Photophysics and Reactivity of Heme Proteins: A Femtosecond Absorption Study of Hemoglobin, Myoglobin, and Protoheme†

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ABSTRACT: On the basis of our time-resolved absorption measurements of hemoglobin (Hb), myoglobin (Mb), and protoheme (PTH), either unligated or ligated with CO, O_2, or NO, we propose a description of the photophysics of heme proteins that encompasses their photodissociation, the origin and fate of the observed short-lived transients, and the appearance of the ground-state, unligated heme proteins. Two distinct species are formed upon ligand photodissociation, which occurs in less than 50 fs. We assign these species to excited states of the unligated heme and label them (for the case of hemoglobin) as Hb^*_I and Hb^*_{II}. We suggest that Hb^*_I is already at least partially domed and has a spin state of at least $S = 1$. Hb^*_I decays in 300 fs to the ground-state unligated heme species, which we consider to be $S = 2$ and at least partially domed. The population of Hb^*_{II} varies with the ligand. It is more significant when the ligand is O_2 or NO than when the ligand is CO. The similarities of the picosecond and femtosecond bleaching and absorption kinetics of HbCO with those of PTHCO (and of HbNO with those of PTHNO) indicate that in this time domain the importance of steric features of the protein are less important than the nature of the ligand itself in the geminate recombination process as well as in the relative amounts of the two heme excited states created. It is suggested that the quantum yield of ligand photodissociation is unity whether the ligand is O_2, NO, or CO. The low yield of photodissociated heme–O_2 or heme–NO compounds as measured on the microsecond time scale is thus attributed to a fast (2.5 ps) recombination of O_2 or NO with Hb^*_{II}. We discuss geminate recombination measurements of cyanomet hybrid hemoglobins with NO and consider these results in terms of α and β subunit heterogeneity. The first picosecond transient absorption spectra of cyanomet-CO hybrid hemoglobins are presented and are compared with the spectra of other heme compounds. The superimposability of the transient spectra on the equilibrium spectra of heme compounds that exhibit minimal or no cooperativity is noted and is compared with the case of cooperative systems where the transient spectra are distorted with respect to the equilibrium spectra. This distortion is interpreted in terms of an interaction of a domed heme with the F helix.

The availability of absorption and Raman spectrometers providing time resolution in the picosecond to femtosecond domains has extended the investigation of the dynamics of heme proteins to the moments just subsequent to ligand dissociation and hence has provided a probe of the interaction of the ligand with the heme (Shank et al., 1976; Chernoff et al., 1980; Martin et al., 1983, 1984a) and the configuration of the heme protein itself (Terner et al., 1981; Dasgupta et al., 1985; Findsen et al., 1985; Petrich et al., 1987) *before* the ligand has had sufficient time to diffuse out of the heme pocket. The growing body of data on heme proteins in this ultrafast time domain has revealed complicated photophysics of the heme that are sensitive to both the structure of the protein (Chernoff et al., 1980; Cornelius et al., 1981; Martin et al., 1983a,b, 1984b) and to the ligand (CO, O_2, and NO) (Chernoff et al., 1980; Martin et al., 1983a,b, 1984a,b; Cornelius et al., 1983; Friedman et al., 1985; Houde et al., 1986).

Here we present and discuss data dealing with the excited-state photophysics of hemoglobin (Hb), myoglobin (Mb), and protoheme (PTH) and the effects of the ligands CO, O_2, and NO on the excited-state photophysics. We also present geminate recombination measurements and the first picosecond transient absorption spectra of cyanomet hybrid hemoglobins and discuss these results in terms of α and β subunit heterogeneity. (A detailed investigation of geminate recombination in heme proteins will be presented elsewhere.) We consider the relevance of these results for an understanding of hemoglobin reactivity, and two basic questions are addressed: (1) Subsequent to dissociation of a ligand from a heme protein, geminate recombination of the ligand with the heme protein is observed. The kinetics of the rebinding process, however, are a sensitive function of the ligand. Early microsecond flash photolysis studies (Gibson & Ainsworth, 1957; Antonini & Brunori, 1971) showed that the apparent quantum yield of photodissociation decreased as the ligand was changed from CO to O_2 to NO. The question then is, how does one explain the significantly lower quantum yield of photodissociation, as measured in the microsecond time scale or in the steady state in O_2 and NO as opposed to CO (Bücher & Kaspers, 1947; Saffran & Gibson, 1977; Noble et al., 1967; Antonini & Brunori, 1971)? In particular, it is important to discover whether energy relaxation to nonphotodissociative states is an important process in the photophysics of heme–O_2 and heme–NO compounds or whether the apparent diminished quantum yield of these compounds is a result of very fast recombination processes. (2) While a considerable amount of data has been amassed regarding the heme photophysics (Chernoff et al., 1980; Martin et al., 1983a, 1984a) and re-

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combination processes [e.g., Chernoff et al. (1980), Cornelius et al. (1981, 1983), Austin et al., (1975), Gibson and Ainsworth (1957), Antonini and Brunori (1971), and Martin et al. (1983a,b, 1984a,b)], one may still ask what information absorption spectroscopy in the ultrafast time domain may yield concerning the heterogeneity of the α and the β subunits of the Hb tetramer and whether the onset of a T-like conformation can be detected in this time domain.

Experimental Procedures

(*A*) *Sample Preparation.* Hb A was prepared from the hemolysate of fresh red blood cells of nonsmoking donors and purified by chromatography on a DEAE-Sephadex column. The purity of the solute was verified by isoelectric focusing that showed a single band migrating at p*I* = 6.95. The Hb was further stripped on remaining contaminants on an ion-exchange column consisting of ion retardation resin AG11A8 and mixed-bed resin AG50 X8-D (Bio-Rad). Hb was concentrated under vacuum and stored in the oxy form in liquid nitrogen. In most circumstances the stock Hb A solution was diluted in 0.1 M potassium phosphate or [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bistris) buffer at pH 7.0 at room temperature. Deoxy samples were obtained by equilibrating the solution under a stream of pure humidified argon until a deoxy spectrum was obtained (Cary 219, Varian). Samples ligated with CO were obtained by equilibrating the deoxy sample under 1 or 0.1 atm of CO in a tonometer. Oxygenated samples were equilibrated under 1 atm of pure oxygen. No dithionite was added, since the fraction of metHb was determined to be less than 2%, as determined spectrophotometrically. NO samples were prepared by equilibration of deoxygenated samples with 1 atm of pure NO. In this case, sodium dithionite (Merck) was added to the solutions to prevent oxidation. Dilute solutions of Mb were prepared from metMb (HH III, Sigma) without further purification and converted to the ferrous form after addition of a 5 M excess of freshly prepared sodium dithionite under strictly anerobic conditions. Excess dithionite was removed by passing the solution on a Sephadex G-25 column. MbCO, MbO_2, and MbNO were prepared in a manner similar to that described above for the ligated hemoglobins.

For the preparation of met valency symmetrical hybrids of Hb, α and β chains were obtained from carboxylated Hb that had been reacted with *p*-(chloromercuri)benzoate according to the method of Bucci and Fronticelli (1965). The chains were separated on DEAE-Sephadex with a NaCl gradient at pH 8. Regeneration of the SH groups of the isolated chains was effected by a β-mercaptoethanol treatment on Sephadex G-25. Bound CO was replaced by oxygen under intense light and equilibration with pure humidified oxygen in the cold. The chains were then oxidized by adding an exact molar excess of $K_3Fe(CN)_6$ in phosphate buffer at pH 7. After oxidation, the hybrids were obtained by adding the carboxylated sister chains. When necessary, a slight molar excess of KCN was added to produce cyanomet α or β hybrids. As a last step, the solutions were centrifuged to remove denatured material and rechromatographed to remove traces of remaining isolated chains and excess anions. The purity of the preparations was verified by isoelectric focusing. The hybrid solutions were then concentrated under pressure and were stored in liquid nitrogen in the CO form.

Protoheme samples were prepared from chlorohemin. A stock solution (2 mM) was obtained in 1 M NaOH and centrifuged to remove precipitates. Working solutions of 0.1 M heme were diluted in a 95:5 (v/v) ethylene glycol/water mixture to obtain predominantly monomeric hemes as recommended by Gibson and Antonini (1963). The percentage of ethylene glycol used was that giving the highest extinction coefficient of a ferrous deoxy heme solution, $\epsilon_{420.5nm} = 13\,800$ M^{-1} cm^{-1}. Hemin was reduced by addition of a 5 M excess of sodium dithionite, which was not removed by chromatography since ferrous heme undergoes rapid reoxidation during this procedure. The reduced ferrous heme solution was equilibrated with 1 atm of either CO or NO for our spectroscopic studies.

Aliquots of the various heme-containing solutions described above were transferred with a gastight Hamilton syringe into 1-mm path length optical cuvettes previously rinsed with the gas phase under study. Optical spectra were measured before and after experiments and indicated no changes. All chemicals used in these studies were of analytical grade.

(*B*) *Laser System and Data Treatment.* The time-resolved absorption spectrometer employed to obtain the results discussed here is comprised of a colliding-pulse, mode-locked ring laser (with four intracavity prisms to compensate for dispersion) whose pulses are amplified to 1 mJ at 10 Hz with a Nd:YAG laser (Quantel). The tunability of the system is achieved by dividing this pulse train in two and generating two white-light continua. One continuum is used for both a probe beam and a reference beam. Excitation wavelengths of 580 nm, for Q-band excitation, were obtained by selecting a 50-Å bandwidth centered at 580 nm from another continuum and by amplifying this portion of the continuum. Excitation wavelengths of 307 nm were obtained by directly frequency doubling the output of the amplifier. The duration of the amplified pulses is 100–150 fs. Such pulse widths provide a time resolution of approximately 50 fs. Our apparatus is described in detail elsewhere (Martin et al., 1983a, 1984a, 1986). Previous studies of the heme photophysics have been complicated by multiple excitations of the heme [for a discussion, see Chernoff et al. (1980) and Martin et al. (1983a)]. For our experimental conditions, the possibility of multiple heme excitations is negligible. For example, using a 100–200-μJ pump pulse at 580 nm and a solution that is 100 μM in heme with a 1-mm pathlength, one observes only 15–20% photodissociation. (As we discuss below, we assume that each absorbed photon results in photodissociation.) Furthermore, the sample was translated through the laser beam at 12 mm/s in order for each laser shot to probe a fresh sample volume. All measurements were performed at room temperature.

