Resonance Raman Characterization of the 7-ns Photoproduct of (Carbonmonoxy)hemoglobin: Implications for Hemoglobin Dynamics†

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ABSTRACT: Resonance Raman spectra are reported for deoxyhemoglobin (deoxyHb) and the (carbonmonoxy)hemoglobin (HbCO) photoproduct Hb* by use of 7-ns YAG laser pulses at wavelengths of 416 and 532 nm, where enhancement is observed for totally symmetric and nontotally symmetric modes, respectively. The frequencies of the porphyrin skeletal modes ν_{10}, ν_2, ν_{19}, ν_{11}, and ν_3 have been determined to be 1602, 1559, 1553, 1542, and 1466 cm^{-1} in Hb*. These frequencies are 2–3 cm^{-1} lower than the corresponding frequencies for deoxyHb. The ν_{19} and ν_{11} frequencies are at the expected values for a C_t–N distance of 2.057 Å, the known core size for a 6-coordinate high-spin Fe^{II}–porphyrin complex. The remaining frequencies, however, deviate from the core size correlations for these modes in the same direction as do those of deoxyHb, suggesting that the porphyrin ring is domed in both species. Thus, the heme structure is similar for deoxyHb and Hb* but is slightly expanded in the latter. The expanded heme in Hb* implies a restraint on the full out-of-plane displacement of the Fe atom, by an estimated ~0.1 Å relative to deoxyHb. This could result from a residual interaction with the CO molecule if the latter remains held by the protein against the Fe atom, in a high-spin 6-coordinate complex. The available spectroscopic evidence suggests that such a complex may be stabilized at 4 K but is unlikely to persist at room temperature beyond the electronic relaxation (0.35 ps) of the electronically excited heme. The longer lived (~100 ns) restraint on the Fe atom displacement is attributed to protein forces on the proximal side. An additional protein interaction, relaxing on a much longer (~1 μs) time scale, is implied by the observations of Friedman and co-workers on the decrease in the Fe–imidazole stretching frequency between the photoproduct and deoxyHb in the R quaternary state. Thus, there are at least two heme-linked tertiary relaxations following photodissociation. The first one allows the Fe to move fully out of the plane to its deoxyHb position, while the second one allows relaxation of an initially elevated Fe–imidazole frequency. Both may be linked to the displacement of the F helix across the heme face.

The molecular mechanism of hemoglobin cooperativity continues to be the focus of intensive scientific scrutiny (Perutz, 1980; Johnson et al., 1984; Viggiano & Ho, 1979; Simolo et al., 1985; Matsukawa et al., 1985; Frauenfelder & Wolynes, 1985; Agmon & Hopfield, 1983; Friedman, 1985). Precisely because more is known about hemoglobin than any other allosteric protein, it is the most fertile testing ground for ideas about how proteins change their shape and respond to the binding or dissociation of ligands. The photolysis of the heme–CO adduct provides a convenient method for the rapid generation of deligated heme and has been used extensively in the study of ligand binding kinetics (Gibson & Ainsworth, 1957; Austin et al., 1973). In addition, photolysis affords a means of examining protein structural changes that attend and follow the loss of ligand from the heme group. Both optical absorption (Martin et al., 1983; Hofrichter et al., 1983; Greene et al., 1978; Shank et al., 1976; Reynolds & Rentzepis, 1982) and resonance Raman spectroscopy (Dallinger et al., 1978; Lyons et al., 1978; Woodruff & Farquharson, 1978; Terner et al., 1980, 1981; Stein et al., 1982; Lyons & Friedman, 1983; Friedman et al., 1982a, 1983; Scott & Friedman, 1984; Ondrias et al., 1983) have been used to monitor the evolution of hemoglobin (Hb) following photolysis of the (carbonmonoxy)hemoglobin (HbCO) adduct. Both techniques are sensitive to the state of the heme chromophore, as it interacts with the surrounding protein, and resonance Raman spectroscopy affords specific structural information via the heme vibrational frequencies (Spiro, 1983).

It is known that a deoxyHb-like absorption spectrum is generated extremely rapidly, with a time-constant of 0.35 ps (Martin et al., 1983). Subsequent evolution of this spectrum proceeds in three identifiable steps (Hofrichter et al., 1983), involving amplitude changes primarily, with time constants of 0.1, 0.8 and 20 μs. The last of these transitions is associated with the R → T quaternary structure change, which has been determined via CO rebinding kinetics (Sawicki & Gibson, 1976). A much earlier rebinding step, a first-order process involving CO molecules that do not leave the protein (recombination), occurs on a time scale of ~170 ns (Hofrichter et al., 1983).

Resonance Raman studies have revealed two tertiary relaxation steps involving vibrational frequencies of the heme group. The stretching frequency of the bond between the Fe atom and the proximal imidazole ligand (Friedman et al., 1982a, 1983), as well as the frequency of the ν_4 porphyrin skeletal model (Lyons & Friedman, 1983) that is responsive to a variety of electronic influences (Spiro, 1983), relaxes with a time constant of ~1 μs in concert with the second optical relaxation. Three skeletal modes, ν_{10}, ν_{11}, and ν_{19}, which are known to correlate with porphyrin core size (Spiro, 1983), are unrelaxed within 20 ns (Terner et al., 1980, 1981) but are relaxed within 300 ns (Stein et al., 1982) of photolysis. This process probably corresponds to the first optical relaxation at 100 ns (Hofrichter et al., 1983). The unrelaxed core size marker frequencies have been suggested (Terner et al., 1980,

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1981) to reflect a restriction by the protein on the excursion of the Fe atom out of the heme plane toward the proximal site. This interpretation has been disputed (Friedman et al., 1982a), however, on the grounds that the large uncompensated nonbonding forces between the imidazole ligand and the pyrrole N atoms occasioned by the departure of the CO molecule should produce instantaneous movement away from the heme plane. Molecular dynamics calculations indicate very rapid (~50–150 fs) motion of the Fe atom out of the heme plane, but they are not inconsistent with some limitation on the extent of the excursion by protein forces (Henry et al., 1985).

