FAST REACTIONS IN CARBON MONOXIDE BINDING TO HEME PROTEINS

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ABSTRACT Using fast flash photolysis, we have measured the binding of CO to carboxymethylated cytochrome *c* and to heme *c* octapeptide as a function of temperature (5°–350°K) over an extended time range (100 ns–1 ks). Experiments used a microsecond dye laser (λ = 540 nm), and a mode-locked frequency-doubled Nd-glass laser (λ = 530 nm). At low temperatures (5°–120°K) the rebinding exhibits two components. The slower component (I) is nonexponential in time and has an optical spectrum corresponding to rebinding from an S = 2, CO-free deoxy state. The fast component (I*) is exponential in time with a lifetime shorter than 10 μs and an optical spectrum different from the slow component. In myoglobin and the separated α and β chains of hemoglobin, only process I is visible. The optical absorption spectrum of I* and its time dependence suggest that it may correspond to recombination from an excited state in which the iron has not yet moved out of the heme plane. The temperature dependences of both processes have been measured. Both occur via quantum mechanical tunneling at the lowest temperatures and via over-the-barrier motion at higher temperatures.

INTRODUCTION

The heme group, a planar organic molecule with a central iron atom, plays a crucial role as active center in biomolecules performing functions ranging from oxygen storage to redox reactions and catalysis. Since most heme proteins contain the same prosthetic group, iron protoporphyrin IX, its reactivity must be controlled by the structure of the protein. To explore the relation between structure and function, we studied the kinetics of ligand binding in heme proteins with flash photolysis over wide ranges in time and temperature. In previous papers we have shown that the overall features of the binding of CO to myoglobin (1), the isolated heme group (2), and the separated hemoglobin chains (3) possess striking similarities. Access to the active center is governed by sequential potential barriers. At sufficiently high temperatures, typically above 300°K, essentially all ligands initially bound at the heme iron overcome these

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barriers after photodissociation and move into the solvent. All ligands in the solvent then compete for the vacant binding site, and the corresponding binding process, called IV, is exponential in time and proportional to the CO concentration in the solvent. At low temperatures, typically below 200°K, the photodissociated CO cannot leave the protein and binding occurs from the immediate vicinity of the heme iron, and is seen as an intramolecular process, called I. The time dependence of I is approximately a power law. We have explained the nonexponential binding by postulating that the activation enthalpy and entropy of the innermost barrier are not sharp, but distributed (1–2). At temperatures below about 30°K, process I does not satisfy an Arrhenius relation, but proceeds through quantum-mechanical tunneling (2–4).

In the present paper, we extend our studies to carboxymethylated cytochrome *c* and heme *c* octapeptide. The features first discovered in globins can again be seen, but additional ones are found, such as a new fast binding process at low temperatures. While all processes observed in the globins can be explained satisfactorily with a simple model, we have not yet been able to establish a simple and unambiguous picture that includes the new results. The present work therefore is written to present the new data and stimulate additional investigations.

EXPERIMENTAL

Cytochrome *c*, a heme protein of mol wt 12,400, is involved in electron transport and does not normally bind CO. The heme group is covalently linked to the protein and the iron is bound to the two axial ligands His^{18} and Met^{80} in addition to the four pyrrole nitrogens of the heme group. The protein can, however, be modified to have the sixth, axial, position available for CO binding. One such modification is the carboxymethylation of Met^{80}, the product of which will be referred to as cm cyt *c* (5). A more extensive modification is tne enzymatic digest of cytochrome *c* by pepsin and trypsin to the heme *c* octapeptide (heme peptide) (6). In the latter, His^{18} is retained as the fifth, axial ligand while the sixth position is free. Horse heart cytochrome *c* (type III) was purchased from Sigma Chemical Co., St. Louis, Mo., and used without further purification. Cm cyt *c* was prepared by the method of Schejter and Aviram (5), which results in carboxymethylation of both methionine residues (at positions 65 and 80) of cytochrome *c*. Heme peptide was prepared from the pepsin-trypsin digest of horse heart cyt *c* (6). Experiments were performed in ethylene-glycol-phosphate pH 7 buffer solutions (3:2 vol/vol) for cm cyt *c* and glycerol-phosphate pH 7 buffer solutions (3:1 vol/vol) for heme peptide. The solutions were saturated with 1 atm CO gas and the heme concentrations $\simeq 200\ \mu M$. The final buffer concentrations were 50 mM for cm cyt *c* and 10 mM for heme peptide. The optical path length of the sample is 0.5 mm.