The transient absorption data were fit (Martin et al., 1983a) to a sum of two exponentials. Because of the presence of geminate recombination, which is slow compared to the time scales of our measurements (except when NO is the ligand), the transient bleaching of the Soret band of the ligated hemoglobins does not recover completely. Hence a constant term was in most instances included in the expression for the bleaching (Figures 2 and 3a). In the discussion that follows concerning the photophysics of heme proteins, several generalizations are made on the basis of our fits to the kinetics of the transient bleaching and transient absorption. While the data could be adequately fit in the manner described above, the physical parameters extracted from the fitting procedure, in particular the weights of the decay components, should be regarded qualitatively. Transient absorption difference spectra were created directly from the decay curves of the transient bleaching and absorption such as those displayed in Figures 1–5. The equilibrium difference spectra were normalized to the maximum of the absorbance of the transient spectra at 7 or 8 ps. Previous results (Martin et al., 1983a, 1984a,b) were obtianed with a 2-D optical multichannel analyzer (OSA,

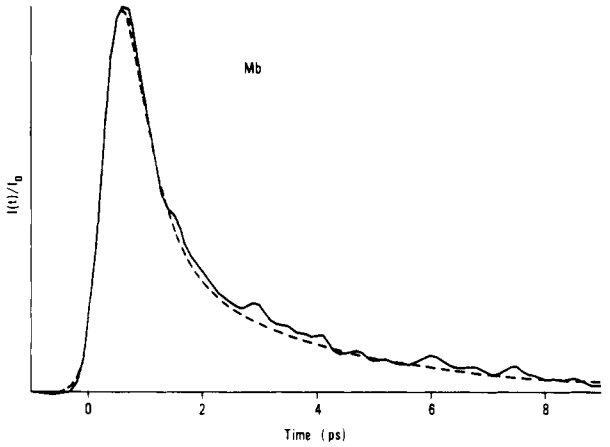

FIGURE 1: Transient bleaching of the Soret band of unligated Mb with λ_{pump} = 580 nm; λ_{probe} = 434 nm. $I(t)/I_0 = 0.65 \exp(-t/300$ fs) + 0.35 exp ($-t/3.2$ ps). The maximum of the bleaching represents an absorption change of 0.30. The data were smoothed.

B.M., Spectronick). The data we present here, as well as the data for the protoheme–CO transient difference spectra presented elsewhere (Martin et al., 1984a), were obtained wavelength-by-wavelength by using two photodiodes positioned at the output slit of a spectrometer, one of the diodes serving as a reference. This is the same arrangement used to obtain the kinetic traces. The optical multichannel analyzer had a smaller linear response than the photodiodes and demanded a very precise optical alignment in order to produce undistorted spectra. Use of the photodiodes was superior in these two respects.

RESULTS

(A) Photophysics of Ligated and Unligated Heme Proteins: Transient Bleaching and Absorption in the Soret Region. Figure 1 presents the transient bleaching of the Soret band for Mb, Figure 2 for HbCO, HbO_2, and HbNO, and Figure 3 for PTHCO and PTHNO. The kinetics of $PTHO_2$ could not be studied because of oxidation of the ferrous heme that precludes reversible binding of O_2. From the cases investigated here, several general observations can be made for any complex of Hb, Mb, or PTH with CO, O_2, or NO.

(1) The kinetics are the same if ligand dissociation is effected with a 307-nm pulse or with a 580-nm pulse.

(2) Bleaching of the Soret band is instantaneous; that is, it occurs in less than 50 fs, the time resolution of our apparatus.

(3) A species with an absorption spectrum characteristic of unligated heme compounds appears in 300 fs.

(4) The recovery of the absorbance of the ligated Soret band can be described by a 2.5–3.2-ps relaxation process and a longer relaxation, attributed to geminate recombination, that ranges from approximately 10 ps in heme–NO compounds to the nanosecond time scale in heme–CO compounds.

In addition, as can be noted from Figure 2, a 300-fs relaxation component is present in the recovery of the absorbance of the ligated Soret band. This absorption recovery must be interpreted carefully since, as we noted above, a Soret band characteristic of unligated hemes appears with a 300-fs time constant. We note that the weight of the 300-fs component observed in the absorption recovery of the ligated Soret band depends on the probe wavelength. Compare Figure 2b with Figure 4, which presents a striking example of the appearance of a "deoxy-like" species with a 300-fs rise time in HbO_2 as one scans through the ligated Soret band and into the unligated Soret band. When the Soret band is probed at 426 nm (Figure 4b), an instantaneous bleaching is at first observed but is later dominated by a new transient absorbance due to the formation

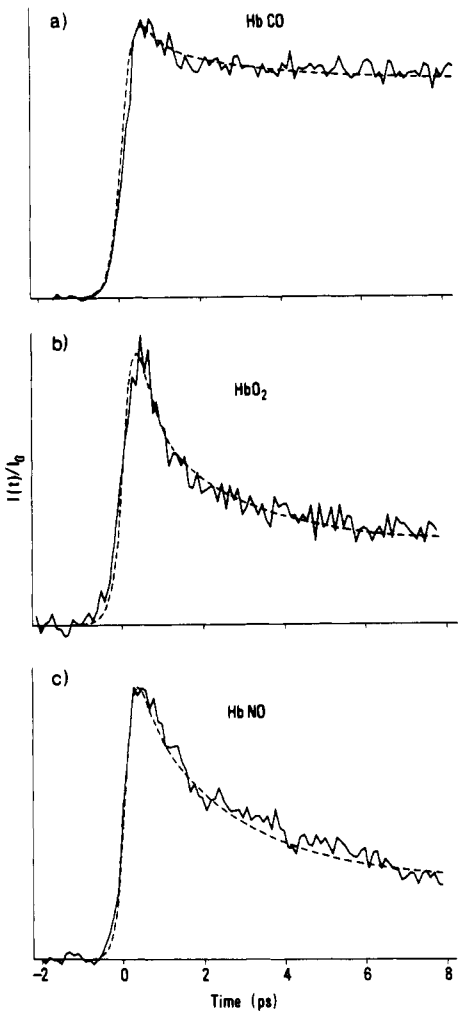

FIGURE 2: Transient bleaching of the Soret band of ligated hemoglobins with λ_{pump} = 580 nm. The probe wavelengths were chosen to correspond to the maximum of the Soret band of the respective ligated hemoglobins. Selection of the probe wavelength in this manner also permits the observation of the appearance of the unligated ground-state hemoglobin that appears in 300 fs and that has a nonnegligible absorption at these wavelengths. This effect is demonstrated in Figure 4. (a) HbCO, λ_{probe} = 419 nm, $I(t)/I_0 = 0.27 \exp(-t/300$ fs) + 0.10 exp($-t/2.5$ ps) + 0.63. The maximum absorption change is 0.34. (b) HbO_2, λ_{probe} = 414 nm, $I(t)/I_0 = 0.45 \exp(-t/300$ fs) + 0.40 exp($-t/2.5$ ps) + 0.15. The maximum absorption change is 0.17. (c) HbNO, λ_{probe} = 416 nm, $I(t)/I_0 = 0.24 \exp(-t/300$ fs) + 0.57 exp($-t/2.5$ ps) + 0.19. The maximum absorption change is 0.12. If one neglects the 300-fs relaxation component for reasons that are discussed under Results and in the caption to Figure 4 and considers the absorption recovery *only* in terms of the *2.5-ps component* and the *constant term* that represents geminate recombination, which is slow on the time scale of these measurements, then the 2.5-ps component comprises roughly 14%, 72%, and 75% of the decay of the transient bleaching in photodissociated HbCO, HbO_2, and HbNO, respectively. The similarity of the bleaching kinetics of HbO_2 and HbNO is not inconsistent with their quantum yields of photodissociation as measured on the microsecond time scale [0.028 and 0.003, respectively (Saffran & Gibson, 1977)]. The factor that is most responsible for the difference in the observed microsecond dissociation yields is the rapid geminate recombination phase of heme–NO compounds (approximately 10 ps, assuming an exponential decay). Heme–O_2 compounds lack this rapid phase. This will be discussed in detail elsewhere.

of the unligated photoproduct (see Figure 11). The 300-fs component in the bleaching kinetics is thus attributed to the appearance of another species that is created subsequent to ligand dissociation and which has a nonnegligible absorption in the region of the ligated Soret band: the unligated, ground-state heme. (See the discussion below, the absorption

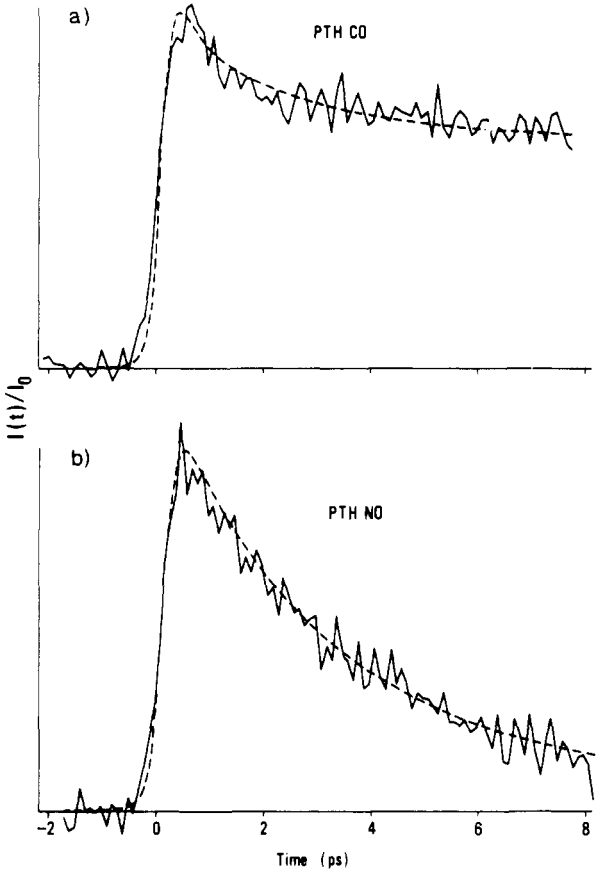

FIGURE 3: Transient bleaching of the Soret band of ligated protohemes with λ_{pump} = 580 nm. (a) PTHCO, λ_{probe} = 410 nm, $I(t)/I_0 = 0.25 \exp(-t/300\text{ fs}) + 0.25 \exp(-t/2.5\text{ ps}) + 0.50$. The maximum absorption change is 0.17. The bleaching of the photodissociated PTHCO shows no significant decay at 400 ps (Martin et al., 1983a). See the captions to Figures 2 and 4 for an explanation of the 300-fs component in (a). (b) PTHNO, λ_{probe} = 401.5 nm, $I(t)/I_0 = 0.65 \exp(-t/2.5\text{ ps}) + 0.35 \exp(-t/7\text{ ps})$. The maximum absorption change is 0.04. The 7-ps component represents geminate recombination of NO with PTH. The similarity among the bleaching kinetics of HbCO and PTHCO and of HbNO and PTHNO are striking. They suggest that purely electronic factors [i.e., the respective electronic states of the ligands (Cornelius et al., 1983)] play a significant role, in addition to the protein matrix, in geminate recombination at room temperature.