Because of the importance of this structural issue, we have redetermined the ν_{10}, ν_{11}, and ν_{19} frequencies for the early HbCO photoproduct using 7-ns YAG laser pulses at 532 nm, which produce complete photolysis [our previous measurements (Terner et al., 1980, 1981) were made with weak pulses from a mode-locked cw laser and produced only partial photolysis, necessitating spectral subtraction], together with polarization measurements. In addition, photoproduct spectra were generated with 416-nm H_2 Raman-shifted YAG pulses, to provide frequencies for other core size marker bands, ν_2 and ν_3. The ν_{11} and ν_{19} frequencies are those expected for a C_t–N (porphyrin center to pyrrole nitrogen) distance of 2.057 Å, the value that has been determined crystallographically (Reed et al., 1980) for an in-plane high-spin Fe^{II} adduct. The other frequencies show deviations from the core size correlations similar to those of deoxyHb, which have been associated with porphyrin doming (Choi et al., 1982a). The 2–3-cm^{-1} frequency downshift of all the porphyrin bands relative to those of deoxyHb indicates an unrelaxed porphyrin core and strongly suggests that the Fe atom has not moved fully to the position it occupies in deoxyHb. Two possibilities for this unrelaxed structure are discussed: (1) restraint on the proximal imidazole by the surrounding protein, which relaxes with a ~100-ns time constant, perhaps via motion of the F helix, and (2) maintenance of a 6-coordinate high-spin heme-CO structure due to protein restraint on the CO molecule, which is released at ~100 ns. The balance of the spectroscopic evidence favors alternative 1, although alternative 2 cannot be ruled out. The nature of the ~1-μs relaxation is also discussed.

Experimental Procedures

Human packed red blood cells were obtained from New Jersey Blood Services, New Brunswick, NJ. Hemoglobin was prepared from it as described by Antonini and Brunori (1971). The deoxy form was made by flushing under nitrogen for extended periods and then adding a slight excess of dithionite. The CO adduct was obtained by flushing deoxyHb with carbon monoxide.

For room temperature Raman spectra, the solutions were flowed through a 1.3-mm internal diameter S1-UV quartz capillary (Wilmad Glass Co.) into a recirculating reservoir that was kept under the appropriate atmosphere, N_2 for deoxyHb and CO for HbCO. The 532-nm 7-ns pulses from a frequency-doubled Nd:YAG laser (Quanta Ray) or the 416-nm pulses obtained by Raman shifting the tripled 355-nm output were focused onto the capillary, and the 90° scattering was collected and focused onto the slit of either a SPEX Triplemate (equipped with a PAR Vidicon) or a 0.5-m SPEX single monochromator (equipped with a PAR Reticon). A sheet polarizer was used to obtain the polarization spectra. The sample concentrations were adjusted to an absorbance of 0.5 in a 1-mm cell at the laser wavelength (see figures for concentrations).

For the low-temperature spectra, a liquid N_2 cold-tip apparatus (Eng et al., 1985) was utilized in back-scattering geometry. The actual temperature was measured by embedding a tungsten–nickel thermocouple in the sample, close to the laser spot. Sample concentrations were 1.5 mM. The collected light was focused into a 1.5-m SPEX 1401 double monochromator with a cooled photomultiplier and photon-counting electronics.

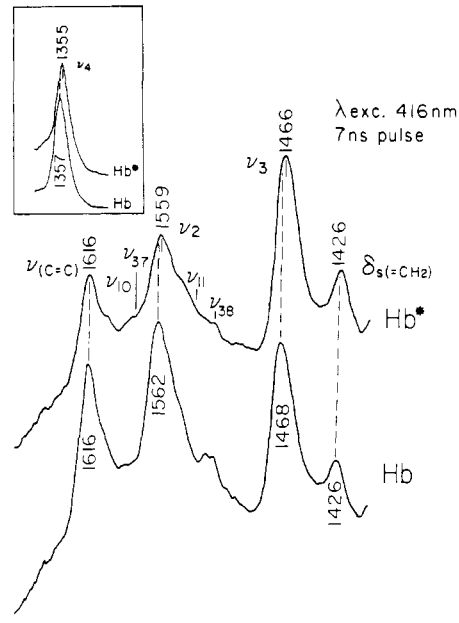

FIGURE 1: Soret band excited RR spectrum of deoxyHb and Hb* with 416-nm, 7-ns pulses. Concentration = 0.1 mM, laser energy = 0.5 mJ/pulse, spectral slitwidth = 8 cm^{-1}, collection time = 10 min, and repetition rate = 10 Hz. (Inset) ν_4 at 10 times reduced scale.

