Subpicosecond resonance Raman spectroscopy of carbonmonoxy- and oxyhemoglobin

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ABSTRACT In this paper we present the resonance Raman spectrum of the carbonmonoxy- (HbCO) and oxyhemoglobin (HbO_2) photointermediates on a 800–900 fs timescale. In the case of HbCO, the frequencies of the so-called core-size markers (1500–1650 cm^{-1}) are characteristic of a deoxylike photoproduct in a high spin state ($S = 2$) with a partially domed heme. The spectrum of the HbO_2 photointermediate, on the other hand, is different, and may be characteristic of an excited-state species. These results are discussed in terms of a reaction scheme previously presented by Petrich, J. W., C. Poyart, and J. L. Martin (1988. *Biochemistry.* 27:4049–4060) and compared with those obtained in the literature on a 30–40 ps timescale. In both molecules a distinct downshift of the ν_4 mode was observed with respect to the equilibrium value, which is indicative of an elevated temperature of the heme after photodissociation.

INTRODUCTION

Hemoglobin (Hb) is probably one of the most thoroughly investigated biological molecules. It is a tetrameric protein, which exists in two different quaternary structures, one of which, designated T, is stable in the absence of ligands like O_2, CO, and NO, and the other, designated R, is stable when ligands are bound (Perutz, 1979). The existence of these two quaternary structures is the basis for the cooperativity of ligand binding: the affinity for oxygen and other ligands increases as the number of ligand molecules already bound increases. A lot of research has been devoted to study the coupling between the heme groups in Hb and the globin moiety during the "switch" from the R to the T state.

The study of the dynamics of the photodissociation of ligated hemoglobins has yielded a wealth of information regarding the process of ligand binding to deoxyhemoglobin (deoxy-Hb) (Antonini and Brunori, 1971; Parkhurst, 1979). The availability of lasers and spectrometers providing time resolution in the picosecond and femtosecond domain has enabled the studies of the primary photophysical processes occurring immediately after ligand photodissociation. The complicated photochemistry and photophysics of ligated hemoglobins have mostly been studied by transient optical absorption spectroscopy (Shank et al., 1976; Greene et al., 1978; Chernoff et al., 1980; Martin et al., 1983, 1984; Janes et al., 1988; Murray et al., 1988; Petrich et al., 1988). The initial photodissociation is assumed to occur in <50 fs and results in two excited-state species, Hb^*_I and Hb^*_{II} (Martin, 1983, 1984; Petrich et al., 1988). Whereas the former decays with a time constant of 350 fs into a deoxylike photoproduct ($Hb^\dagger$), the latter lives much longer (~2.5 ps) and exhibits a much higher reactivity towards the photodissociated ligand. This was assumed to result from a planar heme in this species. Moreover, it was found that the population of Hb^*_{II} from HbO_2 was much larger than that resulting from HbCO (75 vs. 14%). This would partly explain the much lower quantum yield of photodissociation for HbO_2 (~0.05 on a microsecond timescale) compared to that of HbCO (~0.5) (Saffran and Gibson, 1977). The deoxylike species, $Hb^\dagger$, was assumed to be in a high spin state ($S = 2$), although no direct evidence for this was obtained (Martin et al., 1983, 1984; Petrich et al., 1988). Furthermore, a distortion of the spectrum of the deoxylike Hb in the Soret region was interpreted to be indicative of doming of the heme (Petrich et al., 1988).

Although transient absorption spectroscopy has been very widely and fruitfully used to study photodissociation of ligated Hb, it is not very sensitive to structural changes of the heme nor to the influence of the protein environment on the heme, because electronic absorption bands are in general very broad and structureless. Resonance Raman spectroscopy, on the other hand, can yield detailed structural information. It is limited, however, by the bandwidth of the laser, which cannot be much broader than the bandwidth of the Raman bands because otherwise the latter cannot be well resolved. This limits conventional Raman studies to using lasers with a pulsewidth of ~500 fs. Until very recently, resonance Raman spectra of hemoglobins had not been obtained with a time resolution better than 25–50 ps (Coppey et al., 1980; Terner et al., 1980, 1981; Nagumo et al., 1981; Terner et al., 1982; Findsen et al., 1985*a* and *b*; Campbell and Friedman, 1986). In 1987, Petrich et al. presented for the

0006-3495/90/10/931/07 $2.00

first time a subpicosecond, time-resolved resonance Raman study of the ν_4 mode of carbonmonoxyhemoglobin (HbCO). They interpreted a shift of this band as a function of delay time in terms of a rapid vibrational cooling of the heme, allegedly followed by heme doming.