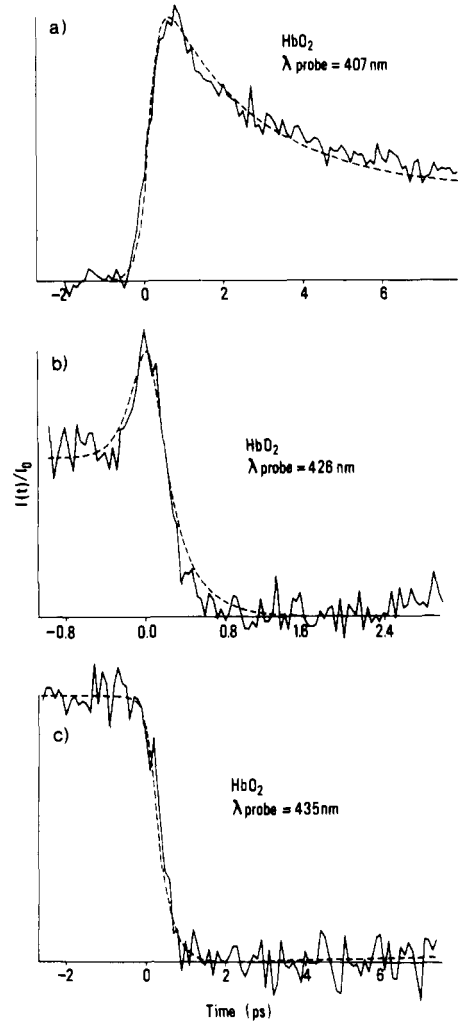

FIGURE 4: Transient bleaching and absorption of HbO_2 with λ_{pump} = 580 nm. The probe beam is scanned through the Soret band of the ligated heme protein and into the Soret band of the unligated species. (a) λ_{probe} = 407 nm, $I(t)/I_0 = 0.02 \exp(-t/300\text{ fs}) + 0.63 \exp(-t/2.5\text{ ps}) + 0.35$. The maximum absorption change is 0.12. In contrast to Figure 2b, here the 300-fs component is negligible because there is little contribution from the unligated Soret band at 407 nm. This permits a more accurate estimate of the relative importance of the 2.5-ps heme excited state which is competitive with the formation of the ground-state deoxy species. Here Hb^*_{II} accounts for 64% of the relaxation of the bleaching of the ligated Soret band. This is in good agreement with the value of 72% determined from Figure 2b. (b) λ_{probe} = 426 nm. (c) λ_{probe} = 435 nm. The maximum absorption change is 0.07. Both (b) and (c) display a long-lived transient absorption that appears with a time constant of 300 fs. (b) differs from (c) in that the probe wavelength permits the observation of not only the unligated Soret region but also the ligated Soret region. [The isosbestic point of HbO_2 and equilibrium unligated Hb is 421.2 nm (Table I).] Hence, we also observe an initial transient bleaching. Thus, (b) provides a convincing illustration that the deoxy-like species is not formed instantaneously.

spectra of Antonini and Brunori (1971), and Figure 11.)

(5) The 2.5–3.2-ps relaxation process is common to all the heme compounds (both ligated and unligated) studied here. Its relative weight, however, is sensitive to the ligand. When the ligand is CO, its contribution is small compared to heme complexes where the ligand is O_2 or NO. In all the heme complexes studied here, *except* for the heme–CO complexes, the 2.5–3.2-ps component comprises roughly 65–75% of the decay of the transient bleaching (Figures 2, 3, and 5). In the unligated heme compounds, this component is observed to be systematically longer (3.0–3.2 ps). It is possible that this difference between the ligated and the unligated heme compounds arises from differences in the geometries of the ground-state ligated and unligated compounds.

(6) The 300-fs and 2.5-3.2-ps processes are present in the bleaching kinetics of the *unligated* heme compounds, and hence these processes are attributed to the relaxation of excited-state unligated hemes (see below).

(7) Figure 5 presents the transient absorption of HbO_2 subsequent to ligand dissociation at 460 and 480 nm. (The absorption maxima of these species are at 455 and 470 nm, respectively.) The absorption at 480 nm appears instantaneously and is well described by a single-exponential decay characterization by a time constant of 300 fs. It is this transient species absorbing at 480 nm that we attribute to the precursor of the ground-state unligated species. The absorption at 460 nm also appears instantaneously, but its decay is dominated by a process characterized by a 2.5-ps time constant. The results were identical with 307- or 580-nm excitation. The presence of the 300-fs and 2.5–3.2-ps absorption decays in all of the heme compounds investigated, including the unligated compounds, is essential to our identification of these processes as relaxations of excited-state unligated hemes.

(*B*) *Transient Absorption Difference Spectra.* (*1*) *HbCO, HbO_2, MbCO, and PTHCO.* The transient absorption spectra

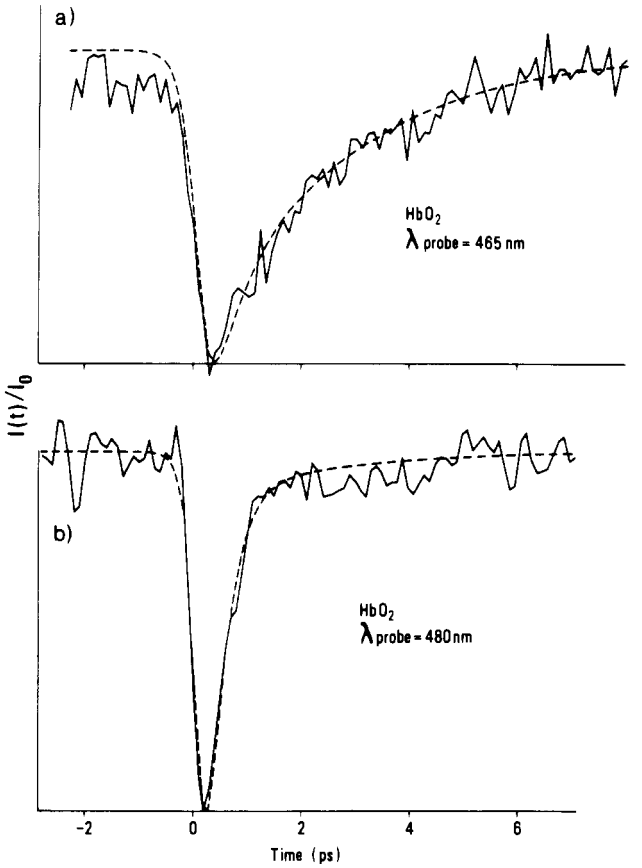

FIGURE 5: Transient absorption in the Soret region in HbO_2. (a) λ_{pump} = 575 nm, λ_{probe} = 465 nm, $I(t)/I_0 = -0.30 \exp(-t/300 \text{ fs}) - 0.70 \exp(-t/2.5 \text{ ps})$. The maximum absorption change is 0.03. (b) λ_{pump} = 580 nm, λ_{probe} = 480 nm, $I(t)I_0 = -0.90 \exp(-t/300 \text{ fs}) - 0.10 \exp(-t/2.5 \text{ ps})$. The maximum absorption change is 0.03.

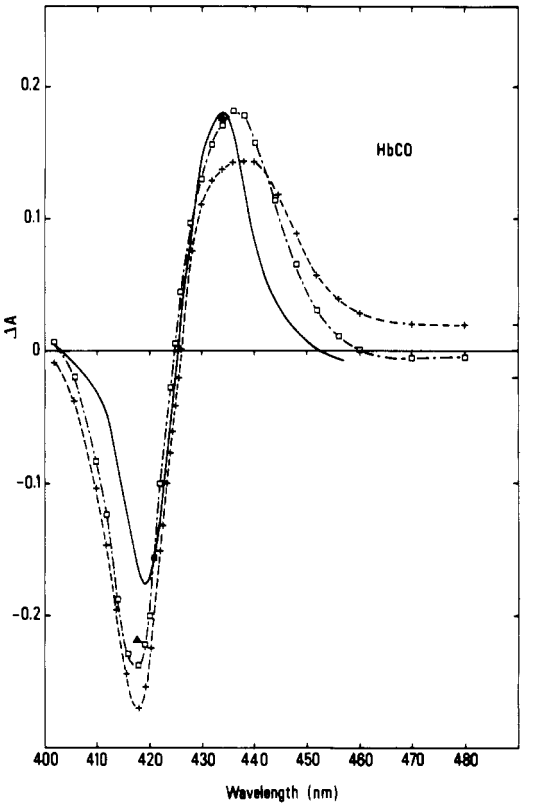

FIGURE 6: HbCO transient difference spectra: (+) 2 ps; (□) 10 ps. The distortion persisting at 250 ps is indicated by a triangle (▲) at the extrema of the difference spectrum. Note the marked asymmetry of the transient spectra compared with the difference spectrum obtained from equilibrium unligated Hb and equilibrium HbCO (—). λ_{pump} = 580 nm.

of the HbCO and the HbO_2 photoproducts (Figures 6 and 7) are fundamentally different from that of the MbCO photoproduct (Figure 8) or that of the PTHCO photoproduct (Figure 9). Both the MbCO photoproduct at 7 ps and the PTHCO photoproduct at 8 ps exhibit difference spectra that are roughly superimposable on their corresponding equilibrium difference spectra. On the other hand, the HbCO and HbO_2 photoproducts yield difference spectra that are significantly distorted *with respect to their equilibrium difference spectra.* See Experimental Procedures for a discussion of the measurement of our absorption spectra.