Results

Figure 1 shows Raman spectra obtained with pulsed excitation of deoxyHb and the HbCO photoproduct Hb* at 416 nm, near resonance with the Soret band [λ_{max} = 430 nm, Hb (419 nm, HbCO)]. The pulse energy (0.5 mJ) was sufficient to produce complete photolysis in the HbCO sample; no bands of HbCO are observed in the spectrum, which resembles that of deoxyHb with slight frequency shifts. The dominant band in the Soret-enhanced (Spiro, 1983) spectrum is due to the ν_4 C–N breathing mode (Abe et al., 1978), at 1357 cm^{-1} for deoxyHb and 1355 cm^{-1} for Hb*. This frequency shift is known to relax in two stages (Lyons & Friedman, 1983), with time constants of ~0.8 and ~20 μs. It is unrelaxed at ~300 ns, when the core size marker frequencies are relaxed (Stein et al., 1982). Hb* downshifts of 2–3 cm^{-1} are also seen in the 416-nm spectra for bands at ~1562 and ~1468 cm^{-1}, assigned to porphyrin skeletal modes ν_2 and ν_3, which are sensitive to the porphyrin core size (Choi et al., 1982a). Other core size sensitive modes expected to be enhanced with Soret excitation (Choi et al., 1982a), ν_{37} at 1584 cm^{-1} and ν_{38} at 1523 cm^{-1}, are insufficiently well resolved to determine their frequency shifts relative to deoxyHb. Traces are seen of ν_{10} and ν_{11}, at 1604 and 1545 cm^{-1}, which are weakly enhanced via Soret excitation but strongly enhanced via Q-band excitation (Spiro, 1983) (see below). Also appearing in the 416-nm spectra are bands arising from vinyl C=C stretching and $=CH_2$ deformation (Choi et al., 1982b) at 1616 and 1426 cm^{-1}. There are no shifts in these frequencies between deoxyHb and Hb*. Thus, the observed frequency downshifts are specifically associated with the core size marker bands ν_2 and ν_3 and with ν_4.

Figure 2 shows similar spectra obtained with 532-nm excitation, near resonance with the deoxyHb and Hb* Q band

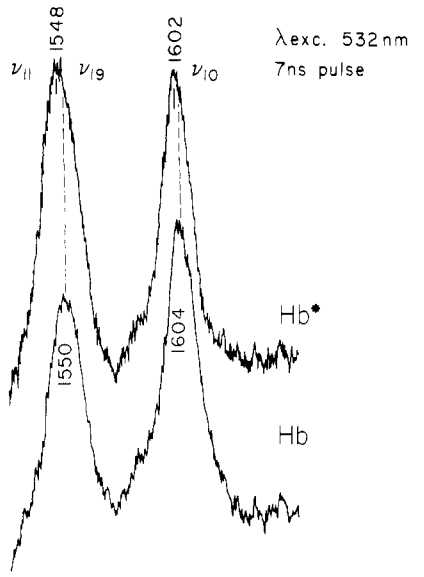

FIGURE 2: Q-band-excited RR spectrum of deoxyHb and Hb* with 532-nm, 7-ns pulses. Concentration = 0.5 mM, laser energy = 1 mJ/pulse, spectral slitwidth = 5 cm^{-1}, collection time = 30 min, and repetition rate = 10 Hz.

($\lambda_{max} \sim 555$ nm). Again, complete photolysis is achieved via the laser pulses (1 mJ); no HbCO peaks are observed. The spectrum contains bands associated with nontotally symmetric skeletal modes (Spiro, 1983), ν_{10}, ν_{19}, and ν_{11}. ν_{10} at 1604 cm^{-1} in deoxyHb shows a clear 2-cm^{-1} downshift in the photoproduct spectrum. ν_{19} and ν_{11} form a broad overlapped band, with a maximum at 1550 cm^{-1} in deoxyHb, shifting to 1548 cm^{-1} in the photoproduct spectrum. To resolve the ν_{19} and ν_{11} frequencies, the spectra were recorded in parallel and perpendicular polarization. ν_{19} and ν_{11} derive from A_{2g} and B_{1g} modes in the idealized D_{4h} symmetry of the porphyrin and should have ideal depolarization ratios of ∞ and 3/4, respectively (Spiro, 1983). The effective symmetry is lowered from 4-fold by the peripheral substituents, especially the conjugated vinyl groups, and by interactions with the protein. Nevertheless, ν_{19} is stronger in perpendicular but weaker in parallel polarization than is ν_{11}, as shown in Figure 3. We carried out a spectral deconvolution with the constraint that the mode frequencies must be invariant to the polarization. [In an earlier procedure, Terner et al. (1981) subtracted the parallel from the perpendicular spectra to obtain ν_{19}, using the criterion of intensity cancellation for the isolated ν_{10} band, on the assumption that both ν_{10} and ν_{11} have 0.75 depolarization ratios. However, Rousseau (1981) has detected an anomalously polarized component for ν_{11}, presumably due to symmetry lowering, which makes this procedure unreliable.] The bandwidths were fixed at 20 cm^{-1} (full width at half-maximum), as determined for the isolated ν_{10} band, and the amplitudes were varied freely while the frequencies were altered systematically and in concert between the two polarization spectra. Figure 3 shows the deconvoluted bands giving the best fit to the data, as determined by the sum of the squares of the residuals. This quantity increased significantly if either or both frequencies were varied by as much as 1 cm^{-1}, in any combination of directions. We estimate the probable error in the frequencies determined in this way to be no greater than 0.5 cm^{-1}. A 2–3-cm^{-1} downshift is seen in both ν_{19} and ν_{11} for the photoproduct relative to deoxyHb. (We note that the spectra obtained in parallel polarization show appreciable background levels associated with some unidentified luminescence process. The background is sufficiently broad not to affect the fitting procedure significantly.)