Since the so-called core-size markers in the region from 1,500 to 1,650 cm^{-1} are especially sensitive to both the position of the Fe atom with respect to the heme plane and its spin state, a study of these resonance Raman bands should be able to confirm the above-mentioned hypotheses. In this work we present a resonance Raman study on a 800–900 fs timescale of the photoproducts of HbCO and HbO_2. We confirm the results of Petrich et al. (1987) obtained for HbCO regarding the ν_4 mode on this timescale. A similar shift was observed for the ν_4 mode of HbO_2. Furthermore, we obtained the resonance Raman spectrum of $Hb^{\dagger}$ resulting from HbCO, and conclusively show the high-spin ($S = 2$) nature of this initial photoproduct and its partially domed heme shape. For HbO_2 on the other hand, even more downshifted Raman bands were observed, which we assigned to Hb^{*}_{II}, and which confirms the planar state of the heme in this excited–state species. The results are discussed and compared to those obtained on a 30–40 ps timescale.

MATERIALS AND METHODS

Sample preparation

Human hemoglobin A was isolated in the oxygenated form (HbO_2) from packed red blood cells, obtained from the Blood Bank at the UCLA Medical Center, according to the method of Antonini and Brunori (1971). For the experiments on HbO_2, this solution was diluted with 0.01 M phosphate buffer (pH 7.4) to a final concentration of 0.6 mM. The deoxygenated form was obtained by stirring a stock solution (concentration 2.90 mM, as measured by the heme absorption at 544 and 576 nm) overnight under nitrogen at 4°C. Carbonmonoxyhemoglobin (HbCO) was obtained by stirring the deoxygenated solution under CO overnight. Before every experiment the stock solution was diluted to a concentration of 0.8 mM with 0.01 M phosphate buffer (pH 7.4), which had been saturated with CO by bubbling for 2 h.

Experimental set-up

The experimental arrangement used for obtaining resonance Raman spectra is similar to that used by Terner et al. (1980, 1981) and Nagumo et al. (1981). However, a different laser system was used: a cavity-dumped dye laser (model 375B, Spectra-Physics Inc., Mountain View, CA) was synchronously pumped by the compressed, frequency-doubled output of a Nd:YAG laser (model 3800, Spectra-Physics Inc., Mountain View, CA). The dye rhodamine 6G in ethylene glycol provided an output power at 578 nm of 20 mW at a dump rate of 800 kHz (25 nJ/pulse). A two-plate birefringent was used as the tuning device. The bandwidth of the laser was found to be ~22–25 cm^{-1}. This is approximately two times the transform–limited bandwidth of a 800–900-fs pulse, as was measured by autocorrelation (model 5-14A, Inrad Inc., Northvale, NJ) assuming a $sech^2$ pulseshape.

The beam was directed via a microscope objective (Zeiss Neofluar 40×) onto a vertically flowing jet, and was thus focused to a ~5–10 μm spot. iecause of the low peak energy of the pulse, tight focusing is necessary to obtain a reasonable amount of photolysis and to eliminate multiple excitation of the sample. Each pulse simultaneously serves as a photolysis and a Raman excitation light source. Spectra of unphotolyzed HbCO and HbO_2 were obtained by defocusing the laser, thus decreasing the power below the threshold needed for photolysis. Difference spectra were obtained by subtracting an arbitrarily scaled diffuse focus spectrum from a tight focus spectrum.

The HbCO sample was kept under a flowing blanket of CO. Both samples were pumped through a syringe needle (diameter 110 μm) by a peristaltic pump (Masterflex, Cole-Parmer Instrument Co., Chicago, IL). The measured flow rate, as calculated from the volumetric flow of 0.25 ml/s and the needle diameter, was 26 m/s. Combined with the 5–10-μm spotsize, this gave a maximum residence time of 0.4 μs. At a dump rate of 800 kHz each pulse should have encountered a fresh volume of unphotolyzed $HbCO/HbO_2$.