(2) Cyanomet Hybrids. The transient absorption spectra of the 5- and 10-ps photoproducts of the cyanomet-CO hybrids, $\alpha_2^{CO}\beta_2^{+CN}$ and $\alpha_2^{+CN}\beta_2^{CO}$ (not shown), are superimposable on their equilibrium difference spectra (Figure 10 and Table I). The difference spectra of the 8-ps PTHCO photoproduct and the cyanomet-CO hybrids serve the important role of a control for the transient absorption spectra measured in heme proteins. The distortion observed in the HbCO difference spectrum gains further credibility when it is compared with the absence of such results in the PTHCO and cyanomet-CO hybrid experiments. Furthermore, distortion in the HbCO difference spectrum has been shown to persist into the microsecond time domain (Hofrichter et al., 1983).

Cyanomet hybrids in which either the α or the β subunit bears CN^- as the ligand have been used in NMR and EPR experiments to study the effect of ligation on tertiary and quaternary structure [e.g., Ogawa and Shulman (1972), Ogata and McConnell (1972a,b), and Banerjee et al. (1973)]. The advantage of working with these valency hybrids is that the cyanomet subunits remain in the ferric state while the other subunits remain in the ferrous state and can be reversibly ligated. We have found that upon photoexcitation of cyanometHb at 580 nm, the relaxation of the transient bleaching of the Soret band is identical with that of unligated heme complexes: that is, it consists of a 300-fs and an ~3-ps component (data not shown). Thus, if the CN^- is photodis-

Table I: Summary of Transient and Equilibrium Spectral Data of Heme Complexes

heme complex	isosbestic point at equilibrium[a] (nm)	isosbestic point of transient species[b] (ps) (nm)	D_{ps}[c]	D_∞[d]	D_{ps}/D_∞	n[e]
HbCO	425.5	424.2 (7)	1.29	0.97	1.33	2.95[g]
HbO_2	421.2	420.5 (7)	0.80	0.67	1.19	2.90[g]
$\alpha_2^{CO}\beta_2^{+CN}$	427.0	427.5 (10)	2.19	2.25	0.97	1.20[h]
MbCO	429.5	429.0 (7)	0.99	1.20	0.82[f]	1.00[i]
PTHCO	416.0	416.0 (8)	1.01	1.05	0.96	

[a] The isosbestic point reported is that obtained from the subtraction of the steady-state spectrum of the unligated heme complex from that of its ligated counterpart. [b] The isosbestic point reported is that obtained directly from the kinetics of bleaching and absorption decay of the transient species. The error in these measurements is ≤1 nm. [c] The absolute value of the ratio of the maximum of the bleaching to the maximum of the absorbance of the difference spectrum created from the transient species. [d] The absolute value of the ratio of the maximum of the bleaching to the maximum of the absorbance of the difference spectrum created from the equilibrium ligated and unligated heme complex. These values are accurate to ~3%. [e] The value of the Hill coefficient, the measure of cooperativity, of the given heme complex. [f] The deviation of this value from 1.00 may be rationalized by a lifetime of Hb^*_{II} that is slightly greater than 2.5 ps. This would explain the less pronounced bleaching near 420 nm at 7 ps (Figure 8). [g] These are values of n_{50} (Bohn, 1979). [h] Nagai (1977). [i] Antonini and Brunori (1971).

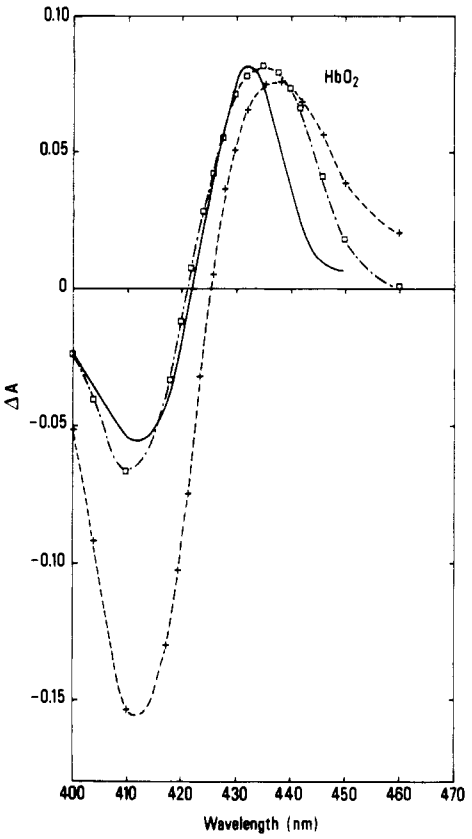

FIGURE 7: HbO_2 transient difference spectra: (+) 500 fs; (□) 7 ps. Equilibrium difference spectrum (—). λ_{pump} = 580 nm.

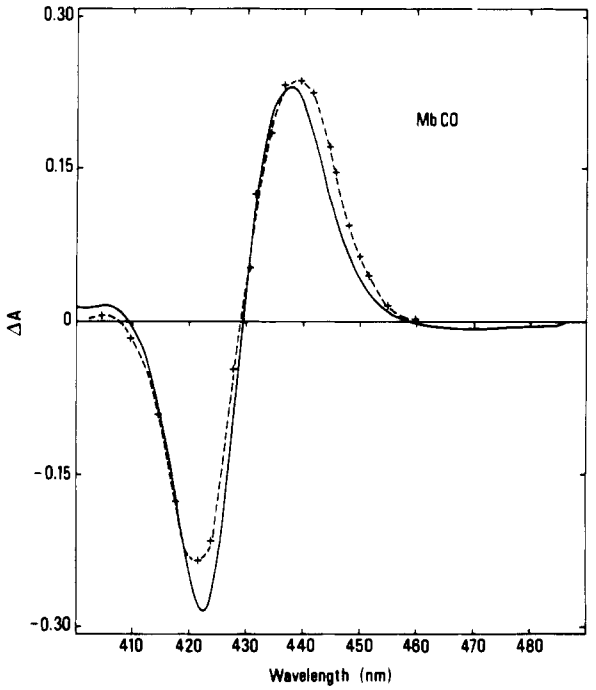

FIGURE 8: MbCO transient difference spectrum at 7 ps (+). Equilibrium difference spectrum (—). λ_{pump} = 580 nm. That the 7-ps spectrum is not exactly superimposable on the equilibrium spectrum around 420 nm and in the longer wavelength region can be attributed to the presence of an Hb^*_{II} species that is slightly longer lived than 2.5 ps.

sociated from the Hb, it quickly recombines in a few picoseconds. Hence, the transient absorption spectra of the $\alpha_2{}^{+CN}\beta_2{}^{CO}$ or the $\alpha_2{}^{CO}\beta_2{}^{+CN}$ hybrids measured at times approximately 5 ps or more subsequent to photodissociation can be interpreted in terms of an Hb molecule where *only* the β or the α subunits, respectively, are photodissociated.

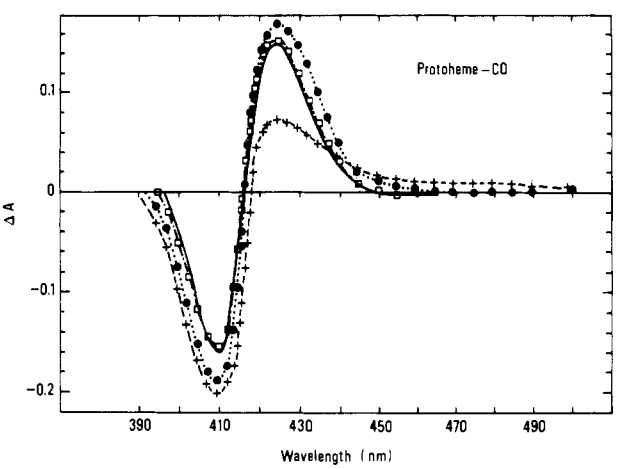

FIGURE 9: PTHCO transient difference spectra: (+) 350 fs; (●) 2 ps; (□) 8 ps. The difference spectrum created from equilibrium unligated PTH and equilibrium PTHCO is given by the solid curve (—) and is superimposable on the 8-ps difference spectrum.

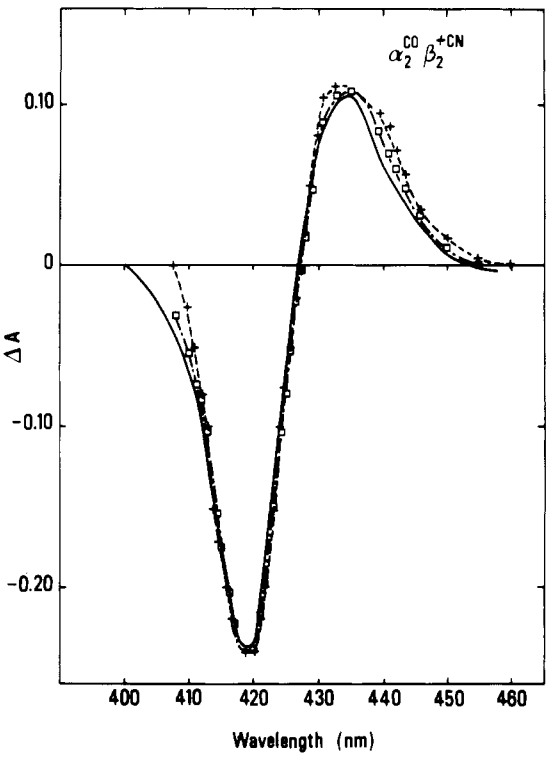

FIGURE 10: $\alpha_2{}^{CO}\beta_2{}^{+CN}$ transient difference spectra: (+) 5 ps; (□) 10 ps. The difference spectrum constructed from equilibrium $\alpha_2\beta_2{}^{+CN}$ and equilibrium $\alpha_2{}^{CO}\beta_2{}^{+CN}$ is given by the solid curve (—). Note that the equilibrium difference spectrum is essentially superimposable on the 5- and 10-ps spectra. λ_{pump} = 580 nm.

Finally, we have measured the geminate recombination kinetics of HbNO, $\alpha_2{}^{NO}\beta_2{}^{+CN}$, $\alpha_2{}^{+CN}\beta_2{}^{NO}$, and MbNO. We briefly discuss these results below in terms of the heterogeneity of the α and β subunits.