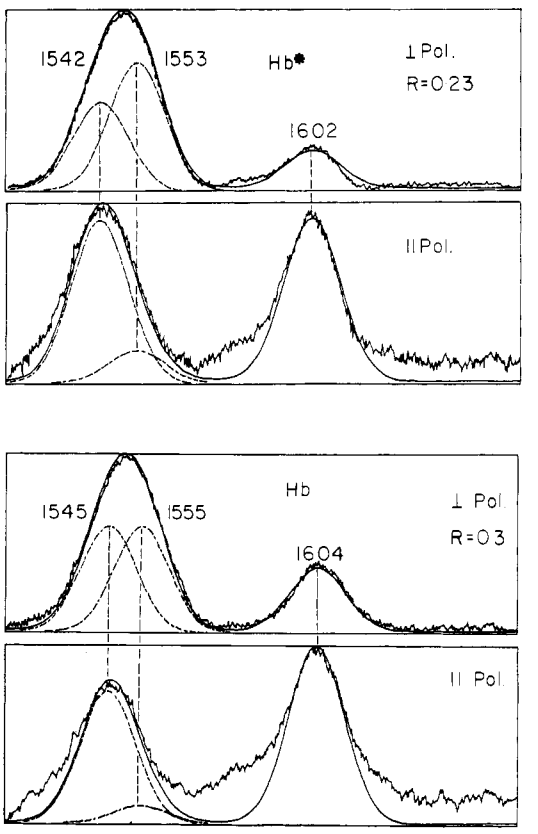

FIGURE 3: Band deconvolution, using computer simulation, of RR spectra recorded in parallel and perpendicular polarization for deoxyHb and Hb*. R is the sum of the squares of residuals: $R = \sum_{\nu=1530}^{\nu=1565}(I_{exptl} - I_{sim})^2$, where I_{exptl} and I_{sim} are the experimental and simulated normalized intensities.

Table I: Observed and Calculated Frequencies (cm^{-1}) for Core Size Sensitive Bands for DeoxyHb and Hb*

band	ν_{calcd} (C_t–N = 2.045 Å)[a]	Hb	$\Delta\nu$, Hb – Hb*	Hb*	ν_{calcd} (C_t–N = 2.057 Å)
ν_2	1557	1562	3	1559	1553
ν_3	1482	1468	2	1466	1476
ν_{10}	1611	1604	2	1602	1605
ν_{11}	1546	1545	3	1542	1542
ν_{19}	1559	1555	2	1553	1553

[a] Calculated via $\nu = K(A - d)$, where $d = C_t$–N (Å) and K (cm^{-1}/Å) and A (Å) are as follows: 390.8, 6.03; 448.3, 5.35; 517.2, 5.16; 344.8, 6.53; and 494.3, 5.20 for ν_2, ν_3, ν_{10}, ν_{11}, and ν_{19}, respectively (Choi et al., 1982).

Resonance Raman (RR) spectra were also recorded for frozen protein solutions at 77 K, with 406.7-cw Kr^+ laser excitation. The HbCO sample was largely unphotolyzed under these conditions, but it was possible to discern downshifts in Hb* relative to deoxyHb of 3 cm^{-1} in ν_4 and 4 cm^{-1} in ν_3. Thus, the protein constraints that determine these shifts are frozen in when photolysis is carried out at 77 K.

Table I lists the core size marker frequencies for deoxyHb and Hb* at 298 K. A 2–3 cm^{-1} downshift is seen for all of them. Also listed in the table are the predicted frequencies, based on the previously established correlations (Choi et al., 1982a), for core sizes of 2.057 and 2.045 Å. These are the C_t–N distances for two model compounds, $(THF)_2Fe^{II}TPP$ and (2-MeImH)Fe^{II}TPP (THF = tetrahydrofuran, 2-MeImH = 2-methylimidazole, TPP = tetraphenylporphine). The protoporphyrin analogue of the latter complex is known to have optical and RR spectra that are essentially the same as those of deoxyHb (Choi et al., 1982a). Unfortunately, we were

unable to obtain RR spectra for $(THF)_2FePP$ (PP = protoporphyrin); solutions prepared in THF by reduction of $Fe^{III}PP(Cl)$ or of $Fe^{III}PP(SbS_6)$ gave RR spectra characteristic of 5-coordination [consistent with photometric titration data (Brault & Rougee, 1974)], as judged by the absence of any spectral change upon addition of 2-MeImH, while attempts to obtain RR spectra of solid samples led to decomposition.

In deoxyHb (Fermi et al., 1984) and (2-MeImH)Fe^{II}TPP (Hoard & Scheidt, 1973, 1974), the Fe atom is out of the pyrrole N plane by 0.5 Å, the Fe–N(pyrrole) bonds are 2.086 Å, and C_t–N = 2.045 Å. (The distances are cited for the more accurately determined small molecule structure.) The frequencies calculated for this core size are al higher than the observed values except ν_2, which is lower than observed. These deviations were noted previously and were suggested to arise from doming of the porphyrin ring (Choi et al., 1982a). In both deoxyHb and (2-MeImH)Fe^{II}TPP, the average plane of the pyrrole N atoms is displaced by ~0.1 Å from the mean heme plane, due to tilting of the pyrrole rings toward the out-of-plane Fe atom. This tilting is expected to weaken the π-conjugation of the porphyrin ring, due to misalignment of the methine and pyrrole p orbitals, and effects on the skeletal mode frequencies are expected, somewhat akin to those observed for low-spin Fe^{II} complexes in which d_{π}–π^* back-bonding alters the π-conjugation (Choi et al., 1982a; Spiro & Burke, 1976). Since the electronic mechanisms are different in these two cases, the frequency shifts are not parallel, but the observation of both positive and negative deviations from the core size correlations is consistent with a π-conjugation effect in both cases.

These same deviations, though smaller in extent, are seen for the Hb* frequencies, even when the comparison is with the values calculated for C_t–N = 2.057 Å, the core size for $(THF)_2Fe^{II}$TPP (Reed et al., 1980). For this molecule, the high-spin Fe^{II} is held by the two axial ligands in the plane of the porphyrin, which is flat. The core size is the largest reported for a Fe porphyrin, and probably represents the maximum extension of the core for high-spin Fe (Scheidt & Gouterman, 1983). Thus, the persistence of the deviations from the calculations indicates that the heme is domed in Hb*, implying that the Fe is displaced from the heme plane and the core size is somewhat smaller than 2.057 Å.