Scattered light was collected at 90° and focused onto the slit of a 0.5-m spectrograph (model 1870, Spex Industries, Inc., Edison, NJ) with a 1,200-grooves-per-mm ruled grating. Resonant scattering was blocked by two 2-63 cut-off filters (Corning Glass Works, Corning, NY). The resolution of the monochromator was determined to be ~3 cm^{-1} with the help of a neon discharge lamp. The latter was also used to calibrate the resonance Raman wavelengths. Vibrational frequencies are estimated to be accurate to ±3 cm^{-1} in the original spectra and to ±3–5 cm^{-1} in the computer-generated "difference" spectra, depending on the "sharpness" of the Raman bands. The detection system consisted of a diode array detector, thermoelectrically cooled to −20°C (model 1455, EG&G Princeton Applied Research Corp., Princeton, NJ) and a model 1460 optical multichannel analyzer (OMA), with a 1462 detector controller and 1462/99 14-bit A/D controller (EG&G Princeton Applied Research Corp., Princeton, NJ).

The "difference" spectra were obtained by subtracting an arbitrarily scaled low-power spectrum from a high-power spectrum. As was noted by Hsieh et al. (1983), caution should be exercised when information is extracted from these "difference" spectra. They are to be used strictly as a guide to interpreting the actual spectral changes. In order to more clearly present the data, in all spectra the background, simulated by a fourth order polynomial fit, was subtracted.

RESULTS AND DISCUSSION

The electronic absorption spectra of the hemoglobins show visible absorption bands around 575 nm (Q-band region) and a very intense band around 425 nm, the Soret or B-band. The different vibrational modes of the hemoglobin, which report on structural changes taking place after light absorption, are enhanced by excitation to either one of these bands. For all spectra presented in this paper excitation was at 578 nm, i.e., in the Q-band.

A vibrational mode which has been shown to be sensitive to the ligation and oxidation state of the heme, is the ν_4 mode. It corresponds to a polarization dependent, totally symmetric stretching mode of the $C_{\alpha}N$ bond of the heme, and is greatly enhanced upon Soret-band excitation. Upon deligation it has been shown to shift from ~1,370 cm^{-1} to ~1,350 cm^{-1}. The frequency of the ν_4 mode of the CO-photointermediate was carefully studied as a function of delay time between 0.2 and 90 ps (Petrich

et al., 1987). It was shown to be downshifted with respect to the equilibrium value of unligated Hb. The time evolution of this downshift was interpreted in terms of a vibrational cooling of vibrationally hot heme.

Although Q-band excitation does not specifically enhance this ν_4 mode, we have been able to see the same effect on the 800–900-fs timescale. In Fig. 1 resonance Raman spectra are shown of the vibrational region between 1,200 and 1,500 cm^{-1} of HbCO and HbO_2 (upper and lower spectrum, respectively). The vibrational frequencies are those found in the literature for HbCO (Woodruff and Farquharson, 1978; Terner et al., 1980, 1981) and HbO_2 (Spiro and Strekas, 1972; Strekas and Spiro, 1972). The ~1,370-cm^{-1} band appears in both spectra as a shoulder on the high energy side of the band around 1,340 cm^{-1}. In Fig. 2 *a* (*upper part*) we have expanded the region around 1,350 cm^{-1} for HbCO. A clear intensity decrease around 1,370 cm^{-1}, and an increase around 1,350 cm^{-1} can be observed. This is clearly evident in the difference spectra in the lower part