Discussion

(*A*) *Excited-State Photophysics.* As shown above, the photodissociation of a ligand from Hb, Mb, or PTH, as monitored by the bleaching of the maximum of the absorption of the Soret band, occurs in less than 50 fs. While the recovery of the absorption of the Soret band in all cases comprises a 2.5–3.2-ps component, the weight of this component varies considerably with the ligand (Figure 2). The instantaneous appearance of species whose absorption maxima are at ap-

proximately 470 and 455 nm and that decay in 300 fs and 2.5–3.2 ps, respectively, for both the ligated and *unligated* heme compounds suggests that these species are excited states of the unligated heme, and thus we denote the 300-fs species as Hb^*_I and the 2.5–3.2-ps species as Hb^*_{II}. (Since the excited states of Mb and PTH exhibit the same behavior as those of Hb, for simplicity we shall refer to all the heme excited states in this paper as Hb^*_I and Hb^*_{II}.) Because the bleaching of the ligated Soret band recovers with a 2.5–3.2-ps component, we consider Hb^*_{II} as an excited-state species whose formation is competitive with ligand photodissociation *as measured on the microsecond or steady-state time scales*. Because the ground-state unligated heme species ($Hb^\dagger$) appears with a 300-fs time constant, we consider Hb^*_I as an excited-state precursor of the ground-state unligated species.

In order to rationalize the quantum yield of ligand photodissociation as it has been determined, for example, in the microsecond and steady-state time domains, we suggest that the quantum yield of ligand photodissociation in all heme complexes of O_2, NO, or CO is unity. This proposal requires the following: (1) We assume that in less than 50 fs subsequent to photodissociation there are no nonradiative processes that are competitive with the formation of the species we have denoted Hb^*_I and Hb^*_{II}. (2) Hb^*_{II} exhibits a higher reactivity toward the photodissociated ligand than does Hb^*_I. (We suggest below that the high reactivity of Hb^*_{II} is due to a planar heme in this species.) (3) The quantum yield of photodissociation in MbCO is approximately unity (Bücher & Kaspers, 1947; Saffran & Gibson, 1977; Noble et al., 1967). The high but smaller yield in HbCO [$\sim$0.5 depending upon the measurement and the experimental conditions such as temperature (Noble et al., 1967; Saffran & Gibson, 1977)] can be attributed to the presence of a slow geminate recombination in HbCO (Hofrichter et al., 1983), whereas geminate recombination is much less pronounced in MbCO (Henry et al., 1983; Friedman & Lyons, 1980).

Because in heme–CO compounds the Hb^*_{II} population is small, a correspondingly high quantum yield of ligand photodissociation is observed on time scales greater than 10 ps subsequent to ligand photodissociation. On the other hand, in heme–O_2 and heme–NO compounds where the population of Hb^*_{II} is significant, the observed quantum yield of dissociation is considerably lower. An additional factor that contributes to the extremely low photodissociation yield (as measured on longer time scales) in heme–NO compounds is the rapid phase ($\sim$10 ps) of geminate recombination (Cornelius et al., 1983; Houde et al., 1986).

The heme protein photophysics are summarized schematically in Figure 11, and the assignments we have just made shall now be discussed in light of complementary experimental and theoretical observations. In the course of this discussion, we treat NO as being similar to O_2 in a first approximation. This is not unreasonable given the similarity of the NO and the O_2 data (Figure 2c and Figure 2b) and the similarity of the absorption spectra of HbO_2 and HbNO (Van Assendelft, 1970).

(*1*) *Photodissociation Channels.* Our observation that the photophysics of Hb, Mb, and PTH with O_2, NO, or CO are the same with excitation wavelengths of 307 and 580 nm is consistent with results from other laboratories (Bücher & Kaspers, 1947; Chernoff et al., 1980). While some investigators have observed differences with the excitation wavelength of photolysis of HbO_2 (Green et al., 1978), these differences were later rationalized in terms of multiphoton absorption processes (Chernoff et al., 1980). Studies of MbO_2 (Reynolds et al., 1981) also revealed a dependence of the photolysis on excitation wavelength, but these results have not since been reproduced. A satisfactory description of the photodissociation channels in ligated heme proteins will therefore place them below the Q-band $\pi \rightarrow \pi^*$ transition. Following the reasoning presented by Chernoff et al. (1980), this suggests that significant excited-state populations exist only in the lowest electronic states of a given spin multiplicity. In this picture, these states are populated faster than photodissociation can occur and hence have been referred to as "bottleneck states" (Chernoff et al., 1980) in the photochemistry.

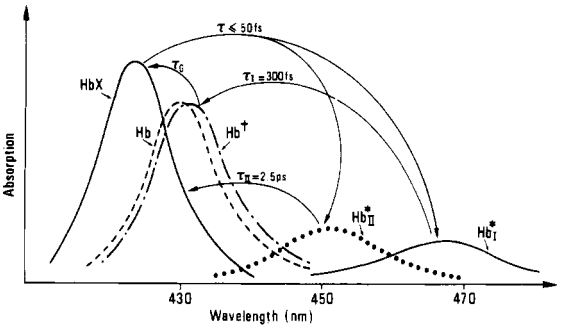

FIGURE 11: Generalized absorption spectra of heme proteins and their photoproducts. The interrelationships among these species are illustrated in the figure and described in more detail in the text. X denotes the ligand CO, O_2, or NO. The excited-state unligated species, Hb^*_I and Hb^*_{II}, are formed in less than 50 fs from the photoexcited HbX, but the amounts of Hb^*_I and Hb^*_{II} formed are dependent on X (see Figure 2). τ_I is the time constant for the formation of the deoxy-like species from Hb^*_I. τ_{II} is the time constant for the decay of Hb^*_{II} to the ground-state species. τ_G is the time constant for geminate recombination and depends on the ligand, the heme protein, and allosteric effectors. The diagram is labeled for the case of hemoglobin, but it is generally applicable for myoglobin and protoheme—providing that the associated shapes and positions of the unligated species are taken into account. The superscript dagger is applicable only to normal hemoglobins and denotes the spectral distortion existing between the newly formed unligated species and the equilibrium T-quaternary state. Compare Figures 6 and 7 with Figures 8–10.

(*a*) *Heme–CO Complexes.* Chernoff et al. (1980) have noted that the ligand field state $HbCO(^1T_1)$ is a species whose dissociation to $Hb(^1A_1)$ and $CO(^1\Sigma)$ is spin-allowed. (In the ensuing discussion, we follow Chernoff et al. and employ octahedral point group notation for both the ligated and the unligated heme complexes.) They, however, dismissed $HbCO(^1T_1)$ as the photodissociative state because recombination of the products is also spin-allowed, and, as has been shown in this work (Figures 2a and 3a) and elsewhere (Martin et al., 1983a), there is no significant recombination of dissociated heme–CO complexes on a picosecond time scale. On the other hand, Chernoff et al. have noted that the ligand field states $HbCO(^3T_1)$ and $HbCO(^5T_2)$ both have spin-allowed dissociation channels that form $Hb(^3T_1)$ and $Hb(^5T_2)$, respectively, whose recombination with $CO(^1\Sigma)$ is spin-forbidden. This suggests, then, that the photodissociative species in heme–CO complexes is a relatively low-lying multiplet ligand field state.

Waleh and Loew (1982a,b) have performed a series of calculations that are suggestive in identifying the photodissociative states in heme–ligand complexes. Their criterion for labeling a state as photodissociative is decreasing energy of the state as a function of iron–ligand distance. They found that for heme–CO complexes (1982a) where the CO is in a bent geometry with respect to the heme plane [the result obtained experimentally (Hanson & Schoenborn, 1981)] a triplet ligand field state ($d_\pi \rightarrow d_{z^2}$) did indeed both fulfill their photodissociative criterion [Figure 3 of Waleh and Loew

(1982a)] and lie below the Q-band $\pi \rightarrow \pi^*$ transition. Based on the above discussion, we shall treat the photodissociative state of heme–CO complexes as a low-lying triplet ligand field state.

(*b*) *Heme–O_2 Complexes.* It has been noted that the absorption spectrum of heme–O_2 complexes is considerably more complicated than that of heme–CO complexes (Eaton et al., 1979; Makinen & Churg, 1983). A large part of this complication has been attributed to charge-transfer transitions from porphyrin π orbitals to iron $d\pi$ orbitals that have been modified by O_2 π electrons (Eaton et al., 1978; Case et al., 1979; Waleh & Loew, 1982b). Chernoff et al. (1980) suggested that HbO_2 photodissociation may proceed via these charge-transfer states as well as through an $HbO_2(^5T_2)$ ligand field state. Since, however, they assumed that the photodissociation of heme–O_2 complexes is small, they concluded that the charge-transfer states were not very photodissociative because they lay near the ground state (10000 cm^{-1}). They also concluded that the $HbO_2(^5T_2)$ state does not contribute significantly to photodissociation because its population from ^1Q involved a second-order spin–orbit interaction.

The calculations of Waleh and Loew (1982b) for heme–O_2 complexes indicate, however, that the porphyrin $a_{1u} \rightarrow d\pi$, $O_2\pi$ and $a_{2u} \rightarrow d\pi$, $O_2\pi$ charge-transfer states both fulfill their criterion for having photodissociative character and, of course, lie below the ^1Q state. Three other photodissociative states were identified, but these lay considerably higher in energy above ^1Q [Figure 1 of Waleh and Loew (1982b)]. Since we consider the photodissociation process to be near unit efficiency *regardless* of the ligand (see above), we suggest that significant populations of low-lying singlet and triplet charge-transfer states (Hoffman & Gibson, 1977) are established in heme–O_2 complexes and that these states are efficient channels for photodissociation. In particular, we suggest that a singlet and a triplet charge-transfer state of HbO_2 dissociate to the energetically accessible $Hb(^1A_1)$ and $Hb(^3T_1)$ ligand field excited-states of unligated hemoglobin [Figure 4 of Chernoff et al. (1980)]. We note, however, that neither the $^1A_1$ nor the $^3T_1$ state has been observed experimentally (Chernoff et al., 1980; Eaton et al., 1979) and that in one calculation (Olafson & Goddard, 1977) the $^1A_1$ state lies considerably higher in energy above the charge-transfer states and hence would be inaccessible.

(*2*) *Origin of the Heme Excited States Hb^*_I and Hb^*_{II}.* A satisfactory discussion of the origin of the heme excited states Hb^*_I and Hb^*_{II} must explain both the near absence of Hb^*_{II} in heme–CO complexes and its significant presence in heme–O_2 (and heme–NO) complexes. Moreover, it is essential that the description of these states be consistent with our suggestion that all of the heme–ligand complexes undergo unit photodissociation. This presumes that in less than 50 fs all of the photophysics and photochemistry discussed in the above section occurs: nonradiative relaxation from the Q band—or the Soret band— to low-lying so-called bottleneck states, ligand photodissociation, and excited-state formation of the unligated heme species.

