Geminate Recombination of Carbon Monoxide Complexes of Hemes and Heme Proteins

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Abstract: Picosecond kinetic studies of complexes of protoheme (PH), protoheme dimethyl ester (PHDME), and chelated protoheme (MCPH) are reported. The photolysis of carbon monoxide (CO) from complexes BHmCO where B is 1-methylimidazole, 1,2-dimethylimidazole, methanol, and 2-propanol all displayed no observable CO return on picosecond time scales in common nonviscous solvents. Myoglobin–CO and chelated protoheme–CO gave similar results. At high viscosity in a glycerol solution, photolysis of 1-methylimidazole HmCO resulted in partial carbon monoxide return to the bound state with a rate constant of 10^9 s^{-1}. These results suggest that the bond-making step in the reaction of CO with five-coordinated hemes and heme proteins has a rate constant around 10^9 s^{-1} compared to $>10^{10}$ s^{-1} for other common ligands. Thus carbon monoxide reactions are the only ones studied which are not diffusion-controlled in normal solvents.

Introduction

Oxygen transport in biological systems and the related kinetics and equilibria of binding dioxygen and other ligands to heme proteins continues to receive much attention.[1-7] Accurately biomimetic model compounds have been prepared, and their reactions with dioxygen, carbon monoxide and several other ligands studied in detail.[1,8,9] Not only have the kinetics, equilibria, and thermodynamics of dioxygen and carbon monoxide binding to R state hemoglobin been duplicated,[10] but the wide variations in these binding equilibria among different heme proteins have also been mimicked through structural variations.[8,11] It is now possible to synthesize and characterize model compounds having wide ranges in both binding constants and in the ratio of the binding constants for carbon monoxide and dioxygen. This has helped to clarify the control of dioxygen affinities in heme proteins.

Those studies on biomimetic models were concerned with overall second order association rate constants, overall first-order dissociation rate constants, and equilibria, as shown in eqs 1 and 2.

$$\mathrm{BHm} + \mathrm{O_2} \underset{k_B^{O_2}}{\overset{k_B^{O_2}}{\rightleftharpoons}} \mathrm{BHmO_2} \qquad (1)$$

$$\mathrm{BHm} + \mathrm{CO} \underset{k_B^{CO}}{\overset{k_B^{CO}}{\rightleftharpoons}} \mathrm{BHmCO} \qquad (2)$$

Meanwhile, other studies of reactions of heme proteins at low temperatures[12] and on very fast time scales[4,5,13-22] showed that it is possible to distinguish multiple fast processes along the pathways of reactions 1 and 2 in proteins, the elucidation of which should provide further understanding of these important reactions. It appears that the reactions of, for example, myoglobin with ligands such as dioxygen or methyl isocyanide occur through a process at least as complex as the four state system of eq 3.[12,18]

$$\underset{\text{bound state}}{\mathrm{MbL}} \overset{h\nu}{\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}} \underset{\text{contact pair}}{[\mathrm{Mb\ L}]} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{protein separated pair}}{[\mathrm{M\|L}]} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underset{\text{separate species}}{\mathrm{Mb} + \mathrm{L}} \qquad (3)$$

Because of such advances in characterizing proteins, we considered it important to subject our biomimetic models to similar analysis and to characterize their fast processes by the same structure, solvent, and ligand variations which we have previously applied to the overall processes.[9,11] In a previous step toward that goal, we showed that the biomimetic model systems chelated protoheme (or the 1-methylimidazole–protoheme complex) and the protein myoglobin have very similar picosecond dynamics after

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Table I

$$BHmO_2 \underset{k^{O_2}_{O_2}}{\overset{k^{-O_2}}{\rightleftharpoons}} BHm \underset{k^{-CO}}{\overset{k^{CO}_{CO}}{\rightleftharpoons}} BHmCO$$

	k^{CO}	k^{-CO}	k^{O_2}	k^{-O_2}	K^{CO}	K^{O_2}	ΔH^{CO}	ΔH^{O_2}
chelated protoheme[a]	3.6×10^6	0.009	2.6×10^7	47	4×10^8	5.5×10^5	−17.5	−14
R state hemoglobin[b]	6×10^6	0.009	6×10^7	17	7×10^8	4×10^6	−17.4	−14