We can make a guess at the probable photoproduct heme geometry in the following way. The core size parameters (Choi et al., 1982a; see Table I) applied to the deoxyHb – photoproduct frequency differences give estimates of the increase in C_t–N from 0.004 Å (ν_{19}) to 0.009 Å (ν_{11}) (the range may reflect a differential influence of doming) with an average of 0.006 Å. If this displacement is added to the C_t–N of the deoxyHb model (2-MeImH)Fe^{II}TPP, 2.045 Å, the photoproduct C_t–N is estimated to be 2.051 Å, half-way to the 2.057-Å value of the in-plane molecule $(THF)_2Fe^{II}$TPP. If the Fe–N(pyrrole) bond-length is also half-way between those of (2-MeImH)Fe^{II}TPP (2.086 Å) and $(THF)_2Fe^{II}$TPP (2.057 Å), i.e. 2.071 Å, then by triangulation we arrive at a displacement of the Fe atom from the mean N(pyrrole) plane of 0.29 Å, 0.13 Å less than the measured value, 0.42 Å, for (2-MeImH)Fe^{II}TPP. This calculation, although very approximate, does indicate that substantial out-of-plane displacement in the photoproduct, perhaps 70% of the deoxyHb displacement, is not incompatible with the core size interpretation of the deoxyHb–photoproduct frequency shifts.

Discussion

Optical transient studies with 0.25-ps laser pulses incident on the CO adducts of Hb, Mb, and protein-free heme show ground-state bleaching within the pulse width and the development of a deoxyheme-like absorption spectrum with a time constant of 0.35 ps (Martin et al., 1983). This process almost certainly involves intersystem crossing to a high-spin ligand field state of the heme, from which the CO dissociates (Greene et al., 1978). If the initially populated porphyrin π–π^* state were itself dissociative (Hoffman & Gibson, 1978), the product of CO loss would be a low-spin 5-coordinate heme–imidazole complex, which would take considerably longer to relax to its high-spin ground state. Thermal spin interconversion rates for Fe^{II} complexes are found in the 10–100-ns range (Beattie et al., 1973, 1978). The initial π–π^* excitation can, however, relax rapidly to a nearby excited ligand field state (Greene et al., 1978) with unpaired electrons in the antibonding Fe $d_{x^2-y^2}$ and d_{z^2} orbitals, which can account for the porphyrin core expansion, typical of high-spin hemes (Scheidt & Gouterman, 1983), seen in the 30-ps RR spectra of Hb (Terner et al., 1980, 1981) and myoglobin (Mb) (Dasgupta et al., 1985). Occupancy of the d_{z^2} orbital leads to CO dissociation, since CO requires a short distance to Fe to engage in the π-back-bonding that is responsible for its binding strength. Quantum mechanical calculations (Zerner et al., 1966; Waleh & Lowe, 1982) confirm that states of the heme–CO complex involving d_{z^2} occupancy are dissociative, whereas the π–π^* states are not. The actual spin state of the initial photointermediate is unknown, although the deoxy-like absorption spectrum generated at 0.35 ps suggests that it is high spin. Photolysis of MbCO at temperatures as low as 1.7 K produces a species with magnetic susceptibility corresponding to $S = 2$ (Roder et al., 1984), indicating that there is no significant barrier to the attainment of the high-spin state.

The first optical transient after the 0.35-ps process has a time constant of 100 ns in Hb (Hofrichter et al., 1983). The present data show that all of the accessible core size marker RR bands of the species present before this relaxation are 2–3 cm^{-1} lower than those in deoxyHb, implying a slightly more expanded core. Although a small frequency shift in any one RR band could be due to a number of effects, the consistent shift pattern for all five core-size marker bands provides strong support for a core size effect. If, for example, there were an electronic effect changing the e_g^* porphyrin orbital occupancy, one would expect a symmetry-related alternation in the magnitudes and signs of the RR frequency shifts (Choi et al., 1982a). The core size shifts relative to deoxyHb have relaxed within 300 ns of photolysis (Stein et al., 1982), and it seems likely, although this has still to be demonstrated, that the core size relaxation coincides with the 100-ns optical relaxation. Moreover, the expanded core is frozen in when photolysis is carried out at 77 K.

The only plausible mechanism for producing a heme core larger than that of deoxyHb, which is already high spin, is to restrain the Fe from its full out-of-plane displacement. As the Fe leaves the plane, the antibonding interaction between the pyrrole N σ orbitals and the half-filled Fe $d_{x^2-y^2}$ orbital is relieved, allowing the core to relax and the Fe–N(pyrrole) bonds to lengthen. This is seen in the comparison between the structure of $(THF)_2Fe^{II}$TPP (Reed et al., 1980), with C_t–N = Fe–N = 2.057 Å, and of (2-MeImH)Fe^{II}TPP (Hoard & Scheidt, 1973), with C_t–N = 2.045 Å and Fe–N = 2.086 Å. There are two possibilities for restraining the displacement: a push from the proximal side or a pull from the distal side. Proximal restraint could be provided by the protein residues and backbone, depending on their rigidity. An attractive force on the distal side could be provided by the CO itself, if it is kept from moving away from the Fe. We turn next to a

consideration of the Fe–CO bond breaking in the 6-coordinate high-spin heme–CO.

