned to test the influence of water on the porphyrin skeletal modes.

The core expansion of the HbCO photoproduct, implied by the core-size marker band downshifts relative to deoxyHb, has been interpreted (Terner et al., 1981) as evidence for restriction of the out-of-plane motion of the Fe atom, five-coordinate deoxyheme (Hoard & Scheidt, 1974a,b) having a smaller core than six-coordinate high-spin planar Fe^{II} hemes (Reed et al., 1980). It was suggested (Terner et al., 1981) that this excursion is limited by protein forces, plausibly associated with the F helix which contains the proximal histidine ligand and which is seen in the deoxyHb crystal structure to be shifted by ~1 Å relative to the heme group (Baldwin & Chothia, 1979; Fermi et al., 1983). This motion of the F helix was suggested to be a reasonable candidate to explain the relatively slow [>20 ns (Terner et al., 1981) but <300 ns (Stein et al., 1982)] relaxation of the core-size frequency shifts. Coupling of the Fe out-of-plane displacement with the F-helix motion could be an important aspect of the mechanism of hemoglobin cooperativity (Perutz, 1970). In this context, the faster relaxation of the Mb core-size markers suggests a faster F-helix

motion, or a weaker coupling to the Fe displacement; the much smaller change in the F-helix position in deoxyMb relative to metMb than in deoxyHb relative to metHb is consistent with this view (Takano, 1977).

It is of interest in this connection that Findsen et al. (1985), using a H_2-Raman-shifted YAG laser at 435 nm in resonance with the heme Soret band, have found that the Fe–histidine stretching frequency, 220 cm^{-1}, is the same in the 30-ps MbCO photoproduct as in deoxyMb. This mode is not enhanced at 532 nm and does not appear in our spectra. When HbCO is photolyzed, ν_{Fe-His} and the core-size marker bands are all initially unrelaxed and subsequently respond to different kinetic steps in the protein relaxation. Thus, ν_{10}, ν_{11}, and ν_{19} relax between 20 and 300 ns after photolysis (Stein et al., 1982), while the relaxation time for ν_{Fe-His} (Friedman et al., 1983) as well as the correlated (Ondrias et al., 1983) skeletal mode ν_4 (Lyons & Friedman, 1981) is $\sim$1 μs, considerably longer than that of the core-size markers. The relaxations of these modes in Mb, although much faster than in Hb, evidently remain kinetically distinct and must be responding to different structural changes. The fact that ν_{Fe-His} is relaxed at 30 ps implies that the Fe–His bond attains the same length as in deoxyHb during this time. Nevertheless, the Fe atom might still be constrained from its full out-of-plane displacement, since this displacement, as well as the porphyrin core size, is determined primarily by the nonbonded contacts between the imidazole ring and the pyrrole N atoms (Olafson & Goddard, 1977; Warshel, 1977). At a given Fe–His distance, the Fe atom can be more or less in the heme plane, the displacement having a detectable effect on the porphyrin core size.

Molecular dynamics simulations (Henry et al., 1985) and subpicosecond transient absorption (Martin et al., 1983) support the view that the out-of-plane displacement of the Fe atom is extremely rapid (<0.4 ps) but are insufficiently accurate to determine to what extent the Fe atom excursion is limited by the protein. The present data suggest that at 30 ps the protein still limits the Fe out-of-plane motion. The relaxation time for the MbCO photoproduct core-size marker bands, which this study has bracketed between 20 ps and 7 ns, should reflect the heme–protein coupling in Mb. Pulse–probe picosecond RR experiments are planned to examine this process.

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