[a] In aqueous cetyltrimethylammonium bromide 20 °C.[9,10] Units for k^-, k, K, and ΔH are s^{-1}, $M^{-1}\,s^{-1}$, M^{-1}, and Kcal/mol, respectively. [b] Isolated α chains have kinetic parameters which match those of chelated protoheme even better than those shown here.[9]

photolysis of their isocyanide complexes.[18,19] The model compounds were adequately described by reaction 4, while the proteins required at least eq 3. The fastest steps, k_2 and k_{-1}, of (3) and (4) appear to be quite similar.

$$BHmL \overset{h\nu}{\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}} [BHm\,L] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} BHm + L \qquad (4)$$

We were not able to observe picosecond or nanosecond recombination for complexes of CO with either myoglobin or our model compounds.[29] However, by assuming that carbon monoxide has diffusion characteristics like dioxygen or methyl isocyanide, that is, the same values of k_2 and k_{-2}, we could use eq 4 and the overall carbon monoxide association rate constant ($k_B^{CO} = 10^7\ M^{-1}\ s^{-1}$)[9] to estimate $k_{-1} = 10^9\ s^{-1}$ by way of (5).[19]

$$k_B^{CO} = \frac{k_{-2}k_{-1}}{k_{-1} + k_2} \qquad (5)$$

Other studies of CO rebinding to iron porphyrins have indicated nanosecond to picosecond rebinding to protoheme in viscous glycerol solutions. These studies were usually carried out in aqueous glycerol solutions containing sodium hydroxide. While some of the data indicate rebinding geminate rates similar to that calculated above, others do not.

Because protoheme in these systems is a dianion and the fifth ligand is not known (carboxylate, water, glycerol, hydroxide, etc.) and the model system lacks the proximal imidazole for true biomimetic behavior, we have investigated more accurately biomimetic systems. The biomimetic nature of our model systems are indicated in Table I. Chelated protoheme is compared with R state hemoglobin.[10]

Having in hand a model system which so accurately mimics R state hemoglobin with regard to overall binding kinetics, equilibria, and thermodynamics, we have extended the study to nanosecond and picosecond time scales to determine the fundamental geminate rate constants for heme proteins having this active site.

In this paper we investigate the elusive geminate recombination of CO to a five-coordinated imidazole–protoheme complex as a model for the corresponding reaction in heme proteins. Since the compound to be photolyzed has been characterized in solution by NMR and its purity established from previously determined equilibrium constants we can be sure of the starting material.[23] The accurate biomimetic nature of our imidazole–heme–CO complexes helps in the interpretation of the data.

Experimental Procedures

Materials. The solvents dimethyl formamide, toluene, and tetrahydrofuran were distilled, the latter two over calcium hydride. Protohemin chloride (Calbiochem), glycerol (Mallincrodt), methanol, 2-propanol (analytical grade), light mineral oil (Whiteworth), and horse heart myoglobin (Sigma) were used as received. Sperm whale myoglobin and chelated protohemin chloride were from previous studies.[3a,b,18,19]

Methods. Sample preparation and picosecond spectroscopy were carried out as previously described[3a,b,18,19] except for those described below. In all the picosecond studies either a flow or cell translation system was used to avoid multiple flashing of the sample. When times are listed in the figures, zero time refers to the time of maximum absorbance excursion at photolysis.

Monochelated Protoheme–CO(MCPH–CO). The hemin was dissolved in the least amount of methylene chloride and added to toluene (2 mL). After passing CO through the solution for 1 h, the heme was reduced with a saturated solution of sodium dithionite–18-crown-6–MeOH (3 μL). The reduced solution was diluted with CO saturated toluene (5–6 mL) so that the Soret absorbance was about 1.5 in a 2-mm quartz cuvette. Ultraviolet visible (UV–vis) spectra were taken before and after the picosecond photolysis to assure the sample was not degraded.