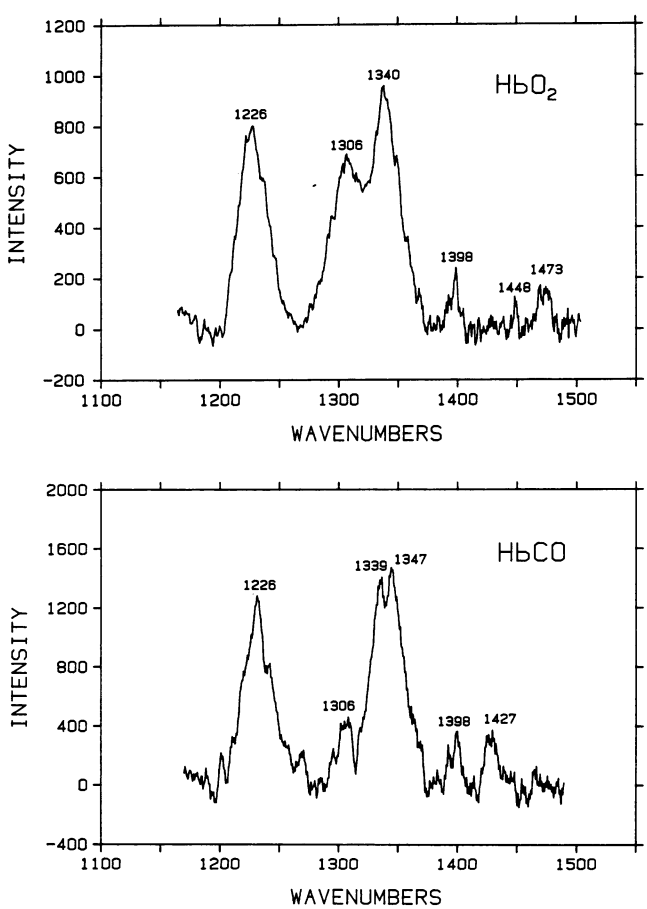

FIGURE 1 Resonance Raman spectra of HbO_2 and HbCO in the region from 1,100 to 1,500 cm^{-1} obtained with tight focusing. The frequencies are in excellent agreement with those found in the literature.

of Fig. 2 *a* obtained by subtracting out the band at 1,339 cm^{-1} which occurs in both spectra. A fit with two Gaussian envelopes is also shown. The equilibrium value of the ν_4 mode (as found in deoxyhemoglobin) on a long timescale is 1,357 cm^{-1}. The downshift with respect to this value was calculated according to the formula: $\Delta\nu = 2^*\nu_{infl} - \nu(Hb) - \nu(HbX)$ (Petrich et al., 1987). In this formula, ν_{infl} is the frequency at the inflection point in the difference spectrum, $\nu(Hb)$ is the equilibrium value in deoxyhemoglobin, and $\nu(HbX)$ is the value in the ligated hemoglobin. The calculated downshift with respect to the deoxy species of 8–9 cm^{-1} for HbCO is in excellent agreement with the value obtained by Petrich et al. (1987) on this timescale. The frequency of the negative band, at 1,372 cm^{-1} (the original HbCO band) has been found for HbCO in a number of studies (Friedman and Lyons, 1980; Asher, 1981). In the case of HbO_2 there is no earlier report on the frequency shift of the ν_4 mode. Similar spectra to those presented for HbCO are shown in Fig. 2 *b* for HbO_2. We find a value for the downshift of 7–8 cm^{-1}, which is slightly lower than that observed for HbCO on the same timescale but shows that a similar process might take place in the two hemoglobin molecules. Dynamical calculations by Henry et al. (1985, 1986) indeed indicate that local heating from the photolysis pulse can be substantial on a timescale of up to 10 ps.

In Fig. 3 resonance Raman spectra are presented of the initial photoproduct after photodissociation of HbCO in the vibrational region between 1,450 and 1,750 cm^{-1}, which contains the core-size markers ν_{11}, ν_{19}, and ν_{10}. These Raman bands are found at 1,545 cm^{-1}, 1,556 cm^{-1} and 1,605 cm^{-1} for deoxy-Hb (Terner et al., 1980, 1981). The spectra shown were obtained with a perpendicular orientation of the polarization. The upper spectrum represents a spectrum in which the laser beam was tightly focused, whereas for the middle spectrum it was defocused. Only in the former case partial photolysis occurs. In the difference spectrum, as shown in the lower part of Fig. 3, the vibrational frequencies of a deoxylike photointermediate are revealed. They appear at 1,540, 1,550, and 1,600 cm^{-1}.

On a timescale of 30–40 ps similar spectra of a deoxylike photoproduct have been reported in the literature (Terner et al., 1980, 1981). The slight, but reproducible downshifts of the frequencies of the core-size markers were shown to persist even up to 15 ns (Dallinger et al., 1978; Woodruff and Farquharson, 1978; Lyons et al., 1978; Terner et al., 1980, 1981; Dasgupta and Spiro, 1986). These results eliminate the possibility that the bands in the femtosecond spectra are due to hot unrelaxed species, and further establish that upon photolysis of HbCO a heme structure results on a timescale of hundreds of femtoseconds, which is similar to that of deoxy-Hb. The frequencies of the core-size markers depend on