High-Spin Hexacoordinate Heme–CO? It is generally assumed that CO is lost from the heme in concert with the initial photoprocess, but this has not been demonstrated. While the state generated at 0.35 ps (Martin et al., 1983) is no doubt dissociative, the actual departure of the CO might be inhibited by the protein, keeping the CO weakly bound to the Fe atom and retarding its displacement. A molecular dynamics calculation by Henry et al. (1985) designed to simulate heme–CO photolysis gave a very rapid (50–150-fs) excursion of the Fe atom from the heme plane and essentially no delay when a full hemoglobin subunit was included in the simulation. The Fe–C interatomic potential was chosen somewhat arbitrarily, however, to provide a very weak attraction and allow the CO to depart from the heme without recoil. The actual attraction of a CO molecule held by the protein against an Fe^{2+} ion might be significantly greater than assumed in the calculation. The situation of CO adsorbed on zinc oxide is instructive. Photoelectron analysis by Solomon and co-workers (Gay et al., 1980) showed the CO to be bound to surface Zn^{2+} ions via the C atom. The measurements showed the expected lack of back-bonding from the d^{10} Zn^{2+} ions. The binding interaction, whose enthalpy is 12.0 ± 0.4 kcal/mol, is dominated by a σ donor interaction from the C end of the molecule, the dipole moment of the coordinated CO rising to ~0.6 D from the free molecule value of 0.112 D. Thus, ion–dipole forces can be significant for CO interacting with a metal ion. Of course, the actual charge on the Fe is much less than 2+, due to electron donation from the porphyrin and imidazole ligands, and may be somewhat smaller than that of the Zn in ZnO.

There is evidence that at sufficiently low temperature the CO can be prevented from leaving the heme. The EXAFS (extended X-ray absorption fine structure) analysis by Chance et al. (1983) shows the C atom still present in the first scattering shell of the MbCO photoproduct at 4 K, a temperature at which geminate recombination is frozen out. [This interpretation of the data has, however, been challenged by Fiamingo and Alben (1986).] Their finding is that photoexcitation is accompanied by a slight expansion, ~0.05 Å, of all bonds to Fe, both axial and equatorial. These changes are in the right direction although somewhat smaller than might be expected for the conversion from a low-spin to a high-spin 6-coordinate heme. [Additional bending of an already bent Fe–CO linkage was also inferred from analysis of the second-shell scattering (Powers et al., 1984).] Since the individual contributors to the first-shell scattering (CO, imidazole, and pyrrole) are not resolved into separate spectral components, their evaluation requires curve fitting and is therefore not as free of ambiguity as one would like. Nevertheless, the analysis showed that the photoproduct scattering could not be accounted for with a 5-coordinate heme or with a mixture of 5-coordinate and low-spin (unphotolyzed) 6-coordinate heme (Chance et al., 1983; Powers et al., 1984).

Magnetic susceptibility measurements on the MbCO photoproduct show a divergence from deoxyHb at temperatures below 5 K, indicating a slightly different zero-field splitting for Mb* (Roder et al., 1984), possibly associated with an interaction between the heme and the CO. The IR spectrum of the MbCO photoproduct (Alben et al., 1982) shows two CO stretching bands, at 2119 and 2131 cm^{-1}, and a minor band at 2144 cm^{-1} due to free CO. Above 13 K, the 2131-cm^{-1} band grows at the expense of the 2119-cm^{-1} band, which disappears. These bands are 25 and 13 cm^{-1} below the free CO frequency; for CO dissolved in nonpolar liquids, the frequency decreases by only a few (~8) wavenumbers (Lascombe et al., 1959). It is not implausible that the 2119-cm^{-1} band represents CO still interacting with the high-spin Fe^{2+} photoproduct, while the 2131-cm^{-1} band to which it converts represents CO bound at a nearby site in the heme pocket. Thus, a variety of physical data are not incompatible with the view that a 6-coordinate high-spin heme–CO photoproduct can be trapped at sufficiently low temperature.

Argade and Rousseau (1985) report that MbCO photoproduct RR spectra generated with cw laser excitation at 4 K show downshifts relative to deoxyMb of 4 cm^{-1} in ν_2 and 2 cm^{-1} in ν_{11}, but these shifts are absent in cw spectra obtained at 20 K or higher temperatures. We recently found that the MbCO photoproduct generated at room temperature with 30-ps 532-nm pulses shows 4-cm^{-1} downshifts of ν_{10} and ν_{11}/ν_{19} (Dasgupta et al., 1985). These shifts are absent in spectra generated with 7-ns pulses, which are identical with those of deoxyHb. Preliminary pulse/probe data indicate that the relaxation occurs at ~200 ps (S. Dasgupta, T. G. Spiro, C. K. Johnson, G. A. Dalickas, and R. M. Hochstrasser, unpublished results). The shifts in question, both at 4 K and in the 30-ps room temperature spectrum, are similar to those seen for the HbCO photoproduct and likewise imply an expanded porphyrin core. It is therefore conceivable that the early photoproduct seen at room temperature is the same high-spin 6-coordinate adduct indicated by the 4 K EXAFS measurements of Chance et al. (1983). In that case the ~200-ps relaxation would be assigned to motions of distal protein residues, which enable the CO to leave the heme.

