Near-Infrared Spectra of *Scapharca* Homodimeric Hemoglobin: Characterization of the Deoxy and Photodissociated Derivatives

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ABSTRACT The near-infrared charge transfer band at 760 nm (band III) has been investigated in deoxy and photodissociated dimeric *Scapharca* hemoglobin. At 300 K, the 10-ns spectrum of the carbonmonoxy derivative photoproduct is shifted by about 6 nm toward longer wavelengths with respect to the deoxy spectrum, both in buffer and in glycerol/buffer solutions. Moreover, the band III peak occurs at about the same wavelength at 300 K and at 10 K for the 10-ns photodissociated derivative, whereas in the deoxy derivative large changes in peak position and linewidth are observed as a function of temperature. These findings suggest that in dimeric *Scapharca* hemoglobin the photoproduct has not relaxed after 10 ns. The complete time dependence of the relaxation process has been studied both in buffer and in glycerol/buffer solutions at room temperature. The relaxation from the photoproduct to the deoxy species occurs on a microsecond time scale, in line with recent optical absorption and resonance Raman measurements.

INTRODUCTION

The hemoglobin from the mollusk *Scapharca inaequivalvis* (HbI) displays unique functional and structural properties. HbI is a highly cooperative homodimer (Hill coefficient of 1.5), whereas in vertebrate hemoglobins, cooperativity is associated with a heterotetrameric assembly. From a structural viewpoint, in HbI the heme-carrying E and F helices do not face solvent as in vertebrate hemoglobins, but form the dimer interface and bring the two hemes almost in direct contact through their propionate groups (Royer et al., 1989, 1990; Royer, 1994). Information on the ligation state is transmitted from one heme to the other through changes in the geometry of the heme peripheral substituents and rearrangements in the heme pocket. Upon ligand binding the heme groups sink deeper in the pocket and, on the proximal side, Phe 97 is extruded from the pocket to the interface. Compared to mammalian Hbs, HbI exhibits a reduced displacement of the proximal histidine (His 101), a significantly smaller quaternary structure change and, possibly as a result of a smaller iron displacement upon ligand binding, a reduced amount of shifting in the F helix (Rousseau et al., 1993; Chiancone et al., 1993). In this framework, the iron-His proximal bond does not seem to play the same role as in vertebrate hemoglobins.

To clarify the relationship between the iron-proximal His bond and ligand binding in HbI, several dynamic studies have been carried out by taking advantage of different spectroscopic markers. The dynamics of the proximal bond relaxation after photolysis of the HbI-CO derivative has been followed directly in transient resonance Raman experiments (Rousseau et al., 1993). These studies show that there is a frequency shift of 6 cm^{-1} between the equilibrium deoxy form of HbI and the 10-ns photoproduct of the CO derivative. In HbA under similar conditions this difference in frequency is almost 20 cm^{-1}, whereas in myoglobin (Mb) there is no difference at 10 ns or even at 30 ps (Friedman et al., 1982). Moreover, unlike HbA, in which the relaxation of the Fe-His bond occurs over an initial tertiary relaxation (10 ns to μs) and is followed by a subsequent clear-cut quaternary relaxation phase (20 μs; Friedman et al., 1982), in HbI relaxation appears to occur in a single phase, on the same time scale as the tertiary relaxation in HbA. In Mb, the relaxation process is much faster with a picosecond phase followed by a realaxation in the nanosecond time scale (Lim et al., 1993; Ansari et al., 1994). Thus the behavior of HbI may be considered as intermediate between those of HbA and Mb with respect to both the above-mentioned spectral shifts and the relaxation properties of the iron-proximal His linkage.

The dynamics of CO rebinding after photolysis has been investigated in optical absorption experiments as a function of temperature by following the spectral changes in the Soret region (Boffi et al., 1994). The activation enthalpy distributions as well as the overall profile of the absorption spectrum were found to be less sensitive to thermal perturbations than in all the other heme proteins studied, consistent with the idea that HbI is a rigid protein in which the dynamic events are localized at the heme pocket. This study also showed that in deoxy HbI the linewidth of the Soret band is characterized by a significantly reduced temperature-dependent broadening compared to HbA and Mb and hence by a reduced temperature-dependent iron displacement out of the heme plane. In addition, the Soret band profile of the low-temperature photoproduct was found to be unusually sharp, indicating that the iron atom is in an

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almost "in plane" position. These observations prompted the present investigation of the near-infrared region. Moreover, the 760-nm band, originating from a porphyrin to iron charge transfer transition, was shown to be sensitive to the local structure of the heme within the heme pocket and in particular to the position of the iron with respect to the heme plane (Sassaroli and Rousseau, 1987; Chavez et al., 1990).