We investigated the binding of carbon monoxide (CO) to cm cyt *c* and to heme peptide with flash photolysis. Consider the heme with CO bound at the iron. The bond between Fe and CO was broken by a light pulse and the subsequent rebinding of CO to the heme followed by monitoring the optical absorbance, $A(t)$, at various wavelengths. The slower components were studied by initiating photodissociation with a 0.1J coumarin 6 dye laser with pulse width 1 μs and wavelength 540 nm. Rebinding was monitored from 2 μs to 1 ks with a transient analyzer with logarithmic time base (7), and below 10 μs also with a storage scope. The fast component was also explored with 20-ps, 530-nm pulses from a frequency-doubled mode-locked Nd-glass laser.

RESULTS

Fig. 1 shows the rebinding of CO to cm cyt *c* and heme peptide after photodissociation by 540 nm light. For comparison, myoglobin (Mb) is also included. The binding is described by plotting the change in absorbance, $\Delta A(t) = A(t) - A(t < 0)$, versus $\log t$. Here, $A(t < 0)$ is the absorbance of the sample before and $A(t)$ the absorbance

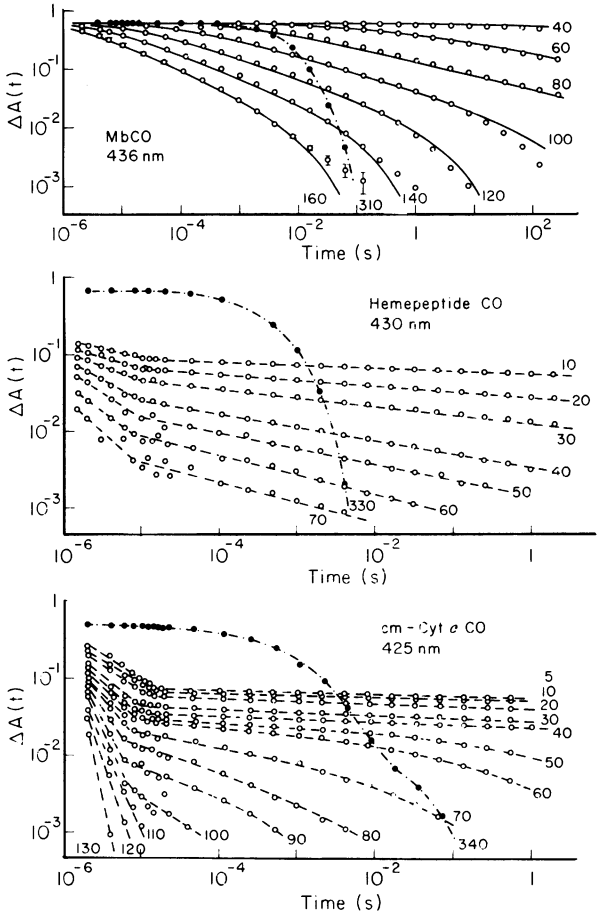

FIGURE 1 Rebinding of CO to myoglobin (Mb), heme peptide, and carboxymethylated cytochrome *c* (cm cyt *c*) after photodissociation by a microsecond laser flash at temperatures below 160°K. For Mb and heme peptide the solvent is glycerol-phosphate buffer, pH 7, (3:1, vol/vol). For carboxymethylated cytochrome *c* the solvent is ethylene glycol-phosphate buffer, pH 7, (3:2, vol/vol). The monitoring wavelengths are indicated in the figure. For heme peptide and carboxymethylated cytochrome *c*, processes I and I* are observed. In Mb only process I is seen. The dashed lines are drawn to guide the eye. The solid curves for Mb are a theoretical fit obtained with a temperature-independent enthalpy spectrum. The high-temperature limits of rebinding are given by the solid circles. For Mb the absorbance changes at high and low temperatures agree. Numbers at ends of lines represent degrees Kelvin.