MCPH–CO in Mineral Oil. Concentrated MCPH–CO toluene solution was made by the above procedure. Mineral oil was degassed by heating near the boiling point under high vacuum and pumped overnight and then saturated with CO. The MCPH–CO toluene solution was added to mineral oil (6 mL) until the absorbance was 1.5 in a 2-mm quartz cuvette. The final composition of the solvent was 95/5 (v/v) mineral oil/toluene. UV–vis spectra were taken before and after the picosecond photolysis to assure the sample was not degraded.

MCPH–CO in DMF. Monochelated protoheme was dissolved in CH_2Cl_2 (100 μL) and added to dimethyl formamide (DMF, Fisher Scientific). The heme solution was saturated with CO and reduced with saturated solution of sodium dithionite–18-crown-6–MeOH (3 μL). The MCPH–CO solution was diluted with CO saturated DMF (5–6 mL) so that the Soret absorbance was 1.5 in a 2-mm quartz cuvette. UV–vis spectra were taken before and after the picosecond photolysis to assure the sample was not degraded.

(1-MeIm)PP(IX)Fe^{II}–CO in Glycerol. Glycerol (4 mL) was degassed by heating under high vacuum for a few hours and placed under argon and then bubbled with carbon monoxide for 1 h. Protoporphyrin(IX)-$Fe^{II}Cl$ (~5 mg) was suspended in a small amount of methanol and then added to 1-methylimidazole (1 mL). The solution was degassed by several freeze–pump–thaw cycles and reduced by saturated solution of sodium dithionite–18-crown-6–MeOH (~2 μL) under carbon monoxide atmosphere. The concentrated 1-methylimidazole–PP(IX)Fe^{II}–CO solution (100 μL) was added to CO saturated glycerol (4 mL). The glycerol sodium dithionite–18-crown-6–MeOH solution was cannulated into a degassed 2-mm quartz cuvette (Precision) for the kinetic runs.

Results

Spectroscopic Identification of Intermediates. In order to identify the photolysis processes we show the spectra of four-coordinated protoheme dimethyl ester, five-coordinated 1,2-dimethylimidazole protoheme dimethyl ester, and six-coordinated 1,2-dimethylimidazole protoheme dimethyl ester–CO in Figure 1.

These spectra were taken in 2% cetyltrimethylammonium bromide solution. We have previously shown that four-, five-, and six-coordinated hemes have the same spectra in this medium as they do in anhydrous toluene.[23a,24] A series of equilibrium difference spectra are shown in Figure 2 for comparison with the

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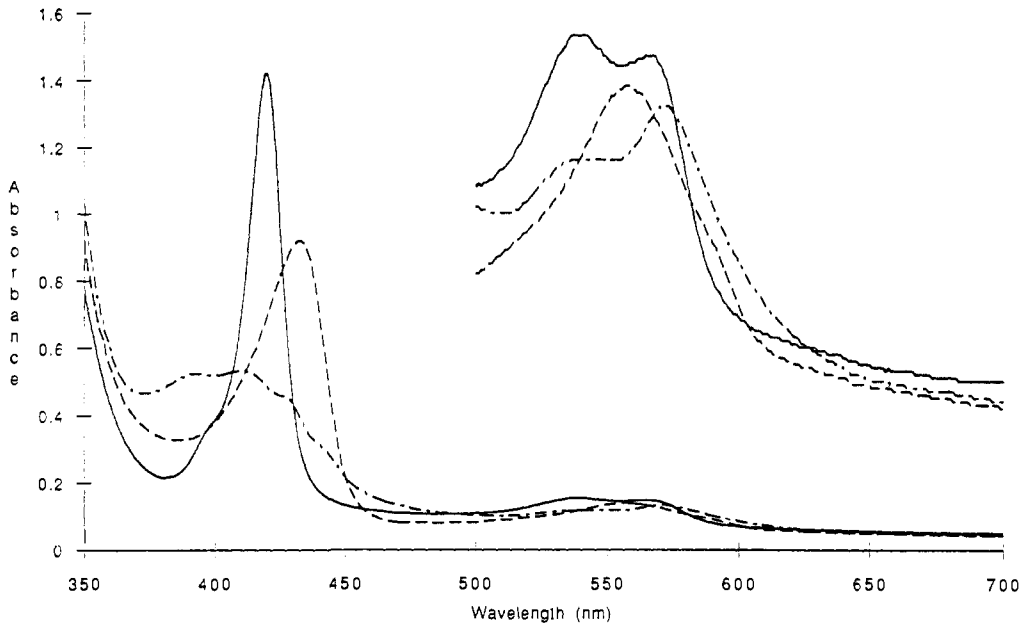