Central to a more precise identification of Hb^*_I and Hb^*_{II} is our above assignment of the photodissociative channels in excited-state ligated hemes. In HbCO we have assigned the dissociative state to a $^3T_1$ ligand field state that can form $Hb(^3T_1)$ and $CO(^1\Sigma)$. We thus attribute Hb^*_I, which gives rise to the short-lived (300 fs) transient absorbance, occurs in all the heme compounds, and is the *predominant* species observed in the photodissociation of HbCO, to an excited-state triplet ligand field state of the unligated heme. For the heme–O_2 (and the heme–NO) complexes we attribute Hb^*_I to a triplet charge-transfer state that similarly produces an excited-state triplet ligand field state of the unligated heme. The population of the species denoted Hb^*_{II} is significant only in heme–O_2 and heme–NO complexes. In heme–CO complexes it comprises a small fraction of the excited-state population (for example, compare Figure 2a with Figure 2b and Figure 2c), and hence we do not include it in the description of the heme–CO photophysics below (eq 1). Following the above reasoning, we suggest that Hb^*_{II} arises from a singlet charge-transfer state of the ligated species which decays to an excited-state singlet ligand field state ($^1A_1$) of the unligated heme. The small amount of unligated Hb observed on time scales longer than 10 ps subsequent to photodissociation of HbO_2 or HbNO can be attributed to a rapid recombination of Hb^*_{II} with O_2 or NO (eq 4).

We may write the reaction schemes for the photophysics and the photochemistry of the heme complexes as follows. We

$$Hb(^5T_2) \xrightarrow{h\nu,\ <50\ fs} Hb(^3T_1) \xrightarrow{300\ fs} Hb(^5T_2) \qquad (1)$$

$$Hb(^5T_2) \xrightarrow{h\nu,\ <50\ fs} Hb(^1A_1) \xrightarrow{3.2\ ps} Hb(^5T_2)$$

$$HbCO \xrightarrow{h\nu} HbCO(^3T_1) \xrightarrow{<50\ fs}$$

$$Hb(^3T_1) + CO(^1\Sigma) \xrightarrow{300\ fs} Hb^\dagger(^5T_2) + CO(^1\Sigma) \qquad (2)$$

$$HbO_2 \xrightarrow{h\nu} HbO_2(^3CT) \xrightarrow{<50\ fs}$$

$$Hb(^3T_1) + O_2(^3\Sigma_g^-) \xrightarrow{300\ fs} Hb^\dagger(^5T_2) + O_2(^3\Sigma_g^-) \qquad (3)$$

$$HbO_2 \xrightarrow{h\nu} HbO_2(^1CT) \xrightarrow{<50\ fs}$$

$$Hb(^1A_1) + O_2(^3\Sigma_g^-) \xrightarrow{2.5\ ps} HbO_2(^1A_1) \qquad (4)$$

have tentatively assigned $Hb(^3T_1)$ to Hb^*_I and $Hb(^1A_1)$ to Hb^*_{II}. For simplicity we write the equations in terms of Hb, although from our data they are generally applicable for Mb and PTH as well. In eq 2 and 3, the species formed from the excited state is labeled $Hb^\dagger(^5T_2)$. Because this species absorbs in the unligated Soret region, it is considered to exist in an $S = 2$ spin state (Martin et al., 1983a) and to be at least partially domed. In other words $Hb^\dagger(^5T_2)$ produced from HbA is a tetramer in the R-*quaternary* conformation with a deligated domed heme and a local heme environment that is evolving toward a *T-like* conformation. The superscript dagger is used to distinguish this newly formed species from the equilibrium T-quaternary state species because of the spectral distortion between them. From the data presented here, we propose that this distortion is peculiar only to heme proteins capable of cooperative ligand binding (compare Figures 6 and 7 with Figures 8–10). Thus, the superscript dagger in the above equations is applicable only to normal hemoglobins. $Hb^\dagger$ attains a spectrum similar to that of the equilibrium T-quaternary state species in a series of steps that take place on the nanosecond to the microsecond time scale (assuming the proper photodissociation conditions; Hofrichter et al., 1983).

The absorption maximum of the excited-state species may yield further information suggestive of assignments for the 300-fs and 2.5-ps reaction pathways proposed above. Wang and Brinigar (1979) have shown in studies of model compounds that a red shift accompanies an increase in the bonding interaction between the heme iron and the proximal histidine (thus giving rise to the doming of the heme plane). Because the absorption maximum of the triplet Hb^*_I (λ_{max} = 470 nm) is the most red-shifted absorption transient observed in heme proteins, we propose that Hb^*_I is characterized by a heme that is at least partially domed. Similarly, the fact that the ab-

sorption maximum of the singlet Hb^*_{II} (λ_{max} = 455 nm) is blue-shifted with respect to that of Hb^*_I suggests that Hb^*_{II} is characterized by a planar heme.

If Hb^*_{II} can indeed be considered planar, the reactivity of the excited-state photodissociative heme species (eq 4) can be rationalized. For example, since the triplet, Hb^*_I, is considered to be a domed heme species, it may relax to the ground-state because the domed heme may present a sizable barrier to ligand recombination. This would be expected to be so even in eq 3, where the recombination of the heme and the oxygen ($^3\Sigma_g^-$) is a spin-allowed process. Attributing the fast decay of Hb^*_{II} to a highly reactive form of the heme supposes not only a planar heme but also a distribution of ligands that have not strayed too far from the binding site.

Considering the differences in the structure and the spin states of ground-state unligated and ligated heme proteins, there is no reason to assume that the Hb^*_I and the Hb^*_{II} species produced from ground-state unligated species are structurally identical with those produced from ground-state ligated species. This is borne out by the slight difference in the decay times of Hb^*_{II} arising from ground-state ligated and unligated species: 2.5 and 3.2 ps, respectively. It is possible that the longer lifetime of Hb^*_{II} arising from ground-state unligated hemes may be due to excess energy that is deposited in the heme and cannot be channeled into photodissociation.

Lastly, we consider a possible alternative assignment for Hb^*_I and Hb^*_{II}: namely, that either Hb^*_I or Hb^*_{II} may be attributed to vibrationally hot *ground-state* unligated hemes. This possibility cannot be unambiguously eliminated since the relaxation times of 300 fs and 2.5 ps are in qualitative agreement with the experimentally determined vibrational relaxation that has been estimated to be complete in less than 10 ps (Petrich et al., 1987). Furthermore, the calculated vibrational relaxation has been shown to occur on a comparable time scale (Henry et al., 1986), although the decay was considerably more complicated than a single exponential (50% cooling in 1–4 ps and the remainder in 20–40 ps). At present, however, we consider Hb^*_I and Hb^*_{II} as excited-state unligated hemes arising from specific photodissociative channels of the excited-state ligated heme complexes for the following reason. From our time-resolved Raman measurements of the HbCO photoproduct we estimated a heme temperature of 218 K at 0.9 ps (Petrich et al., 1987). The absorption spectrum of the Soret band is known, however, to be relatively insensitive to temperature. For example, the maximum of the Soret band of cytochrome *c* shifts from 413 nm to only 415 nm going from 110 to 298 K, and its fwhm broadens by less than 10% (Schomaker et al., 1984). Thus, the red shift of the Soret band with temperature observed by Schomaker et al. is considerably smaller than that observed for Hb^*_I or Hb^*_{II} with respect to equilibrium ligated heme proteins (see Figures 4 and 10). We conclude, therefore, that Hb^*_I and Hb^*_{II} are excited-state unligated hemes and are *not* vibrationally hot ground-state species.

(*3*) *Deoxy-like Species, Hb*†. By deoxy-like we mean a heme compound that is 5-coordinate, high-spin (S = 2, or perhaps S = 1, as is suggested for Hb^*_I), and at least partially domed. For *all* the heme compounds investigated, even the unligated species, the induced absorption in the Soret region characteristic of the unligated species in question appears with a rise time of approximately 300 fs. This rise time corresponds to the decay time of the species Hb^*_I and hence is consistent with our above interpretation of Hb^*_I as the precursor of the ground-state unligated species (eq 1–3 and Figure 2–5). A 300-fs rise time in the unligated Soret region *in itself*, however, is not sufficient to determine any specific structural information concerning the newly formed unligated ground-state heme compounds. Earlier we had briefly discussed the relationship between a red shift and a domed, 5-coordinate, S = 2 heme species. Red shifts in the Soret region of heme compounds have been well correlated with the strength of the interaction between the heme Fe and the proximal histidine (or nitrogenous bases simulating the proximal histidine) (Wang & Brinigar, 1979). On the basis of this work, it was suggested elsewhere that the red shift of the isosbestic point (Martin et al., 1984a) and the distortion (Petrich et al., 1987) of the transient difference spectra with respect to the steady-state difference spectra in heme proteins, notably in the HbCO photoproduct, were indicative of the appearance of a domed, unligated heme. As we noted above, however, further investigation has shown that within our experimental error there is no red shift of the isosbestic point of the transient difference spectrum with respect to the steady-state difference spectrum for any of the heme complexes we have studied at times where the excited states of the unligated heme have relaxed. An important feature of our older measurements that does remain in our newer ones is the distortion of the transient difference spectrum. We have quantified the distortion of the difference spectrum by the parameter, D, which is the absolute value of the ratio of the maximum of the bleaching to the maximum of the absorbance. The interesting quantity is the value of D_{ps}/D_{∞}, the quotient of the transient ratio and the steady-state ratio (Table I). This value is greater than unity for heme proteins that exhibit cooperativity.