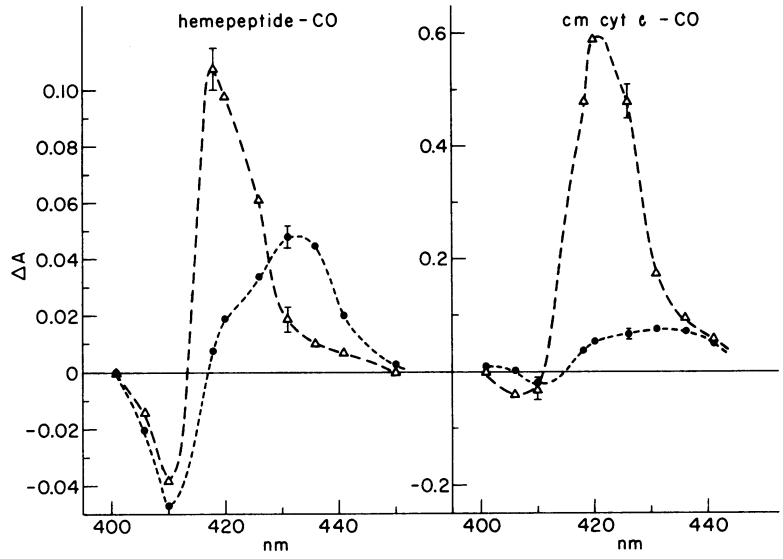

FIGURE 2 Soret spectral changes of processes I* (Δ) and I(•) at 40°K for heme peptide and carboxymethylated cytochrome *c*. If I follows a power-law behavior to microsecond times, the change in absorbance for I, ΔA_I, can be extrapolated and subtracted from the total absorbance to give ΔA_{I^*} for I*. For heme peptide the absorbance changes are calculated at $t = 2$ μs, for cm cyt *c* at $t \approx 0.5$ μs. The optical interference filters for the monitoring light have a full width at half-maximum of 7 nm.

at time *t* after photodissociation. The data in Fig. 1 exhibit pronounced similarities and some characteristic differences among the three cases. At high temperatures, Mb and heme peptide show a single prominent process (IV), which is exponential in time and proportional to the CO concentration. Cm cyt *c* has two pH-dependent components corresponding to rebinding to low-spin and high-spin ferrous cm cyt *c* (8, 9). At low temperatures, all three show a process (I) that is nonexponential in time, but in addition cm cyt *c* and heme peptide display a fast process that we call I* (one-star). While for Mb the maximal absorption change is the same for processes I and IV, in cm cyt *c* and heme peptide I is much less intense than IV.

Fig. 2 gives the absorbance change for processes I and I* for cm cyt *c* and heme peptide at 40°K as a function of wavelength. The optical difference spectra for I and I* are clearly different.

The time dependence of I* is obtained by subtracting the extrapolated contribution of I from the total absorbance change. The resulting absorbance changes for cm cyt *c* at several temperatures are plotted versus time in Fig. 3. Within errors, process I* is exponential in time, $\Delta A_{I^*}(t) = \Delta A_{I^*}(0) \exp(-k^* t)$ and $\Delta A_{I^*}(0)$ temperature-independent below about 130°K. In contrast to I*, process I approximately follows a power law in time, $\Delta A_I(t) \approx \Delta A_I(0)(1 + t/t_0)^{-n}$, where t_0 and n are temperature-dependent parameters. I* and I thus have very different time dependences.