MATERIALS AND METHODS

Scapharca HbI was purified as described previously (Chiancone et al., 1981). Flash photolysis experiments were carried out on 2 mM HbI solutions in 0.1 M phosphate buffer at pH 7.0 or 65% glycerol/phosphate saturated with 1 atm CO and sealed in a 0.5-mm cell. The time-resolved near-IR absorption system consisted of two electronically synchronized Nd:YAG lasers. The firing of one laser with respect to the other can be electronically controlled with 5-ns precision. One laser is used to generate 532-nm pulses (8 ns, 10 Hz, 10 mJ/pulse), which are used to photodissociate the HbI-CO samples. The second laser is used to generate a nanosecond near-IR continuum for probing the photoproduct population. The near-IR continuum is generated by focusing 532-nm pulses into a capillary containing a flowing mixture of laser dyes chosen to produce a smooth, broad continuum in the 700- to 800-nm region. The transmitted light is dispersed by an ISA 0.24 meter single spectrograph and detected with an unintensified optical multichannel array detector (Princeton Instruments). A closed-cycle Janis cryosystem is used to maintain the sample at cryogenic conditions. Experiments as a function of temperature were carried out in 65% (v/v) glycerol, 0.1 M phosphate buffer at pH 7.0. A small amount of sodium dithionite was added to the solution before starting measurements.

The near-infrared spectra were measured on a Jasco 650 spectrophotometer with 0.4 nm bandwidth, 1 s time constant, and 40 nm/min scan speed. A 1-cm-path metacrylate cuvette was used. The optical dewar and temperature control apparatus have already been described (Cordone et al., 1986). The moments of the overall absorption band were calculated from the spectral profile $A(u)$, after subtracting the tangent between the minima at each temperature. Within the framework of the so-called narrow-band approximation (Dexter, 1958), the zeroth (M_0), first (M_1), and second (M_2) moments of the absorption band are defined as

$$M_0 = \int A(\upsilon)\mathrm{d}\upsilon \tag{1}$$

$$M_1 = \int \upsilon A(\upsilon)\mathrm{d}\upsilon / M_0 \tag{2}$$

$$M_2 = \int \upsilon^2 A(\upsilon)\mathrm{d}\upsilon / M_0 - M_1^2 \tag{3}$$

The temperature dependence of the moments thus obtained has been analyzed, within the limits of the harmonic approximation, taking into account the coupling of the electronic transition with a "bath" of soft vibrational modes. In the range of 300–10 K, the following expressions hold:

$$M_1(T) = D + F\coth(\hbar\langle\upsilon_l\rangle/2k_BT) \tag{4}$$

$$M_2(T) = A\coth(\hbar\langle\upsilon_l\rangle/2k_BT) + C^2 \tag{5}$$

where $\hbar$ and k are the Planck and the Boltzmann constants, respectively; $\langle\upsilon_l\rangle$ is the average frequency of the "bath" of soft modes; the parameters C^2 and D contain temperature-independent contributions to the observed linewidth and peak position, respectively; and the parameters A and F are related to linear and quadratic coupling constants of the electron-vibration coupling (Cupane et al., 1995). Recently (Gilch et al., 1995) the deconvolution of band III both for HbA and Sw-Mb was performed in terms of sub-bands attributed to different conformers, in analogy to what was obtained for the Fe-His stretching vibration as detected by Raman spectroscopy (Gilch et al., 1993). In the present case the fitting procedure adopted, i.e., the method of the moments of the band, tends to outline the differences between photodissociated and equilibrium deoxy derivatives; indeed, in the absence of Raman Fe-His stretching measurements as a function of temperature, an analysis in terms of sub-bands appears to be purely speculative.

RESULTS

Spectrum of the HbI-CO photoproduct at 300 and 10 K

The spectrum of the 10-ns photoproduct at 300 K has been measured under the same conditions and with the same experimental apparatus (see Materials and Methods) as the 10 K one. The spectrum of the photoproduct is centered at about 766 nm both at 300 and at 10 K (Fig. 1, *A* and *B*), in contrast with the deoxy HbI spectrum, which shifts from 759.5 nm to 753 nm upon lowering the temperature (see Fig. 4).