There are, however, serious difficulties with the 6-coordinate high-spin heme–CO model. One is the recent observation by Friedman and co-workers that the stretching frequency for the bond between the Fe atom and the proximal imidazole, ν_{Fe-ImH}, has the same value, 220 cm^{-1}, for the MbCO photoproduct generated with 30-ps 416-nm pulses as for deoxyMb (Findsen et al., 1985). Since the two axial bonds of the proposed 6-coordinate high-spin adduct form a coupled mechanical system, it is unlikely that ν_{Fe-ImH} would be found at the same frequency as in 5-coordinate deoxyheme. For hemoglobin there is a shift in the photoproduct ν_{Fe-ImH} (Friedman et al., 1982a), but identification of the early photoproduct with 6-coordinate high-spin heme–CO remains implausible because the first relaxation, 100 ns, is an unduly long time for a small molecule like CO to remain restricted by surrounding protein residues, which are themselves fluctuating rapidly. Recombination of the heme with CO within the protein occurs at ~170 ns for about 30% of the photolyzed heme (Hofrichter et al., 1983), but Ansari et al. (1986) conclude on the basis of extrapolation from low-temperature data that this process corresponds to diffusion from the protein matrix, whereas recombination from the heme pocket itself is some 40 times faster. If this is correct, then the putative 6-coordinate high-spin heme–CO would have to relax to low-spin heme–CO at least this fast and could not be involved in the 100-ns process. Another difficulty is that the departure of the CO is expected to alter the heme electronic energies somewhat, yet the 100-ns optical transient of Hb* involves an amplitude change, primarily, with very little shift in the Soret band wavelength (Hofrichter et al., 1983). For all these reasons, we think it unlikely that the early photoproduct RR spectrum is associated with a 6-coordinate high-spin heme. Whatever the situation at low temperatures, it is likely that the high-temperature motion of the CO away from the heme group is concerted with the electronic relaxation to high-spin heme at 0.35 ps after photoexcitation and that subsequent relaxations

involve the resulting 5-coordinate heme and its linkage to the protein.

Proximal Restraint. If the restriction on the out-of-plane movement of the Fe that is implied by the core expansion seen for Hb* and Mb* cannot be attributed to interaction with CO, then a proximal restraint must be invoked. Friedman et al. (1982a) have argued against the restraint hypothesis, pointing to the large uncompensated nonbonded repulsions between the proximal imidazole and the pyrrole N atoms, which are occasioned by the departure of the CO and which act to propel the Fe atom out of the plane. There may, however, be protein forces that oppose the nonbonded repulsions. These forces are not expected to support more than a few kilocalories per mole of opposing energy, but this may be enough to restrict the Fe motion and leave the core detectably expanded. As discussed under Results, the Hb* data do not exclude a partial excursion of the Fe atom from the heme plane. The molecular dynamics calculations of Henry et al. (1985) show very rapid Fe movement but give a slightly smaller amplitude when the protein is included; as suggested under Results, a diminution in the out-of-plane displacement of only ~0.1 Å is not inconsistent with the estimated core size expansion of Hb*.

In looking for structural elements that might account for a protein restraint on the Fe movement, one is struck by the fact that the entire F helix, to which the proximal imidazole is attached, moves ~1 Å across the heme face when the crystal structures of ligated and deoxyHb are compared (Baldwin & Chothia, 1979). For Mb (which lacks a tetrameric structure), the F-helix motion associated with ligation is much less, ~0.1 Å (Takano, 1977). It therefore seems reasonable to suppose that the F helix, acting as a more-or-less rigid body, provides internal restraint to the motion of the Fe. In Mb, this restraint relaxes much more rapidly (~200 ps) than in Hb (~100 ns), reflecting a weaker coupling of the two motions, as evidenced by the structure data. Henry et al. (1983b) have suggested that the 100-ns Hb transient is associated with departure of the CO from the heme surroundings (as opposed to the heme itself), because of its near coincidence with the ~170-ns recombination process. It is possible that a shift in the F helix at this stage is concerted with opening of the heme pocket.

We next turn our attention to later events in the Hb* relaxation associated with shifts in ν_{Fe-ImH} and ν_4, as explored by Friedman and Rousseau and their co-workers (Lyons & Friedman, 1983; Friedman et al., 1982a, 1983; Scott & Friedman, 1984; Ondrias et al., 1983). When excited with 7-ns laser pulses in the Soret region, Hb* gives a low-frequency RR spectrum showing ν_{Fe-ImH} to be at 230 cm^{-1}, significantly higher than its position in R-state deoxyHb, 222 cm^{-1}, as determined by chemical modifications (Nagai et al., 1980; Ondrias et al., 1982) or kinetic isolation (Stein et al., 1982). The R-state frequency is in turn higher than the T-state frequency, 215 cm^{-1}. [The broad asymmetric band in deoxyHb is actually a composite of α- and β-chain contributions, 207 and 220 cm^{-1}, as shown with valency (Nagai & Kitagawa, 1980) and Fe,Co (Ondrias et al., 1980) hybrid Hb's.] Also, ν_4 is 2 cm^{-1} lower in the 7-ns Hb* spectrum than in the deoxyHb spectrum (Lyons & Friedman, 1983) (Figure 1). ν_4 and ν_{Fe-ImH} have been shown to be inversely correlated in Hb (Friedman et al., 1982). A pioneering pulse-probe RR study by Lyons and Friedman (1983) showed relaxation of the Hb* ν_4 in two steps, the larger one (1.5 cm^{-1}) occurring at 0.8 μs and the smaller one (0.5 cm^{-1}) occurring at 20 μs. ν_{Fe-ImH} also appears to relax with roughly these time constants (Friedman et al., 1983; Irwin & Atkinson, 1981), although there is appreciable variation in the rates with solution conditions (Scott & Friedman, 1984). The 0.8- and 20-μs relaxations coincide with the second and third optical transients found by Hofrichter et al. (1983). The 20-μs step is associated with the R → T quaternary transition of deligated Hb (Sawicki & Gibson, 1976), and the 0.8-μs transient must be associated with a prior tertiary step.

Two points seem particularly significant about the ν_{Fe-ImH} variation:

(1) The frequency is substantially higher in Hb* than in R-state deoxyHb or in deoxyMb, implying an actual strengthening of the Fe–ImH bond relative to a relaxed deoxyheme, as well as a weakening of it in T-state deoxyHb. The frequency differs for Hb* generated from hemoglobin variants having different R-like or T-like characteristics (Friedman et al., 1983; Scott & Friedman, 1984), but it is always higher than the relaxed frequency for the variant under study.