Figure 1. UV–vis spectra of protoheme dimethyl ester in 0.1 M phosphate buffer at pH 7.3 containing 2% CTAB: (- - -) reduced with sodium dithionite solution in the same solvent; (- -) the same reduced solution in the presence of 0.6 M 1,2-dimethylimidazole; (—) the same solution in the presence of 0.6 M 1,2-dimethylimidazole and 1 atm of CO.

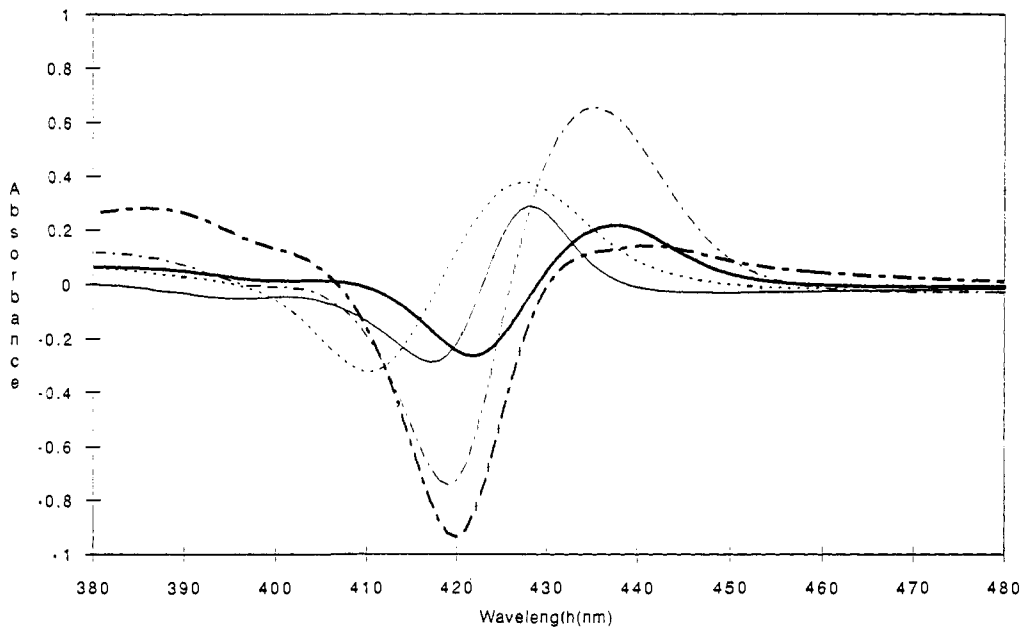


Figure 2. The Soret equilibrium difference spectra between (— · —) protoheme dimethyl ester and 1,2-dimethylimidazole protoheme dimethyl ester–CO; (- - -) (1,2-dimethylimidazole) protoheme dimethyl ester and 1,2-dimethylimidazole protoheme dimethyl ester–CO; (—) HHMb(deoxy) and HHMb–CO; (—) bis(1-methylimidazole) protoheme dimethyl ester and 1-methylimidazole protoheme dimethyl ester–CO; (- -) (2-propanol) protoheme dimethyl ester and (2-propanol) protoheme dimethyl ester–CO.

picosecond difference spectra obtained upon photolysis.