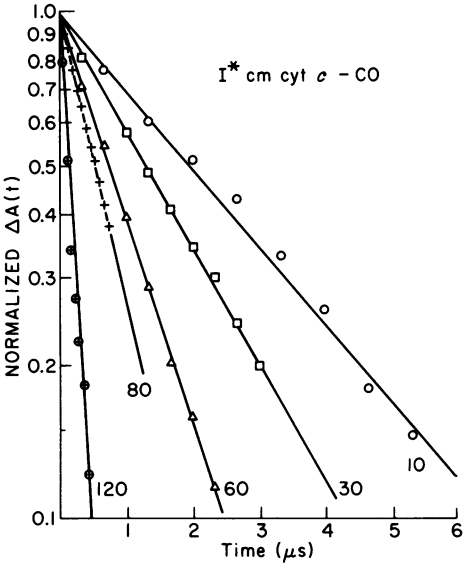

FIGURE 3

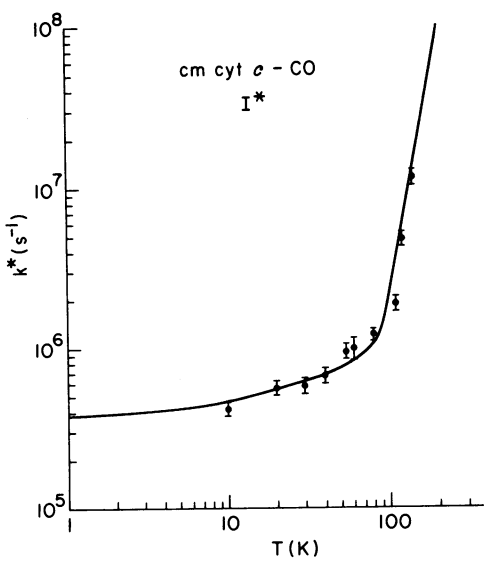

FIGURE 4

FIGURE 3 The absorbance change due to I* for cm cyt *c* plotted as a function of time. Photodissociation is induced by a 20 ps, 530 nm pulse from a frequency-doubled mode-locked Nd-glass laser. Monitoring wavelength is 425 nm. Because of pulse-to-pulse energy variation of the laser, the different temperatures have been normalized to the same change in absorbance at $t = 0$. Data taken with the microsecond dye laser at a constant energy indicate that the initial absorbance change due to I* is indeed independent of temperature below 130°K. Numbers at ends of lines represent degrees Kelvin.

FIGURE 4 Rate parameter k^* for rebinding of CO via process I* for cm cyt *c* as a function of temperature. The solid line is a fit to $k^* = A + BT^{\alpha} + Ce^{-E/k_BT}$, which yields $A = 3.8 \times 10^5\ s^{-1}$, $B = 4.8 \times 10^3\ s^{-1}\,°K^{-\alpha}$, $C = 7.8 \times 10^9\ s^{-1}$, $E = 7.5$ kJ/mol, $\alpha = 1.1$. The data shown here have been obtained with the Nd-glass laser. Corresponding experiments with the dye laser (Fig. 1) give similar results and in particular show that k^* is temperature-independent below 10°K.

The temperature dependence of the rate parameter k^* for I* in cm cyt *c* is given in Fig. 4. The rate is approximately temperature-independent up to about 20°K, varies approximately linearly with temperature up to 100°K, and then appears to become exponential in $1/T$. Since process I is not exponential in time, it cannot be characterized fully by one rate parameter. The general features of the temperature dependence of I can be obtained by considering $k_{0.75} = 1/t_{0.75}$, where $t_{0.75}$ is the time at which $\Delta A(t)$ drops to 0.75 $\Delta A(0)$. The parameter $k_{0.75}$ depends only weakly on temperature up to about 40°K and then becomes exponential. Up to at least 100°K, $k_{0.75}$ is much smaller than k^*. The rates and temperature dependences of processes I and I* thus are very different.