Time dependence of the HbI-CO photoproduct

A series of measurements have been carried out at room temperature on the HbI photoproduct to follow the time dependence of its relaxation to deoxy HbI in buffer and in 65% glycerol/buffer solutions. The time evolution of the spectrum in buffer is presented in Fig. 2. The spectral changes after photodissociation have been analyzed in terms of band moments (Eqs. 1–3). The time dependence of the zeroth and first moments is given in Fig. 3, *A* and *B*, respectively. The time course of the zeroth moment is clearly biphasic. The fast process, which accounts for about

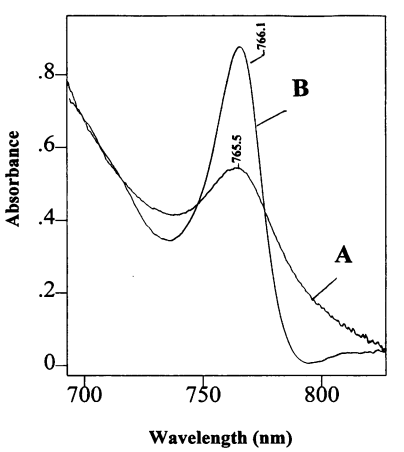

FIGURE 1 Comparison of the near-infrared spectra of the HbI-CO photoproduct at 10 K (*A*) and 300 K (*B*). Spectra were measured in 65% glycerol/buffer solutions on the same sample.

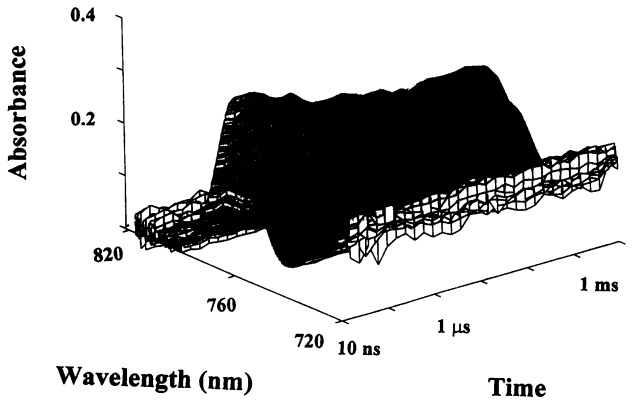

FIGURE 2 Time dependence of the near-infrared spectra of the HbI-CO photoproduct. A set of 35 spectra measured at different time delays after photolysis is shown. Protein concentration was 2 mM in 0.1 M phosphate buffer (pH 7.0) and in the presence of 1 atm CO.

20% of the total spectral intensity in buffer, occurs on the same time scale as the tertiary relaxation process followed in the Soret region (Chiancone et al., 1993). In the presence of glycerol, the amplitude of the fast phase is increased to 25–30% of the total signal, and the rate appears to be slowed down slightly. The slow phase corresponds to CO rebinding from the solvent; its rate is decreased by a factor of 2 in the presence of 65% glycerol. The time course of the first moment, which monitors the peak shift due to the relaxation of the photoproduct to the deoxy spectrum, appears to be monophasic. Again, the presence of glycerol appears to decrease the relaxation rate. The second moment (band linewidth) is time independent within experimental error (data not shown).

Temperature dependence of the 760-nm band in deoxy HbI

The near-infrared absorption spectrum of deoxy HbI has been measured in the region between 700 and 850 nm as a function of temperature from 300 to 10 K, every 10 K. The whole set of spectra is shown in Fig. 4. The overall band intensity (M_0) increases upon lowering the temperature (Fig. 5 *A*), as observed in other hemoproteins (Cordone et al., 1990). The analysis of the first and second moments of the absorption band in terms of Eqs. 4 and 5 is given in Fig. 5, *B* and *C*, respectively. With an increase in temperature, a large increase in linewidth (M_2) is paralleled by a shift of the peak position (M_1) from 13,280 cm^{-1} (753 nm) to 13,160 cm^{-1} (759.5 nm).