Low Viscosity Studies. To determine the extent of carbon monoxide return after myoglobin–CO photolysis on the picosecond time scale we have taken spectra from 0 to 50 ps (Figure 3) or 60 to 1500 ps (Figure 4). There is no appreciable change of the Soret band. Therefore, less than 5% of carbon monoxide returns in the first 1500 ps. The photolysis of the complex, 1,2-dimethylimidazole–protoheme dimethyl ester–CO in tetrahydrofuran, Figure 5, displays no relaxation except for the long wavelength absorption. This is expected since the corresponding methyl isocyanide complex (with this sterically hindered trans base) did not show isocyanide return.[3a]

Photolysis of the chelated protoheme–CO complex was studied in three solvent systems (Figures 6–8). In all solvents there was a small change with a half-life of about 3 ps in the Soret region (420 nm) (see the insets in each figure) but no change at the five-coordinate region (435 nm) after photolysis. The time scales were extended from 200 ps to 8 ns in separate experiments as illustrated in Figure 9. Clearly no observable return is seen over this time range. Therefore we do not detect any recombination of CO over the time range 0–8000 ps in either myoglobin or the model compound chelated protoheme.

The model system 1-methylimidazole–protoheme dimethyl ester–CO was prepared in pure 1-methylimidazole as solvent, an experiment designed to observe base binding. Carbon monoxide pressure of 400 psi was applied to assure complete formation of the carbon monoxide complex (BHmCO). Picosecond photolysis (Figure 10) revealed an interesting spectral evolution in which little or no change occurred in 20 ps. There followed a slower change in which a maximum at ~430 nm appeared. In order to clarify the processes responsible for this change we prepared the static difference spectra: five coordinated chelated proto-