I and I* also differ in their dependence on the energy of the laser pulse. In Fig. 5 are plotted ΔA_I and ΔA_{I^*} for cm cyt *c* versus the energy of the laser flash. Both de-

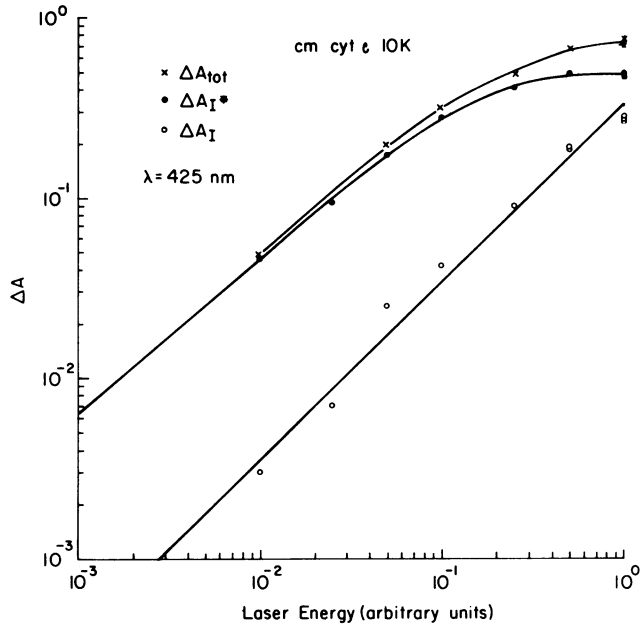

FIGURE 5 Plot of the total change in absorbance ΔA_{tot} and the absorbance changes ΔA_I and ΔA_{I^*} due to I and I* at a monitoring wavelength of 425 nm as a function of microsecond laser energy. The laser energy is varied by inserting neutral density filters in the laser beam. The experiment is performed at $T = 10°K$ with cm cyt *c*. I* saturates at a lower laser energy than I.

pend linearly on laser energy for low relative laser energies, but saturate at different values of laser energy. In the range of laser energies obtained in this experiment, the saturation of I* is observed, whereas I is not yet saturated.

The evidence presented so far indicates that two different processes, I and I*, occur in the low-temperature rebinding of CO to cm cyt *c* and heme peptide. Do both processes coexist and compete in the same system or do two classes of biomolecules exist, one with I and the other with I*? To decide between these alternatives, we have performed two experiments. In the first, we repeated photodissociation at time intervals such that all recombination via I* had taken place before the next flash. In the first alternative, such multiple flashing pumps CO into the state corresponding to process I; the intensity of I* should decrease, that of I increase with each successive flash. In the second alternative, no pumping occurs. Experiments on both heme peptide and cm cyt *c* show that the intensity of I* decreases and I increases with multiple flashes hence favoring the first alternative. To quantitate these results, a second experiment was performed on cm cyt *c* at 10°K. First the sample, monitored with a very weak light, was photodissociated with a flash from the dye laser. The very weak monitoring light causes no photodissociation and the absorbance of the sample before the flash is characteristic of the CO bound species. The absorbance changes of I and I* resulting from the flash are measured. Next the sample was allowed to come to equi-

librium with a bright monitoring light and its absorbance recorded. Since the strong light photodissociates some of the cm cyt *c*-CO systems, the absorbance of the sample will move towards the value characteristic of the ligand-free protein. The sample was then exposed to a laser flash and the absorbance changes ΔA_I and ΔA_{I^*} were measured. This procedure was repeated for various monitoring light levels. ΔA_I and ΔA_{I^*} are plotted versus the value of the absorbance of the sample before the flash, A, in Fig. 6. Before the flash, the biomolecules are either in the CO-bound state or in the long-lived state giving rise to process I. The monitoring light is not intense enough to populate a state as short-lived as the one producing process I*. Hence if I and I* occur in separate biomolecules, we would expect ΔA_{I^*} to be independent of the monitoring light for the light levels used. Fig. 6 shows that ΔA_{I^*} decreases with increasing monitoring light intensity. If I and I* occur in the same biomolecule and are created from a common state by the laser flash we would expect the ratio $\Delta A_{I^*}/\Delta A_I$ to be independent of monitoring light. Fig. 6 shows that both ΔA_{I^*} and ΔA_I do decrease with increasing monitoring light levels, but not at the same rate. Thus processes I and I* must occur in the same biomolecule in at least a fraction of the sample.