DISCUSSION

The 760-nm band, present in five coordinate ferrous heme derivatives, has been assigned to a plane polarized charge transfer transition from the porphyrin π system to the iron d_{xy} orbital ($a_{2u}(\pi) \rightarrow d_{xy}$) (Eaton and Hofrichter, 1978) or alternatively to an iron $d_{x2y2} \rightarrow e_g(\pi^*)$ orbital promotion (Makinen and Churg, 1983). In both cases, structural rearrangements leading to changes in the energy of the iron d orbitals will affect the energy of the observed transition. Hence, the dynamic behavior of the 760-nm band will reflect the fine structural modifications within the iron atom coordination sphere in response to chemical and physical

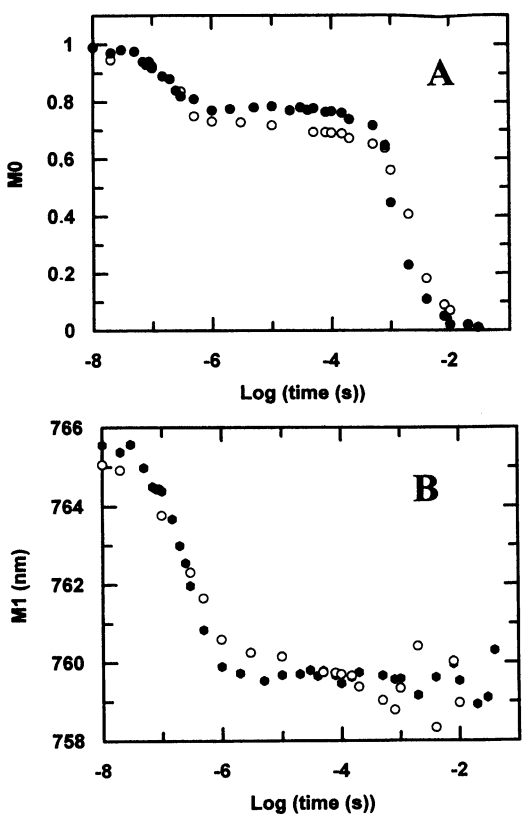

FIGURE 3 Time dependence of the zeroth (*A*) and first (*B*) moment of the HbI-CO photoproduct. ○, Data in 0.1 M phosphate buffer at pH 7.0. ●, Data in 65% buffer/glycerol solutions. The M_0 values as a function of time were normalized to the value obtained for the 10-ns photoproduct.

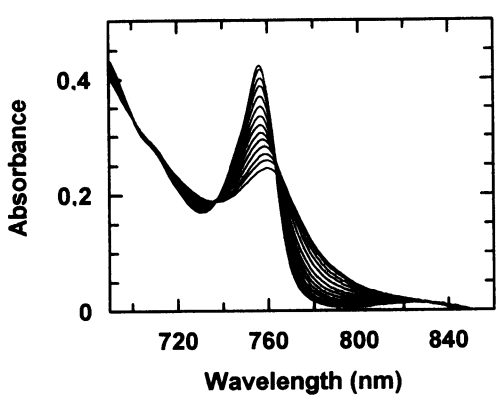

FIGURE 4 Temperature dependence of the near-infrared spectrum of the deoxy HbI derivative. Spectra were obtained in 65% buffer/glycerol solutions. Temperature ranges are between 300 K (*lowest curve* at 760 nm) and 10 K (*highest curve* at 760 nm). Only one spectrum for each 20 K is shown.

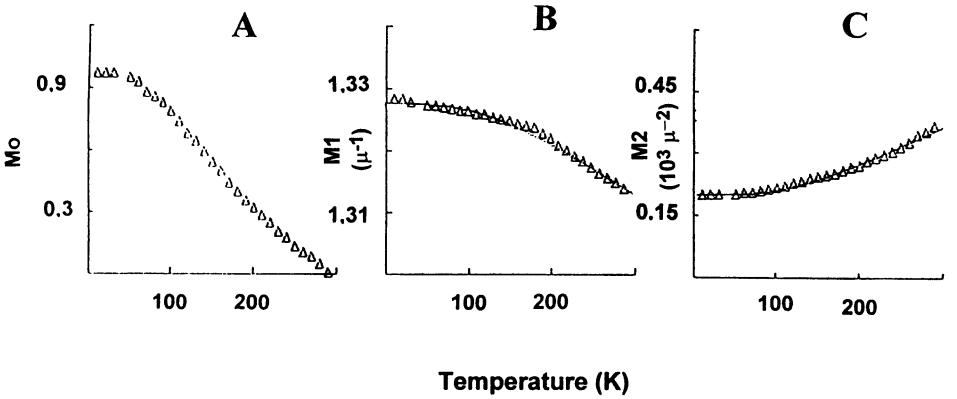

FIGURE 5 Temperature dependence of the zeroth (*A*), first (*B*), and second (*C*) moments of the HbI deoxy spectrum. Continuous lines represent the fitted curves in terms of Eqs. 4 and 5, with $\upsilon_1 = 290\ cm^{-1}$.

perturbations. The temperature dependence of the band lineshape is influenced by the coupling of the electronic transition with the thermally populated low-frequency vibrational modes of the protein matrix (Srajer et al., 1986; Cupane et al., 1995). On the other hand, the time evolution of the CO photoproduct allows a direct inspection of the changes in iron coordination and the subsequent protein relaxation.