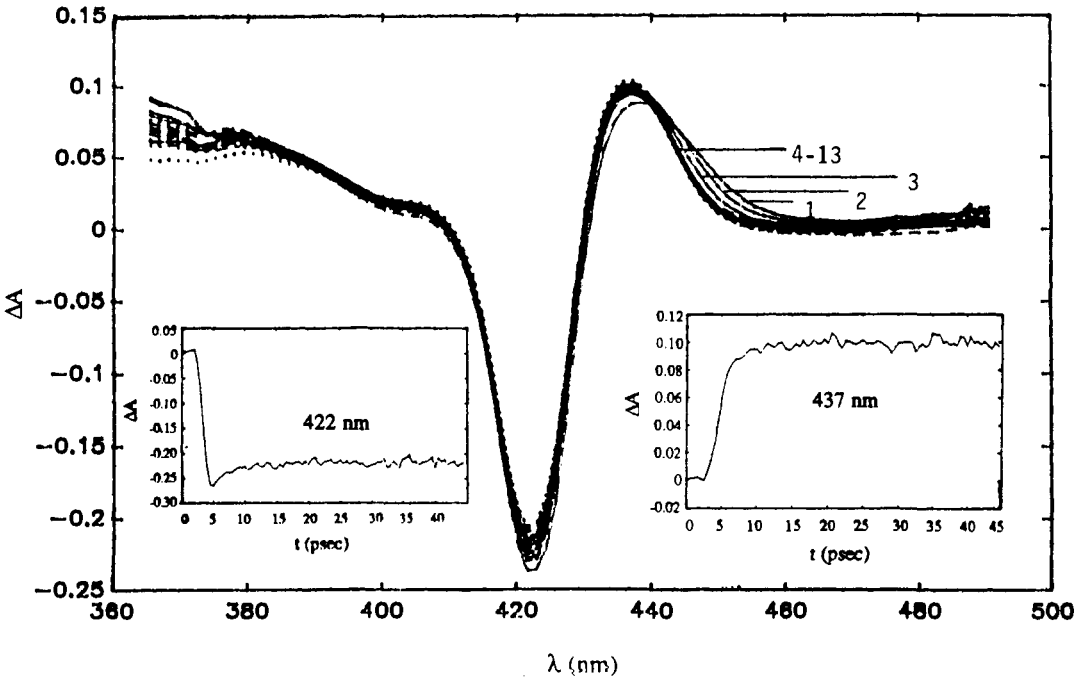


Figure 3. Picosecond transient absorption difference spectra for horse heart myoglobin–CO in 0.05 M bis-tris buffer, 0.1 M (NaCl), [Mb] = 1.5×10^{-5} M, and [CO] = 9.8×10^{-4} M. The numbers 1–13 label the curves recorded at 0, 4, 8, 12, 16, 20, 24, 26, 32, 36, 40, 44, and 48 ps after photolysis. The insets show the absorbance changes vs time at 422 and 437 nm. The absorbances at 422 and 437 nm remained constant to 50 ps, the longest time in this experiment.

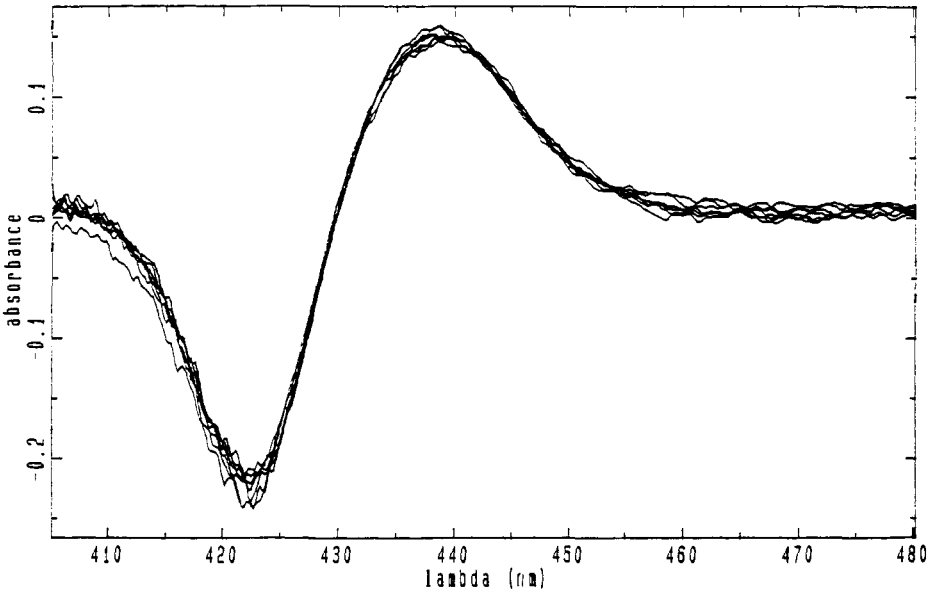


Figure 4. Picosecond transient absorption difference spectra for SWMbCO. Transient absorption difference spectra were recorded at 60, 100, 200, 400, 600, 1000, and 1500 ps.

heme–chelated protoheme–CO (BHm–BHmCO) (A) and bis-(1-methylimidazole)–protoheme dimethyl ester–chelated protoheme–CO (B_2Hm–BHmCO) (B). Fractions of A and B were added together to give the series of spectra shown in Figure 11. Although there are small wavelength differences, the spectroscopic changes are similar to those in Figure 10. We therefore interpret these spectra to show that the BHm which is formed by photolyzing BHmCO does not recombine with CO in 0–1 ns time scale. Instead, the bimolecular recombination of 1-methylimidazole occurs at a rate which is consistent with its bimolecular rate constant (2×10^8 M^{-1} s^{-1}) and its concentration. At lower concentrations of 1-methylimidazole the change at 430 nm was absent.