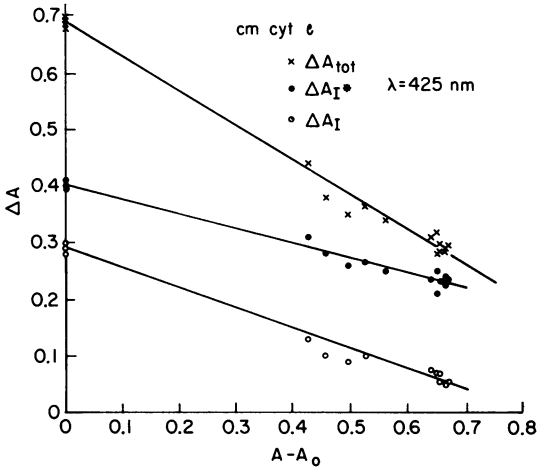

FIGURE 6

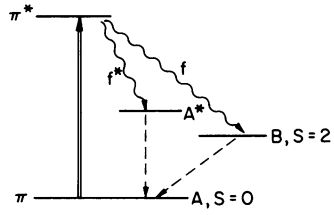

FIGURE 7

FIGURE 6 The total change in absorbance ΔA_{tot} and the absorbance change ΔA_I and ΔA_{I^*} due to I and I* resulting from a microsecond laser flash plotted versus $A - A_0$. Here, A is the absorbance of the sample before the flash and A_0 the absorbance before any photodissociation at all has occurred. The experiments are performed at $T = 10°K$ and a monitoring wavelength of 425 nm. Different values for A were obtained by equilibrating the sample with different intensities of light. Experimental details are given in the text. Note that $\Delta A_{I^*}/\Delta A_I$ is not independent of A. The solid lines drawn through the data indicate that ΔA_{I^*} and ΔA_I do not vanish for the same value of A.

FIGURE 7 States involved in the photodissociation of cm cyt *c*-CO and heme peptide-CO. The double lines indicate transitions involving radiation, the wavy lines radiationless transitions, and the dashed lines CO. The proposed mechanism is described in the text.

INTERPRETATION OF PROCESSES I AND I*

In previous papers (1–3), we have interpreted the kinetic data for the binding of CO to Mb, protoheme, and the separated hemoglobin chains in terms of a simple model. In particular, process I is assumed to represent direct rebinding after photodissociation and process IV the case where CO leaves the protein and moves into the solvent. Here, we will not be concerned with IV, but only with the intramolecular processes I and I*. Is it possible to construct a model in which both I and I* can be explained in a natural way? We have not found a model that explains all observed facts simply and convincingly, but we will present one that may be a good starting point for further improvements. We assume that two different types of molecules exist; in the first type, both I and I* coexist and compete, in the second, only process I takes place. We will restrict our attention to the first type because it is here where new features arise.

The observation of two processes with different optical spectra in the same protein implies the existence of three states. We denote the states, shown in Fig. 7, by A, A*, and B. The existence and properties of A and B are well established through X-ray, optical, and Mössbauer experiments. In A, CO is bound at the heme iron which is in a spin $S = 0$ ($d_{xy}^2 d_{\pi}^4$) state and lies in the heme (xy) plane. In B ("deoxy"), the ligand has moved away, the iron has changed to $S = 2(d_{xy}^2, d_{\pi}^2, d_{z^2}^1, d_{x^2-y^2}^1)$ and in most systems moved out of the heme plane by about 0.05 nm. In A and B, the electronic structure of the heme ring is in its ground state, denoted by π. The state A* is required to account for I*. The experimental results can now be interpreted as follows: Initially, the system is in the ground state A. The laser flash excites the electrons of the heme. At a laser wavelength of about 540 nm the excitation causes the promotion of an electron from the highest filled porphyrin π orbital to the lowest empty porphyrin π orbital ($\pi \rightarrow \pi^*$). Photoexcitation is followed by rapid radiationless transitions, leading to state B and to the transient state A* with probabilities f and f^*, respectively. Binding from A* produces process I*, from B process I.