The persistence of this distortion in HbCO into the microsecond time regime (Hofrichter et al., 1983) leads us to distinguish the deoxy-like species that is formed in 300 fs from the equilibrium unligated Hb by the superscript dagger. [The distortion at 400 ps, our maximum possible delay time, cannot easily be measured in HbO_2 because of the several hundred picosecond phase of geminate recombination (Chernoff et al., 1980; Martin et al., unpublished results; Friedman et al., 1985)]. Because there is no, or only very little, distortion between the difference spectra of the PTHCO, the MbCO, and the cyanomet-CO hybrid photoproducts and their corresponding equilibrium difference spectra, we suggest that the spectral differences observed between the HbCO photoproduct and equilibrium unligated Hb are a result of the interaction of a domed, or partially-domed, heme with an F helix that forms part of an allosteric core (Gelin & Karplus, 1977). In other words, we suggest that the spectral distortion of the HbCO photoproduct is related to the transmission of the strain energy from the heme which initiates the R to T transition (Petrich et al., 1987). The spectral distortions are not expected to disappear until the environment about the heme has relaxed. This, then, explains the absence of spectral distortions in the photoproducts of PTHCO, MbCO, and the cyanomet-CO hybrids. For a physical description of the origins of these spectral distortions, the reader is referred to the discussion and the references cited in Petrich et al. (1987) as well as to Baldwin and Chothia (1979), Gelin and Karplus (1977), Findsen et al. (1985), and Wang and Brinigar (1979).

The presence of a red shift of the isosbestic point in the transient spectra is not essential for an interpretation of the distortion of the spectra of the HbCO photoproduct given the lack of distortion as measured by our quantity, D, in the spectra of the photoproducts of PTHCO, MbCO, and the cyanomet-CO hybrids. Hence, the absence of the red shift does not alter any of the conclusions we have presented elsewhere (Martin et al., 1983a,b; Petrich et al., 1987).

Lastly, Rousseau and Argade (1986) have discussed their 4 K Raman spectra of MbCO in terms of the "functionally important motions" proposed by Frauenfelder and co-workers (Ansari et al., 1985) to describe the structural dynamics of heme proteins. Rousseau and Argade ascribe the 300-fs appearance of the deoxy-like product in transient absorption experiments to a room temperature process that converts a planar, high-spin, 5-coordinate heme to a partly domed, high-spin, 5-coordinate heme with *no* observable intermediate. This picture should be clarified in view of the results presented here and elsewhere (Martin et al., 1984a) that reveal a clear separation between the rapid (<50 fs) photodissociation event and the formation of the deoxy-like product via an intermediate excited-state species (Hb^*_I or Mb^*_I). (See Figures 2, 4, 5, and 11.)

(*B*) *Distortion of the Transient Absorption Spectrum of the HbCO Photoproduct.* The 7-ps spectrum of the HbO_2 photoproduct shows a distortion similar to that of the 7-ps spectrum of the HbCO photoproduct. The absence of distortion in the 8-ps PTHCO photoproduct spectrum and of the 7-ps spectrum of the MbCO photoproduct with respect to their equilibrium difference spectrum strongly suggests that the origin of the spectral distortion in the HbCO photoproduct lies in the tetrameric nature of hemoglobin (Figures 6–8 and Table I). An investigation of the transient absorption spectra of the cyanomet hybrids (Figure 9 and Table I) is particularly useful in testing this idea. Ogawa and Shulman (1972) have shown that in these hybrids the allosteric equilibrium is shifted toward the R state so that the R to T transition occurs neither upon dissociation of O_2 from $\alpha_2^{+CN}\beta_2^{O_2}$, except in the presence of organic or inorganic phosphates, nor upon dissociation of O_2 from $\alpha_2^{O_2}\beta_2^{+CN}$, except in the presence of inositol hexaphosphate (IHP). Furthermore, Brunori et al. (1970) have shown from their oxygen equilibrium curves that the behavior of $\alpha_2\beta_2^{+CN}$ and $\alpha_2^{+CN}\beta_2$ lies between that of isolated α and β chains and that of normal hemoglobin. Specifically, the Hill coefficient, n, which is a measure of the cooperativity of a system, is less than or equal to 1.2 in $\alpha_2\beta_2^{+CN}$ and $\alpha_2^{+CN}\beta_2$ (Brunori et al., 1970; Nagai, 1977). For the reaction with oxygen in the isolated α and β chains, as well as for Mb, n is 1.0, and in normal Hb, $n \sim 2.9$ [and varies slightly with pH (Bohn, 1979)]. The data of Figures 6–10 and Table I indicate that the heme proteins exhibiting cooperativity possess transient spectra that are distorted with respect to their equilibrium difference spectrum.

As we indicated earlier, it is very unlikely that given our specific photolysis conditions we generate a significant population of hemoglobin molecules that are fully dissociated. Because we do not generate a large population of fully dissociated R-state hemoglobin molecules, we cannot expect to produce a significant population of molecules that will on a longer time scale undergo an R to T transition. This has been shown by Hofrichter et al. (1983): Hemoglobin molecules that are only 20% dissociated do not exhibit the 20-μs relaxation that is characteristic of an R to T transition. This does not imply, however, that photodissociation of one heme in a hemoglobin tetramer cannot induce structural changes at the tertiary level. The 30-ps relaxation observed in transient Raman experiments (Petrich et al., 1987) and the distortion of the spectra of the HbCO and HbO_2 photoproducts that we present here indicate that such structural changes are induced. The more probing question is whether or not these changes are related to the onset of a T-like structure. A comparison of our absorption spectra of the HbCO photoproduct up to 250 ps (Figure 6) with those shown in Figure 4a of Hofrichter et al. (1983) up to 3 μs strongly suggests that there is such a relation. The distortion observed between their 10-ns photoproduct spectrum and the spectrum of their relaxed T structure is nearly the same as the distortion we observe between our 7-ps photoproduct spectrum and our equilibrium spectrum. These similarities exist not only between the ratios of the extrema of the difference spectra but also in the spectral broadening of the photoproduct in the long-wavelength region. The persistance of this broadening into the microsecond time regime is, furthermore, strong evidence that our photoproduct spectra at 7 ps are not distorted by heme excited states, vibrationally hot hemes, or nonlinear phenomena.

The work of Ogata and McConnell (1972a,b) and of Szabo and Karplus (1972, 1975) has suggested that the interactions coupled to the β chains are stronger than the corresponding interactions involving the α chains, and a considerable amount of data has been collected in support of this idea [e.g., Edelstein (1971), Ogawa and Shulman (1972), Ogata and McConnell (1972a,b), Banerjee et al. (1973), Perutz et al. (1976), Inubushi et al. (1986), Nagai and Kitagawa (1980), and Scott et al. (1983)]. In light of the evidence indicating the inequivalence of the α and β chains in hemoglobin reactivity, the equivalence of the transient absorption spectra of the cyanomet-CO hybrids with their equilibrium difference spectra and with each other is surprising. One does, however, observe a difference in the geminate recombination kinetics of the cyanomet hybrids with NO. For example, the geminate recombination kinetics of HbNO, $\alpha_2^{NO}\beta_2^{+CN}$, $\alpha_2^{+CN}\beta_2^{NO}$, and MbNO can all be fit reasonably well to a double exponential with 14- and 300-ps components. What is significant is that the weight of the 14-ps component decreases in the order HbNO $\gtrsim \alpha_2^{NO}\beta_2^{+CN} > \alpha_2^{+CN}\beta_2^{NO} >$ MbNO. The geminate recombination kinetics of HbNO and $\alpha_2^{NO}\beta_2^{+CN}$ are nearly identical. On the other hand, the geminate recombination kinetics of $\alpha_2^{+CN}\beta_2^{NO}$ are intermediate between those of HbNO and MbNO. The order of this series is consistent with the above notion that the influence exerted by the β chains on the α chains is stronger than that of the α chains on the β chains.

Since the geminate recombination process of HbNO can be described by exponentials of 14 and 300 ps, a time scale that is too fast to include any quaternary structure change of the protein (Hofrichter et al., 1983), we must conclude that the effects exerted by the different subunits in our geminate recombination data lie at the level of the tertiary structure. It is also important to note that the HbNO result of an *apparent* biexponential geminate recombination cannot be given the simple interpretation of a different recombination rate for the α and the β chains since both of the cyanomet-NO hybrids as well as MbNO have geminte recombination kinetics that can be described by the same two time constants.

Conclusions

In this paper we have attempted to synthesize our data concerning the photophysics and reactivity of heme proteins with existing experimental and theoretical observations. Our results and conclusions are summarized in the following:

(1) We observe identical kinetics upon photodissociation of ligated heme compounds whether the excitation wavelength is 307 or 580 nm. The quantum yield of photodissociation in heme compounds is approximately unity whether the ligand is CO, O_2, or NO. The photodissociation yields measured for heme–O_2 and heme–NO compounds in the steady state or on long time scales can be attributed to a significant presence of Hb^*_{II} as well as to a rapid geminate recombination ($\sim$10 ps) in heme–NO compounds.

(2) Hb^*_I and Hb^*_{II} are formed in less than 50 fs and are attributed to excited states of the unligated heme. A consequence of eq 2 and 3 for the photochemistry of heme compounds is that Hb^*_I exists in at least an $S = 1$ spin state and is at least partly domed.

(3) The amount of stable, ground-state unligated heme protein that is formed upon photodissociation is inversely proportional to the population of the species Hb^*_{II}.

(4) The 2.5-ps decay of Hb^*_{II} arising from ground-state ligated hemes is attributed to a homogeneous distribution of ligands in the heme pocket that rapidly recombines with a *planar* heme.

(5) The 300-fs decay of Hb^*_I is accompanied by the appearance of a deoxy-like species, $Hb^\dagger$ (Martin et al., 1983a). $Hb^\dagger$ is considered to be an $S = 2$ species and at least partially domed. The distortion of the spectrum of $Hb^\dagger$ formed from photodissociated HbCO (and of photodissociated HbO_2) with respect to equilibrium unligated Hb is interpreted in terms of an increased interaction between the heme and its proximal environment and is consequently suggestive of a doming of the heme. We cannot, however, specify when the heme attains its fully domed configuration.

(6) The slow relaxation of the distortion of the transient absorption spectrum of the HbCO photoproduct is attributed to the constraint in the hemoglobin tetramer. This conclusion is borne out by the absence of such distortion with respect to the equilibrium difference spectra in PTHCO, MbCO, and the cyanomet-CO hybrids.

(7) The similarity of the bleaching kinetics of HbCO and PTHCO and of those of HbNO and PTHNO (Figures 2 and 3) indicate that at room temperature a significant contribution to the barrier to ligand rebinding is contributed by electronic factors such as the spin state of the ligand, as suggested by Cornelius et al. (1983).

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REFERENCES

Ansari, A., Berendzen, J., Browne, S. F., Frauenfelder, H., Iben, I. E. T., Sanke, T. E., Shyamsunder, E., & Young, R. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5000.