We have included studies of protoheme dimethyl ester in pure alcohol solvents because other researchers have reported carbon monoxide reactions in glycerol–water mixtures.[4,5,7,12] In these pure solvents the species photolyzed is predominantly the six-coordinated CO complex, eq 6 being shifted far to the right in the pure solvent (R = alkyl or H).[24] See Figure 1 for definitive evidence against the presence of the four-coordinated heme as a photolysis product.

$$\mathrm{ROH} + \mathrm{HmCO} \rightleftharpoons \overset{\mathrm{H}}{\mathrm{ROHmCO}} \qquad (6)$$

Figure 12 displays time-resolved spectra after photolysis of the 2-propanol–protoheme dimethyl ester–CO complex. There is little or no CO or solvent return up to the last spectrum of 231 ps. Although there is the usual fast decrease in absorbance in the 450–470 nm range (finished by 6 ps) neither the Soret bleaching at 416 nm nor the five-coordinated heme absorbance at 431 nm showed any change.

High Viscosity Studies. In an attempt to slow diffusion from the contact pair to the 10^9 s^{-1} range we have studied the picosecond

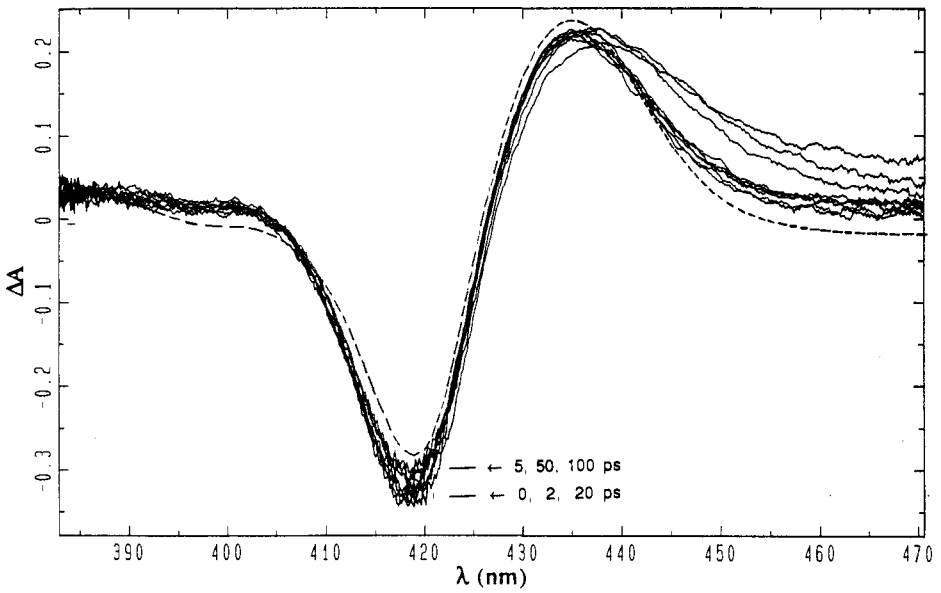


Figure 5. Picosecond transient absorption difference spectra for 1,2-dimethylimidazole protoheme dimethyl ester–CO in THF (DMI = 0.05 M) satuated with CO at 1 atm (—), along with the equilibrium difference spectrum of 1,2-dimethylimidazole protoheme dimethyl ester and 1,2-dimethylimidazole protoheme dimethyl ester–CO in 2% CTAB saturated with CO at 1 atm in 0.1 M phosphate buffer at pH of 7.3 (– –). Transient spectra were recorded at 0, 2, 5, 10, 20, 50, 315, and 2000 ps. The absolute scale of equilibrium difference spectra is different from that of transient spectra.