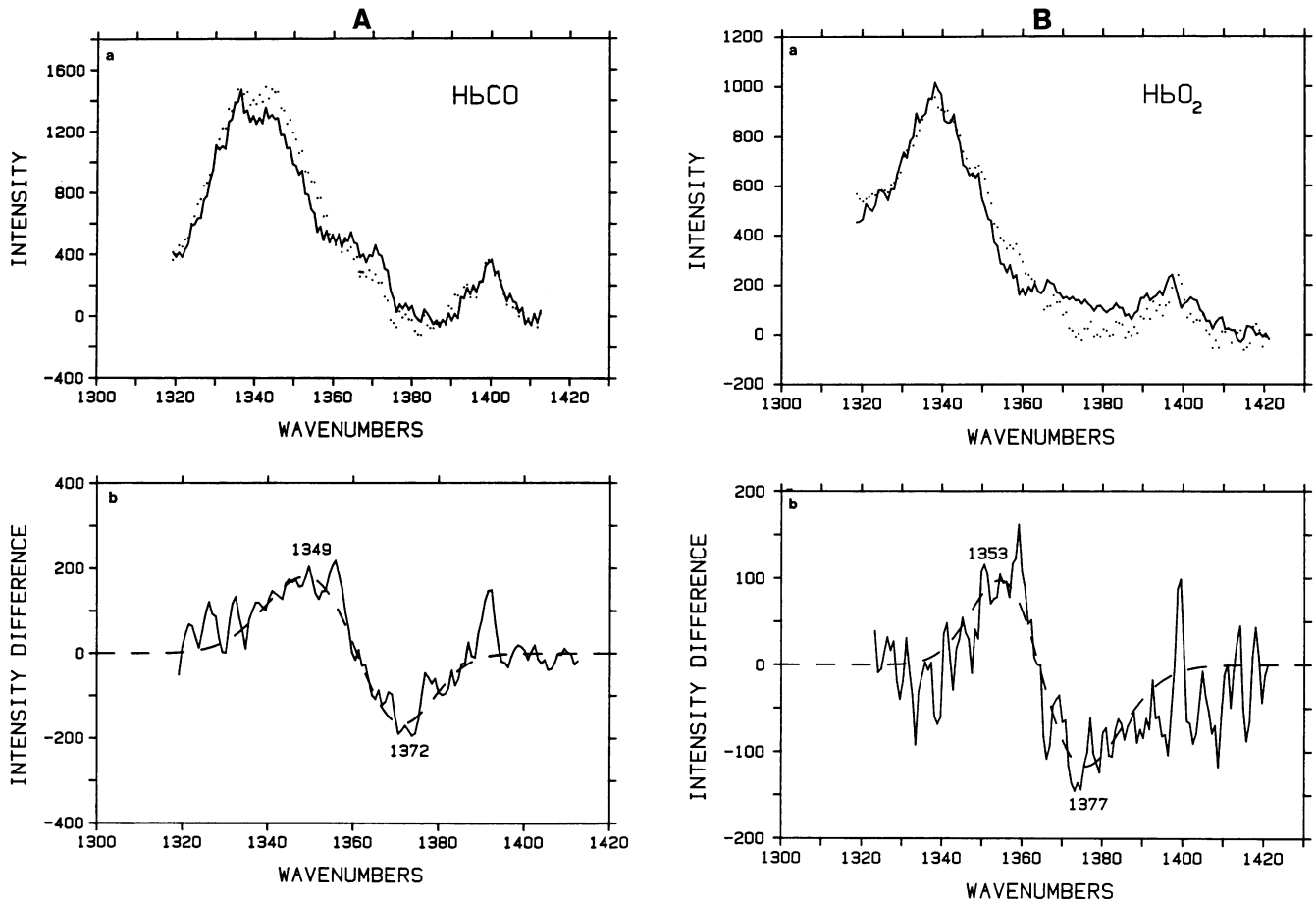

FIGURE 2 (*A, a*) Expanded view of part of the spectra presented in Fig. 1 for HbCO. The spectra were obtained at tight (*dotted line*) and diffuse (*solid line*) laser focus. In the former case, the increase of the region ~1,350 cm^{-1} and decrease ~1,370 cm^{-1} can be clearly distinguished. (*A, b*) Difference spectra of the same region shown in the upper part of this figure for both molecules studied. Upon focusing, distinct photolysis is found to occur for both molecules, indicated by a shift of the ν_4 mode from ~1,370 to ~1,350 cm^{-1}. (*B, a*) Expanded view of part of the spectra presented in Fig. 1 for HbO_2. The spectra were obtained at tight (*dotted line*) and diffuse (*solid line*) laser focus. In the former case, the increase of the region ~1,350 cm^{-1} and decrease ~1,370 cm^{-1} can be clearly distinguished. (*B, b*) Difference spectra of the same region shown in the upper part of this figure for both molecules studied. Upon focusing, distinct photolysis is found to occur for both molecules, indicated by a shift of the ν_4 mode from ~1,370 to ~1,350 cm^{-1}.