The states and transitions in Fig. 7 can be interpreted in terms of the molecular orbital calculations of Zerner et al. (10). According to these authors, the transition $d_{\pi} \rightarrow d_{z^2}$ provides a plausible pathway for photodissociation. We thus tentatively identify state A* with the iron electronic configuration d_{xy}^2, d_{π}^3, $d_{z^2}^1$ which, by Hund's rule, has $S = 1$. The d_{π} in state A contributes to the bonding of CO to Fe, while the d_{z^2} in A* is antibonding. CO is thus less tightly bound in A* than in A and can dissociate. In A*, the orbital $d_{x^2-y^2}$ is empty; since it is occupation of this orbital that makes the out-of-plane position favorable for the iron, Fe will very likely still be in the heme plane in the $S = 1$ state. The CO can then rebind quickly via process I*. Some support for the assignment comes from the optical spectra, Fig. 2. For heme peptide the difference spectrum for I agrees with the difference spectrum calculated from the separately measured carbonmonoxy and high spin-deoxy states. For cm cyt c, the difference spectrum for I peaks at the same wavelengths as the separately measured carbonmonoxy and high spin-deoxy states, but is much too broad. The broadness of

this spectrum, may, in part, be due to the existence of molecules with only a process I rebinding. The spectrum for I* in both heme peptide and cm cyt *c* resembles the difference spectrum between CO-bound and the low spin-deoxy conformations of the proteins.

Photodissociation, in the proposed model, involves a number of steps: first, excitation $\pi \rightarrow \pi^*$; second, radiationless transfer of energy from π^* to Fe; and third, dissociation of the CO. Details of the second and third steps are not yet fully understood. In the model of Zerner et al., the second step involves the transition $d_\pi \rightarrow d_{z^2}$, but recent calculations[1] and measurements (11) show that the $d_\pi \rightarrow d_{z^2}$ transition occurs at an energy at least an electron volt higher than the $\pi \rightarrow \pi^*$ transition at 540 nm. It is, however, still possible for the $\pi \rightarrow \pi^*$ excitation to couple to $d_\pi \rightarrow d_{z^2}$ via radiationless transfer of energy. It has also been suggested recently that the $\pi \rightarrow \pi^*$ transition itself directly leads to dissociation (12). In any case, in the scheme of Fig. 7, two different pathways are involved, one leading to A*, the other to B.

PROBLEMS

The results obtained so far with cm cyt *c* and heme peptide raise a number of questions that will require extension of the experiments to better samples, lower temperatures, shorter times, higher accuracy, and other systems, and will also call for other techniques. We briefly outline some of the open problems here.

How are I and I* related? In Fig. 7, we have proposed a simple scheme for the rebinding processes in cm cyt *c* and heme peptide. Is this scheme correct? Do transitions A* → B and B → A* exist? Another problem emerges from Fig. 1. While I is much slower than I* at low temperatures, it speeds up more quickly with increasing temperature. Will it overtake I* or will the two processes merge? Is the return B → A a one-step transition or is an intermediate state, which may be A*, involved?

Is the explanation invoking two types of biomolecules, one with I* and I, the other with I only, correct? Can the experimental data be explained with an alternate model, based on only one type, but with additional transitions among the various states?

At low temperatures, only a fraction of heme peptide or cm cyt *c* molecules move to the deoxy state B after photodissociation. At high temperatures, above about 340°K, all rebinding occurs via the solvent and in our model via state B. How and at which temperature does the switch from the predominant direct rebinding A* → A to the indirect sequence B → A occur? One possibility invokes the transition A* → B at higher temperatures; another that motion to B becomes dominant when the molecule can easily relax and change conformation.

In Mb and the separated α and β chains of hemoglobin, we have not observed process I* (1, 3). Why is I* absent, or very small, in these proteins? One explanation

[1]Eisenstein, L., D. R. Franceschetti, and K. L. Yip. Iterative extended Hückel studies of some pyridine-Fe(II)-porphin complexes. In preparation.

invokes the different anchorage of the proximal histidine that connects the iron to the protein backbone. In the globins, the histidine is connected to the F helix which may exert some tension on the iron and pull it out of the heme plane after photodissociation. In cm cyt *c* and heme peptide, the protein backbone is linked covalently to the heme and may thus hold the iron more tightly in the heme plane.