(2) There is no elevation in this frequency for Mb* (Findsen et al., 1985). Thus, the photolysis-induced Fe–ImH bond strengthening seems to be associated with the tetrameric character of Hb, although this has yet to be tested by examining Hb* for isolated subunits.

It is clear that ν_{Fe-ImH} does *not* correlate with core expansion; the two relaxations follow different time courses in Hb*, while Mb* shows core expansion but no ν_{Fe-ImH} elevation. This result is surprising if proximal protein restraint is responsible for the core expansion, since the same protein forces might be expected to influence ν_{Fe-ImH}. It seems likely, however, that pushing the Fe toward the heme plane, thereby expanding the core, takes less energy than does compressing the Fe–ImH bond significantly and that the core size marker frequencies are simply more sensitive to the proximal restraint than is ν_{Fe-ImH}. Some structural factor other than this restraint is required to explain the variation of ν_{Fe-ImH} in Hb.

The Fe–ImH bond is normal to the heme plane in ligated Hb but is tilted by 7° in deoxyHb (Baldwin & Chothia, 1979). A result of this tilt is a rather close nonbonded contact between the imidazole C(ϵ)H and the pyrrole(I) N atom. Gelin and Karplus (1977) proposed a significant role for this contact in the mechanism of the R → T transition, although Fermi et al. (1984) have recently questioned its importance on the basis of a new high-resolution crystal structure. Friedman and co-workers (Friedman et al., 1982a; Scott & Friedman, 1984) have suggested that the variations in ν_{Fe-ImH} and in ν_4 are associated with this imidazole tilting. If the C(ϵ)H···N repulsion were significant, then it would be expected to weaken the Fe–ImH bond upon tilting. A difficulty with the tilting hypothesis is that deoxyMb actually has a tilt angle, 11° (Takano, 1977), larger than that of deoxyHb, yet it has ν_{Fe-ImH} = 220 cm^{-1} vs 215 cm^{-1} for deoxyHb. Champion and coworkers (Bancharoenpaurpong et al., 1984) have proposed that the downshift in deoxyHb is due to the tilt in combination with a rotation of the imidazole away from an orientation eclipsing the pyrrole(I) and pyrrole(III) N atoms (Baldwin & Chothia, 1979). In the eclipsed orientation associated with the R state, the tilt of the Fe–ImH bond would permit delocalization of the electron occupying the Fe d_{z^2} orbital into the porphyrin π^* orbital, thereby relieving the antibonding interaction between the d_{z^2} and ImH orbitals; in a staggered orientation (T state), the antibonding interaction is more fully expressed, and the Fe–ImH bond is weakened. Another suggestion that had earlier been advanced is that the lower ν_{Fe-ImH} in deoxyHb might be due to weaker H bonding between the imidazole N(γ)H and a backbone carbonyl, model studies having shown a strong correlation between these bonding features (Stein et al., 1980). NMR evidence does not support this proposal

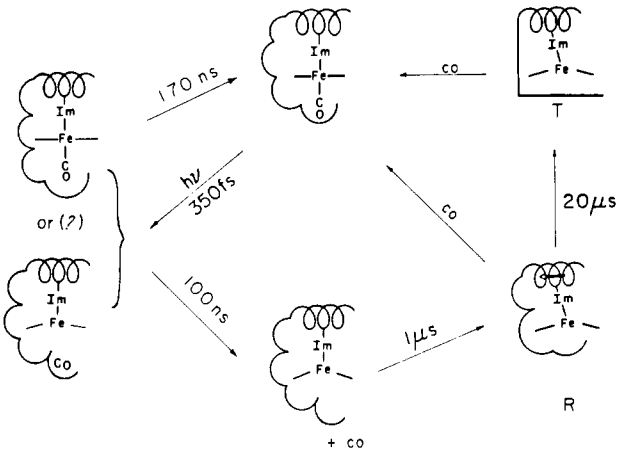

FIGURE 4: Suggested photolysis scheme for HbCO and subsequent tertiary and quaternary changes (see text).

(LaMar & deRopp, 1982), however, and it was found that ν_4 is invariant to H bonding in model compounds, whereas it correlates inversely with ν_{Fe-ImH} in Hb (Friedman et al., 1982b).

Whatever the explanation for the ν_{Fe-ImH} variation, it seems likely to be connected with the large F-helix motion seen in Hb but not in Mb. Neither the time scale of this motion nor its trajectory is known. Although the slowing down of the full out-of-plane Fe displacement in Hb relative to Mb may be due to tighter coupling with the F helix, as suggested above, the 100-ns transient that we associate with this displacement may only involve a small helix displacement. Other, and perhaps more complex, motions may be associated with the 0.8- and 20-μs transients, leading to the full $\sim$1-Å displacement of the F helix across the face of the heme (Baldwin & Chothia, 1979).

SUMMARY

In Figure 4 we sketch a diagram, somewhat akin to that of Henry et al. (1983b), for the proposed sequence of events attending HbCO photolysis. Initial photoexcitation is followed by a 0.35-ps electronic relaxation to a high-spin heme in which the CO is either still attached to the Fe or moved to a nearby site in the heme pocket. The latter is more likely, although the former structure may be trapped at sufficiently low temperature. Proximal constraints on the Fe atom that inhibit its full out-of-plane displacement relax at 100 ns, probably via a small displacement of the F helix; at the same time the CO leaves the vicinity of the heme, or geminately recombines. A larger motion of the F helix is suggested to occur at 1 μs, which permits the elevated ν_{Fe-ImH} to relax. Finally, the R $\rightarrow$ T quaternary transition at 20 μs involves a further shift of the F helix and the generation of tension in the Fe–ImH bond, as reflected in the lowering of ν_{Fe-ImH}. While speculative, this scheme is consistent with the data currently available and may stimulate further experiments to test and refine the structural inferences.

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