the ligation of the heme and on the spin state of the iron. Because deoxy-Hb is known to have a high spin ($S = 2$), the photointermediate must also be in the $S = 2$ state. The further downshift of the core-size markers with respect to deoxy-Hb can be explained by the fact that the Fe atom is apparently more constrained in the heme plane following photoexcitation than in deoxy-Hb, in which it has been shown to be fully out-of-plane.

Excited-state spin conversion is known to be very rapid, because it takes place via intersystem crossing. On the basis of femtosecond transient absorption spectroscopy Petrich et al. (1988) postulated the formation of a $^5T_2$ $Hb^{\dagger}(^3T_1)$ on a timescale of 300–350 fs from an excited-state precursor, Hb^*_I. The subpicosecond resonance Raman spectra presented here show that spin conversion is indeed extremely fast, i.e., <800 fs. The downshift of the frequencies of the core-size markers, however, seems to imply that the Fe atom is still only partially out of the plane. It should be noted that on the timescale of the experiments presented here, Raman scattering from the excited-state species Hb^*_I (Petrich et al., 1988) cannot contribute more than 10% because of its short lifetime (300–350 fs).

A completely different situation exists for the Hb^*_{II} excited state. This was shown to live much longer (~2.5 ps). However, in the case of HbCO, it is not formed in large amounts on this timescale (~14%) (Petrich et al., 1988); therefore we did not observe its spectrum after photodissociation of HbCO. For HbO_2 on the other hand, Hb^*_{II} is the main product and is expected to show up in the

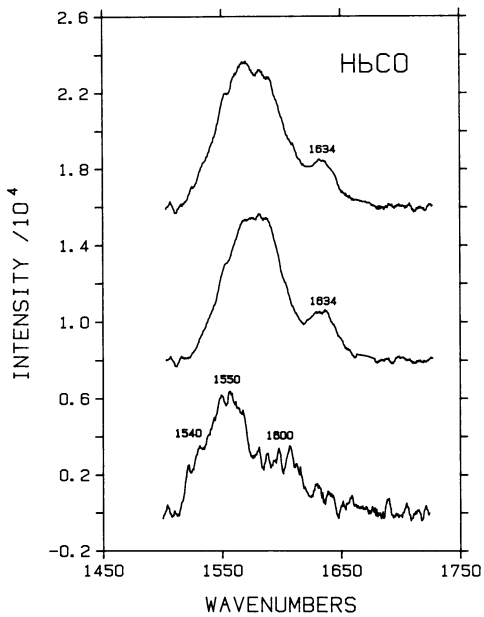

FIGURE 3 Resonance Raman spectra of HbCO and its photodissociation products on the 800–900-fs timescale in the core-size region (between 1,450 and 1,750 cm^{-1}). The upper and middle spectrum were obtained with tight and diffuse focus, respectively. Only in the former case an appreciable photolysis yield is obtained. The lower spectrum is a difference spectrum, which shows the Raman modes of a deoxylike photoproduct, $Hb^{\dagger}$.