Temperature dependence of the 760-nm band

A distinct feature of the near-infrared band is its sensitivity to thermal perturbations. In fact, the temperature dependence of the 760-nm band in Sw-Mb differs from that of the visible and Soret lines, because the band narrowing and peak shift are accompanied by a large increase in intensity upon lowering temperature (Cordone et al., 1990; Srajer and Champion, 1991). Such changes in the oscillator strength can be thought to reflect thermal depopulation of the iron d ground-state orbitals (Srajer and Champion, 1991) or changes in the overlap between ground and excited states of the charge transfer transition arising from changes in the position of the iron atom with respect to the heme plane (Cupane et al., 1988). The HbI deoxy derivative is not an exception in that the 760-nm band undergoes large changes in intensity, linewidth, and peak position upon decrease in temperature (Fig. 4). In particular, the overall intensity of the spectrum (Fig. 5 *A*) increases by a factor of 2 from 300 K to 10 K, a much larger effect with respect to that reported for temperature-dependent changes in the Soret and visible regions (Boffi et al., 1994). The line sharpening (M_2) and peak blue shift (M_1) effects upon decrease in temperature have been accounted for by the thermal depopulation of the protein low-frequency modes of the heme-protein-solvent complex (Leone et al., 1994). The temperature dependence of the Soret band of both the CO and deoxy derivatives indicated that the average frequency of the soft modes is higher in HbI than in all of the other hemoproteins studied, and the anharmonic contributions to the thermal broadening effects are greatly reduced (Boffi et al., 1994). The fit to the data shown in Fig. 4, in terms of Eqs. 4 and 5 (Fig. 5, *B* and *C*), yields a value of 290 cm^{-1} for υ_1 in HbI, a value that is significantly higher than that observed for Sw-Mb (180 cm^{-1}). This finding, on the one hand, points to an unusual rigidity of the heme pocket in HbI, in agreement with previous results (Boffi et al., 1994), and on the other indicates that the temperature-induced changes in intensity cannot be ascribed to protein matrix-dependent broadening effects (anharmonic motions). The sensitivity of the near-infrared band to the position of the iron atom with respect to the heme plane deserves further comment. A semiquantitative relationship between the iron out-of-plane displacement and the Soret band linewidth in hemoprotein deoxy derivatives has been proposed by Srajer and Champion (1991). The temperature-dependent asymmetric broadening of the Soret band has been explicitly correlated to the iron coordinate with respect to the heme plane (Q_o) scaled by a factor (b) that is proportional to the force constant of the proximal bond. Calculations carried out on the deoxy HbI Soret line temperature dependence according to this relationship revealed a remarkable thermal insensitivity of the $Q_o\sqrt{b}$ value, which has been attributed to the relative inertia of the iron atom, which is unlikely to move from its equilibrium position upon decrease in temperature (Boffi et al., 1994). In this framework, the spectral changes associated with the near-infrared band do not appear to be directly correlated to the iron coordinate with respect to the heme plane.

The HbI-CO photoproduct, effect of temperature, and dynamics of relaxation

A large number of spectroscopic observations have been carried out on the photoproduct of Sw-Mb and on HbA-CO to characterize the relationships between recombination and relaxation processes after photolysis and establish a temporal sequence of the structural rearrangements that accompany the relaxation process. In Sw-Mb, the absorption spectrum of the photolyzed protein appears within a fraction of a picosecond after photodissociation (Martin et al., 1983; Petrich et al., 1987; Xie and Simon, 1991). Recently Lim et al. (1993) showed that the position of band III in the Mb-CO photoproduct evolves nonexponentially toward its equilibrium value, with a large fast phase in the first few tens of