Antonini, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reactions with Ligands*, North-Holland, Amsterdam.

Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., & Gunsalus, C. (1975) *Biochemistry 14*, 5355.

Baldwin, J. M., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175.

Banerjee, R., Stetzkowski, F., & Henry, Y. (1973) *J. Mol. Biol. 73*, 455.

Bohn, B. (1979) Thesis, Université de Paris.

Brunori, M., Amiconi, G., Antonini, E., Wyman, J., & Winterhalter, K. H. (1970) *J. Mol. Biol. 49*, 461.

Bucci, E., & Fronticelli, C. (1965) *J. Biol. Chem. 240*, PC55.

Bücher, T., & Kaspers, J. (1947) *Biochim. Biophys. Acta 1*, 21.

Case, D. A., Huynh, B. H., & Karplus, M. (1979) *J. Am. Chem. Soc. 101*, 4433.

Chernoff, D. A., Hochstrasser, R. M., & Steele, W. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5606.

Cornelius, P. A., Steele, W. A., Chernoff, D. A., & Hochstrasser, R. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7526.

Cornelius, P. A., Hochstrasser, R. M., & Steele, W. A. (1983) *J. Mol. Biol. 163*, 119.

Dasgupta, S., Spiro, T. G., Johnson, C. K., Dalickas, G. A., & Hochstrasser, R. M. (1985) *Biochemistry 24*, 5295.

Eaton, W. A., Hanson, L. K., Stephans, P. J., Sutherland, J. C., & Dunn, J. B. R. (1978) *J. Am. Chem. Soc. 100*, 4991.

Edelstein, S. J. (1974) *Biochemistry 13*, 4998.

Findsen, E. W., Friedman, J. M., Ondrias, M. R., & Simon, S. R. (1985) *Science (Washington, D.C.) 229*, 661.

Friedman, J. M., & Lyons, K. B. (1980) *Nature (London) 284*, 570.

Friedman, J. M., Scott, T. W., Fisanick, G. J., Simon, S. R., Findsen, E. W., Ondrias, M. R., & Macdonald, V. W. (1985) *Science (Washington, D.C.) 229*, 187.

Gelin, B. R., & Karplus, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 801.

Gibson, Q. H., & Ainsworth, S. (1957) *Nature (London) 180*, 1416.

Gibson, Q. H., & Antonini, E. (1963) *J. Biol. Chem. 238*, 1384.

Greene, B. I., Hochstrasser, R. M., Weisman, R. B., & Eaton, W. A. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5255.

Hanson, J. C., & Schoenborn, B. P. (1981) *J. Mol. Biol. 153*, 117.

Henry, E. R., Sommer, J. H., Hofrichter, J., & Eaton, W. A. (1983) *J. Mol. Biol. 166*, 443.

Henry, E. R., Eaton, W. A., & Hochstrasser, R. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8982.

Hoffman, B. H., & Gibson, Q. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 21.

Hofrichter, J., Sommer, J. H., Henry, E. R., & Eaton, W. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2235.

Houde, D., Petrich, J. W., Rojas, O. L., Poyart, C., Antonetti, A., & Martin, J. L. (1986) in *Ultrafast Phenomena* (Fleming, G. R., & Siegman, A. E., Eds.) Vol. V, p 419, Springer, Berlin.

Inubushi, T., D'Ambrosio, C., Ikeda-Saito, M., & Yonetani, T. (1986) *J. Am. Chem. Soc. 108*, 3799.

Makinen, M. W., & Churg, A. K. (1983) in *Iron Porphyrins* (Lever, A. B. P., & Gray, H. B., Eds.) Vol. 1, p 141, Addison-Wesley, Reading, MA.

Martin, J. L., Migus, A., Poyart, C., Lecarpentier, Y., Astier, R., & Antonetti, A. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 173.

Martin, J. L., Migus, A., Poyart, C., Lecarpentier, Y., Astier, R., & Antonetti, A. (1983b) *EMBO J. 2*, 1815.

Martin, J. L., Migus, A., Poyart, C., Lecarpentier, Y., Astier, R., & Antonetti, A. (1984a) in *Ultrafast Phenomena* (Auston, D. H., & Eisenthal, K. B., Eds.) Vol. IV, p 447, Springer, Berlin.

Martin, J. L., Migus, A., Poyart, C., Lecarpentier, Y., & Antonetti, A. (1984b) in *Hemoglobin* (Schnek, A. G., & Paul, C., Eds.) p 173, University of Brussels, Brussels.

Martin, J. L., Breton, J., Hoff, A. J., Migus, A., & Antonetti, A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 957.

Nagai, K. (1977) *J. Mol. Biol. 111*, 41.

Nagai, K., & Kitagawa, T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2033.

Noble, R. W., Brunori, M., Wyman, J., & Antonini, E. (1967) *Biochemistry 6*, 1216.

Ogata, R. T., & McConnell, H. M. (1972a) *Proc. Natl. Acad. Sci. U.S.A. 69*, 335.

Ogata, R. T., & McConnell, H. M. (1972b) *Biochemistry 11*, 4792.

Olafson, B. D., & Goddard, W. A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1315.

Perutz, M. F. (1979) *Annu. Rev. Biochem. 48*, 327.
Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A., & Simon, S. R. (1976) *Biochemistry 15*, 378.
Petrich, J. W., Martin, J. L., Houde, D., Poyart, C., & Orszag, A. (1987) *Biochemistry 26*, 7914.
Reynolds, A. W., Rand, S. D., & Rentzepis, P. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2292.
Rousseau, D. L., & Argade, P. V. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1310.
Saffran, W. A., & Gibson, Q. H. (1977) *J. Biol. Chem. 252*, 7955.
Schomaker, K. T., Bangcharoenpaurpong, O., & Champion, P. M. (1984) *J. Chem. Phys. 80*, 4701.
Scott, T. W., Friedman, J. M., Ikeda-Saito, M., & Yonetani, T. (1983) *FEBS Lett. 158*, 68.
Shank, C. V., Ippen, E. P., & Bersohn, R. (1976) *Science (Washington, D.C.) 193*, 50.
Szabo, A., & Karplus, M. (1972) *J. Mol. Biol. 72*, 163.
Szabo, A., & Karplus, M. (1975) *Biochemistry 14*, 931.
Terner, J., Stong, J. D., Spiro, T. G., Nagumo, M., Nicol, M. F., & El-Sayed, M. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1313.
Van Assendelft, O. W. (1970) *Spectrophotometry of Hemoglobin Derivatives*, Royal Vangorcum, Netherlands.
Waleh, A., & Loew, G. H. (1982a) *J. Am. Chem. Soc. 104*, 2346.
Waleh, A., & Loew, G. H. (1982b) *J. Am. Chem. Soc. 104*, 2352.
Wang, C. M., & Brinigar, W. S. (1979) *Biochemistry 18*, 4960.

Ruthenium–Iron Hybrid Hemoglobins as a Model for Partially Liganded Hemoglobin: NMR Studies of Their Tertiary and Quaternary Structures†

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ABSTRACT: Diruthenium-substituted Ru–Fe hybrid hemoglobins (Hb) were synthesized by heme substitution from protoheme to ruthenium(II) carbonyldeuteroporphyrin in the α or β subunits. As the carbon monoxide coordinated to ruthenium(II) is not released under physiological conditions, deoxygenated Ru–Fe hybrid derivatives [$\alpha(Fe)_2\beta(Ru–CO)_2$ and $\alpha(Ru–CO)_2\beta(Fe)_2$] can serve as models for half-liganded Hbs. On the basis of proton NMR spectra of hyperfine-shifted proton resonances, these Ru–Fe hybrid Hbs have only small structural changes in the heme environment of the partner subunits at low pH. The proton NMR spectra of the intersubunit hydrogen-bonded protons also showed that the quaternary structures of the two complementary hybrids both remain in the "T-like state" at low pH, suggesting that the T to R structural conversion is induced by ligation of the third ligand molecule. Marked conformational changes in the heme vicinity are observed at high pH only for $\alpha(Ru–CO)_2\beta(Fe)_2$, and its quaternary structure is converted into the "R state"; the $\alpha(Fe)_2\beta(Ru–CO)_2$ hybrid does not undergo this change. This implies that the free-energy difference between the two quaternary states is smaller in the α-liganded hybrid than in the β-liganded one.

Hemoglobin (Hb) has long served as a paradigm for cooperative ligand binding in proteins (Antonini at al., 1971; Edelstein, 1975; Dickerson & Geis, 1983). On the basis of numerous experimental studies, particularly X-ray crystallography (Perutz, 1976, 1979) and nuclear magnetic resonance (NMR) spectroscopy (Ho et al., 1975; Shulman et al., 1975), a number of specific mechanisms to account for the cooperativity have been proposed (Monod et al., 1965; Koshland et al., 1965; Gelin & Karplus, 1977; Warshel, 1977; Perutz, 1976). However, detailed understanding of the control mechanism of ligand affinity in Hb may well be achieved only by analyzing the tertiary and quaternary structures and functional properties of the protein as a function of the degree of ligation. Therefore, the most uncompromising problem that we encounter in studying the quaternary and tertiary structural change induced by ligation is physical and chemical characterizations of Hb species at the intermediate state of ligation, which have been very elusive owing to the difficulty in isolating such species.

To gain an insight into structural characterizations of the partially liganded Hb, the half-liganded Hbs have been studied by utilizing valency hybrid Hb (Ogawa & Schulman, 1972), metal hybrid Hb (Inubushi et al., 1983, 1986; Blough et al., 1980, 1982; Simolo et al., 1985; Shibayama et al., 1987), and intersubunit cross-linked Hb (Miura & Ho, 1982, 1984). However, there has not been an ideal model which is stable enough to study the structure of partially liganded Hb in detail, which would be requisite in characterizing the effect of partial ligation on the properties of individual subunits and the tetrameric Hb molecule as well. Ogawa and Shulman (1972) used $Fe^{III}–CN^-$ heme as a structural model for an oxyheme, but it was very difficult to deoxygenate the Fe(III)–Fe(II) hybrids due to autoxidation. In this sence, iron–ruthenium symmetric hybrid Hbs in which the α or β subunit contains Ru^{II}–CO porphyrin, with the other subunit having a deoxygenated iron(II) porphyrin, could be ideal models for testing the properties of the intermediate ligand binding state. Ru^{II}–CO porphyrin has the following unique properties. (1)

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