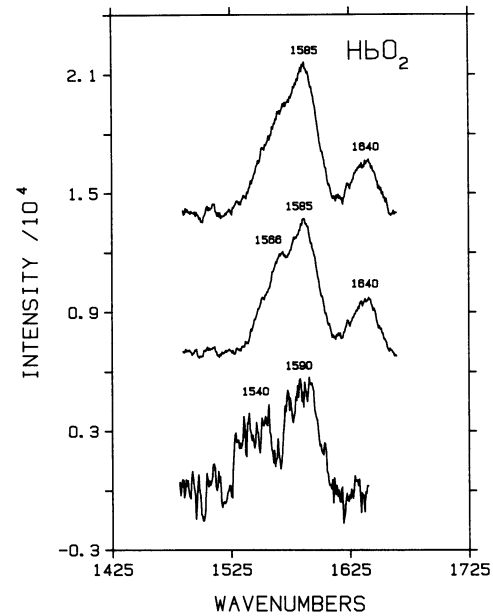

FIGURE 4 Resonance Raman spectra of HbO_2 and its photodissociation products on the 800–900-fs timescale in the core-size region (between 1,450 and 1,750 cm^{-1}). The upper and middle spectrum were obtained with tight and diffuse focus, respectively. Only in the former case an appreciable photolysis yield is obtained. The lower spectrum is a difference spectrum, which shows the Raman modes of an excited-state photoproduct, Hb^*_{II}. Note the much larger downshift of the frequencies in this spectrum, compared to those obtained in Fig. 3 for HbCO.

resonance Raman spectra. This can indeed be seen in Fig. 4 in which the spectra in the core-size region are presented for HbO_2. The difference spectrum (*lower part*) shows even more downshifted bands than those of Fig. 3 (*lower part*), which were attributed to $Hb^{\dagger}$. This additional downshift cannot be attributed to a further expansion of the porphyrin core. In $(THF)_2FeTPP$ (THF = tetrahydrofuran, TPP = tetraphenylporphin) the Fe atom has been shown to be fully in the plane of the heme, and this molecule shows the same frequencies as the HbCO-photoproduct (Reed et al., 1980). Alternatively, the larger downshifts might be explained by assuming that the molecule is in an excited state. The ν_{10} and ν_{11} modes, found in the HbO_2 photoproduct spectrum at ~1,540 cm^{-1} and ~1,590 cm^{-1} respectively, have been shown to decrease when the porphyrin π^* orbital is occupied, i.e., in an excited state (Spiro and Strekas, 1975; Kitagawa et al., 1975). A similar spectrum has been obtained by Terner et al. (1982) using 25-ps pulses. They attributed the spectrum to that of an electronically excited high-spin heme. The spectra of the HbO_2 photoproduct of Nagumo et al. (1981), obtained with ~50-ps pulses, were similar, however, to the HbCO photoproduct. Terner et al. (1982) speculated that the excited-state species they observed was due to electronically excited deoxy-Hb, directly formed from the photodissociation of photoexcited HbO_2. They assumed that this excited-state species was the spectral intermediate found by Chernoff et al. (1980) in a transient optical absorption experiment. On the basis of the rather broad spectra, Terner et al. (1982) concluded that on the timescale of their experiment they were indeed observing a mixture of prompt and delayed photoproducts. Instead of being sharper, our spectrum presented in Fig. 4 taken at shorter times is broader than the spectra obtained by Nagumo et al. (1981) and by Terner et al. (1982). This and the difference in timescale between their and our experiments (30–50 ps vs. 800 fs) suggests that the broadening in our spectrum is most likely a result of scattering from vibrationally unrelaxed species, in accordance with the observations made for the ν_4 mode.

We attribute the spectrum of Fig. 4 (*lower part*) to that of Hb^*_{II}. It was noted by Petrich et al. (1988) that the reactivity of Hb^*_{II} towards ligands should imply a planar heme because a partially domed heme might present a sizable barrier towards ligand recombination. If so, the resonance Raman spectra presented here might be those of the planar heme in Hb^*_{II}. Both Figs. 3 and 4 give structural information on the earliest photointermediates of both HbCO and HbO_2 on this timescale.

In summary, we have studied the resonance Raman spectra of the photointermediates of HbCO and HbO_2 on an 800–900-fs timescale. The ν_4 mode was studied, and a distinct shift with respect to the equilibrium value of deoxy-Hb was observed upon photodissociation. For HbCO a good agreement was found with results obtained in an earlier time-resolved study by Petrich et al. (1987). Furthermore, distinct differences were found between the two photoproducts in the region of the core-size markers (around 1,550 cm^{-1}). These differences could be accounted for in terms of the reaction scheme presented by Petrich et al. (1988), and conclusions could be drawn on the structure and spin state of these very early intermediates.

R. van den Berg would like to thank Steve F. Vetorino for a thorough training on the femtosecond laser systems and the Shell Research Laboratory, Amsterdam (Koninklijke/Shell-Laboratorium, Amsterdam) for financial support.

This work was supported by the Department of Energy (Office of Basic Energy Sciences) under Grant DE-FG03-88ER13828.

Received for publication 16 April 1990 and in final form 11 June 1990.

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