What are the properties of A*? In particular, what is its spin? Hopefully, fast susceptibility or pulsed Mössbauer experiments can provide more information.

It is interesting to speculate on why process I* has an exponential time dependence, whereas I is nonexponential. We attribute the nonexponential time dependence of process I to different conformational states of the protein, each with a different activation enthalpy for rebinding of CO via I. The different conformational states would then have the same value for the activation enthalpy for rebinding via process I*. According to our interpretation of the nature of processes I and I*, the iron $d_{x^2-y^2}$ orbital should then be more influenced by the protein state than the d_{z^2} orbital.

Fig. 1 shows that in cm cyt *c* the rebinding curve for I at 30°K is nearly parallel to the one at 5°K, but 5°K is shifted by about a factor of two towards larger values of ΔA. We have observed this behavior in various samples. If correct, it would indicate that the transition $\pi^* \rightarrow$ B becomes more favorable at very low temperatures.

Tunneling extends to remarkably high temperatures in process I*. The component proportional to T is measurable to about 120°K. It is possible that a component proportional to a higher power of T follows before the Arrhenius regime is reached. Why does tunneling extend to such temperatures?

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DISCUSSION

BRILL: We would like to comment, first, upon the inner barrier energy distributions found to be a general property of heme proteins and heme derivatives (1, 2); second, on a possible relation between level broadening seen in frozen solutions and the activation energy of electron transfer reactions; and third, on the use of glass-forming solvent systems.

Analysis of electron paramagnetic resonance (EPR) orientation studies of ferric hemoglobin single crystals provides orientation disorder angles and hyperfine component line widths.[1] Agreement of the latter with the predictions of a dipolar broadening computation (based upon the three-dimensional structure of horse ferric hemoglobin) suggests that the various contributions to the line width in the crystals are known quantitatively within a small range of uncertainty.[2] Extension of these considerations to frozen solutions leads one to expect significant narrowing of the EPR lines, while the data show a broadening. On the assumption that the latter arises from energy level broadening associated with a distribution in structures, we have analyzed the frozen solution EPR spectra of many ferric heme-protein complexes in terms of Gaussian distributions in the spin-Hamiltonian parameters.[3] The internal consistency of the derived data suggests that this is the appropriate explanation of the phenomenon. The four parameters involved in fitting the spectra are the rhombic-to-axial symmetry ratio E/D and its root mean square (rms) deviation $\sigma_{E/D}$, the coefficient η for the admixture of quartet states (related to iron out-of-planarity) and σ_η. We view $\sigma_{E/D}$ and σ_η as reflecting conformational modes, with amplitudes given by the equipartition principle at the temperature at which they are frozen into metastable states (minima of secondary potentials which modulate the primary, approximately harmonic, potentials) (3). There is little leeway in specifying the four parameters; departures of 5% in E/D, 10% in $\sigma_{E/D}$, 4% in η, and 20% in σ_η significantly alter the quality of the fit. Likewise, the constant line width below 50°K in the frozen solution EPR spectra from low-spin ferric cytochrome-*c* has been attributed to a ±6% spread in rhombic potential (4). Distribution of spin-Hamiltonian parameters is not unique to heme proteins; this phenomenon has also been observed in iron-sulfur proteins (5) and copper blue proteins (6).

Information of this kind is related to protein reactivity. In the case of the cupric site in blue proteins, we have proposed that the EPR spectral distribution can be used to obtain the force

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[1]Hampton, D. A., and A. S. Brill. 1978. Crystalline state disorder and hyperfine component line widths in ferric hemoglobin chains. To be published.

[2]Brill, A. S., and D. A. Hampton. 1978. Quantitative evaluation of contributions to electron paramagnetic resonance line widths in ferric hemoglobin single crystals. To be published.

[3]Brill, A. S., F. G. Fiamingo, and D. A. Hampton. 1978. Characterization of ferric energy levels in alcohol complexes of myoglobin and hemoglobin. To be published.