picoseconds, and a residual relaxation that extends into the nanosecond time regime. Lambright et al. (1991) and Ansari et al. (1994) showed that the rate of this residual relaxation is reduced by increasing the concentration of glycerol. Because the Fe-His band is fully relaxed before this band III relaxation occurs (Friedman et al., 1982), it is likely that the latter is derived from the relaxation that involves either movement of water into the heme pocket and/or changes in the propionate geometry. Support for the latter comes from transient Raman experiments on myoglobin, which show that at 10 ns the propionate-sensitive bands are not fully relaxed, whereas the Fe-His bond is (Friedman, unpublished experiments). Distal effect contributions have also been taken into account to explain the different rate of conformational relaxation obtained in Mb mutants in specific positions on the distal pocket (Lambright et al., 1993). Conversely, large changes in the band III position have been observed in the HbA-CO photoproduct in aqueous solutions and at room temperature in the time scale between 10 ns and a few milliseconds. In particular, between 35 ps and 60 ns, the photoproduct is at 765 nm, it relaxes at 763 nm in a few microseconds (tertiary relaxation), and further relaxes to the deoxy-HbA spectrum, at 759 nm within 200 μs (quaternary relaxation) (Dunn and Simon, 1991).

It is of particular interest to compare the room temperature photoproduct in 65% glycerol to that obtained at 10 K. In Sw-Mb, the 10 K photoproduct is peaked at 758 nm, whereas a value of 770 nm has been reported for HbA. Dunn and Simon (1991) proposed that the low-temperature band III intermediate at 770 nm can be associated with a pentacoordinate heme in which the iron is still in the heme plane and the proximal His is still in an R-like conformation. Hence, the low-temperature band III position represents, to a first approximation, the "time 0" absorption spectrum that would be generated on the femtosecond time scale at room temperature. In HbI, the near-infrared spectra of the photoproduct at room temperature and at 10 K are at almost the same energy (Fig. 1). If one assumes that the 10 K photoproduct corresponds to the spectrum of the unrelaxed protein, this finding suggests that no temperature-dependent relaxation occurs in HbI within 10 ns after photolysis. Alternative interpretations may be given, but only a picosecond investigation will provide a definite answer. At variance with HbI, both Mb and HbA show pronounced temperature-dependent shifts in band III and in the Raman spectrum (Srajer and Champion, 1991) that are consistent with temperature-dependent relaxation. A likely explanation is that for Mb and HbA the relaxations occurring within 10 ns involve a small elastic shift of the F helix as the upright histidine tilts slightly upon movement of the iron out of the heme plane (Friedman et al., 1982). At cryogenic temperatures the coupling between the iron-histidine bond and the F helix is likely to be altered as the movement of the F helix is impaired. In this framework, the results on HbI are consistent with a small rapid initial iron movement upon photolysis being decoupled from any F helix motion.

The behavior of HbI-CO upon photolysis is shown in Fig. 2, and the time course relative to the overall intensity of the 760-nm band (M_0) and its peak position (M_1) are reported in Fig. 3 (*A* and *B*, respectively). Inspection of Fig. 3 shows that a 20% decrease in intensity occurs with a half-time of about 0.5 μs. No further relaxation can been detected up to the millisecond time scale, where second-order ligand rebinding from the solvent occurs. This observation strongly supports the previous data obtained in flash photolysis experiments (Chiancone et al., 1993) and is in agreement with transient resonance Raman experiments (Rousseau et al., 1993). In both cases only one relaxation process was observed in the microsecond time scale. It should be pointed out that transient Raman experiments have shown a 6 cm^{-1} increase in the frequency of the iron-His bond in the 10-ns photoproduct, whereas the increase is considerably larger (20 cm^{-1}) in tetrameric hemoglobins (Rousseau and Friedman, 1988). In HbA, this shift has a clear structural and functional counterpart in that it monitors the large histidine motion coupled to the protein tertiary and quaternary rearrangements. On this basis, it has been inferred that in HbI the iron-His linkage is not necessarily the main conduit for heme-heme interaction, in line with recent high-resolution crystallographic data, which show very small quaternary changes and no tilt or distortion of the imidazole plane with respect to the heme upon ligand binding (Royer, 1994). It follows that the large changes in intensity and peak position of the near-infrared band cannot be directly correlated to the proximal bond relaxation. A possible origin of the wavelength change can be found in the changes in the heme geometry, because it would involve the energies of both iron d and porphyrin π orbitals, coupled to the ligand-linked shift of the heme within its pocket. This hypothesis is consistent with all of the available data and, in particular, with the transient resonance Raman observation (Rousseau et al., 1993), which shows that all of the heme low-frequency modes, sensitive to the peripheral substituents' out-of-plane movements, relax in a concerted way after photodissociation.

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