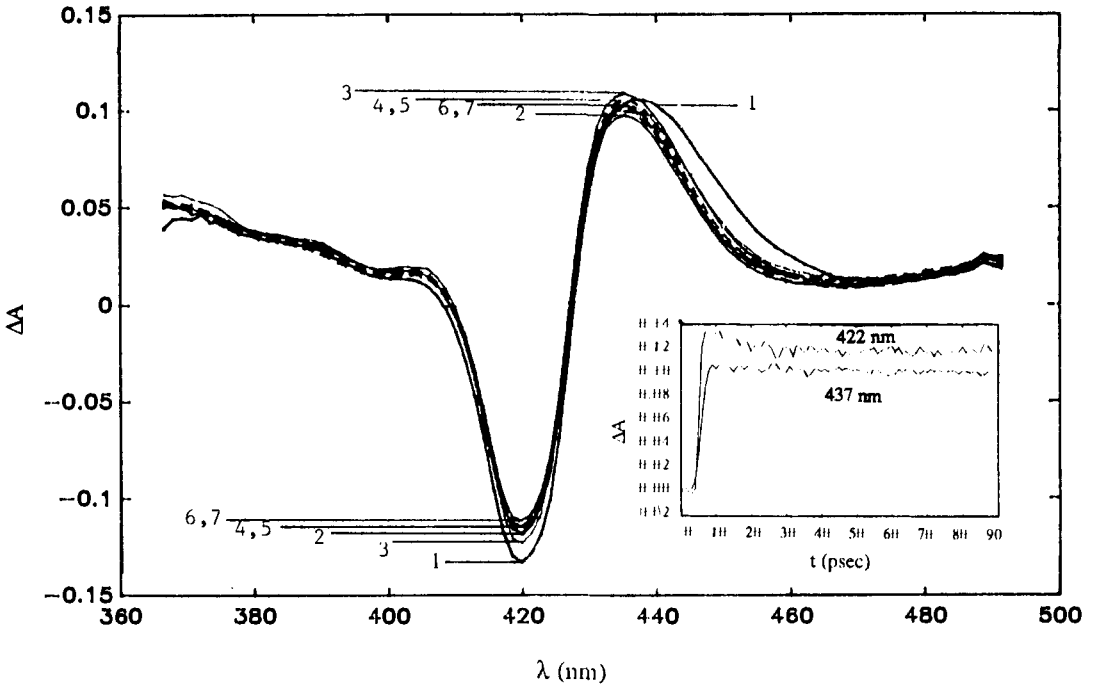


Figure 6. Picosecond transient absorption difference spectra for monochelated protoheme–CO in toluene, [heme] = 1×10^{-5} and [CO] = 5.6×10^{-3} M. The numbers 1–7 label the curves recorded at 0, 9, 18, 27, 36, 45, and 54 ps after photolysis. The insets show the absorbance changes at 422 and 437 nm vs time. The absorbances remained constant to 200 ps, the longest time in this experiment.

photolysis of the 1-methylimidazole–protoporphyrin(IX) Fe^{II}–CO complex in glycerol and aqueous glycerol solvents. The spectra of all solutions characterize the stated complexes.

Figures 13 and 14 show time-resolved spectra after photolysis in glycerol containing 0.4 M 1-methylimidazole. At this concentration of 1-methylimidazole the only species present is the complex 1-methylimidzole–heme–CO, and the presence of the 1-methylimidazole improves the solubility of the heme complex and prevents aggregation. Although the spectra in Figures 13 and 14 are poorly resolved, they demonstrate CO return long after the excited state relaxation is over.

Discussion

Characterization of the Starting Heme Complex. In order to understand the processes which occur after photolysis we must first establish two things: (1) the identity of the species photolyzed and (2) the absence of interfering processes. Unlike photolysis of such stable compounds as a ketone or chromium hexacarbonyl,[25] the several species involved in liganded iron(II) porphyrins are in dynamic equilibria[24] and reliable methods of structure determination in the solutions of interest are necessary.

We,[23] and others,[1b,8] have studied several heme systems including chelated protoheme and protoheme dimethyl ester–imidazole (or deuteroheme systems) in detail. We have characterized the carbon monoxide complexes by NMR spectroscopy[23e] and have determined all of the rate constants shown in Scheme I[1b,8,23] along with separately measured equilibria for these systems.[23,26] The NMR of chelated protoheme–CO at 10^{-2} M at once characterizes the structure and shows that the heme does not aggregate in solution in good organic solvents.

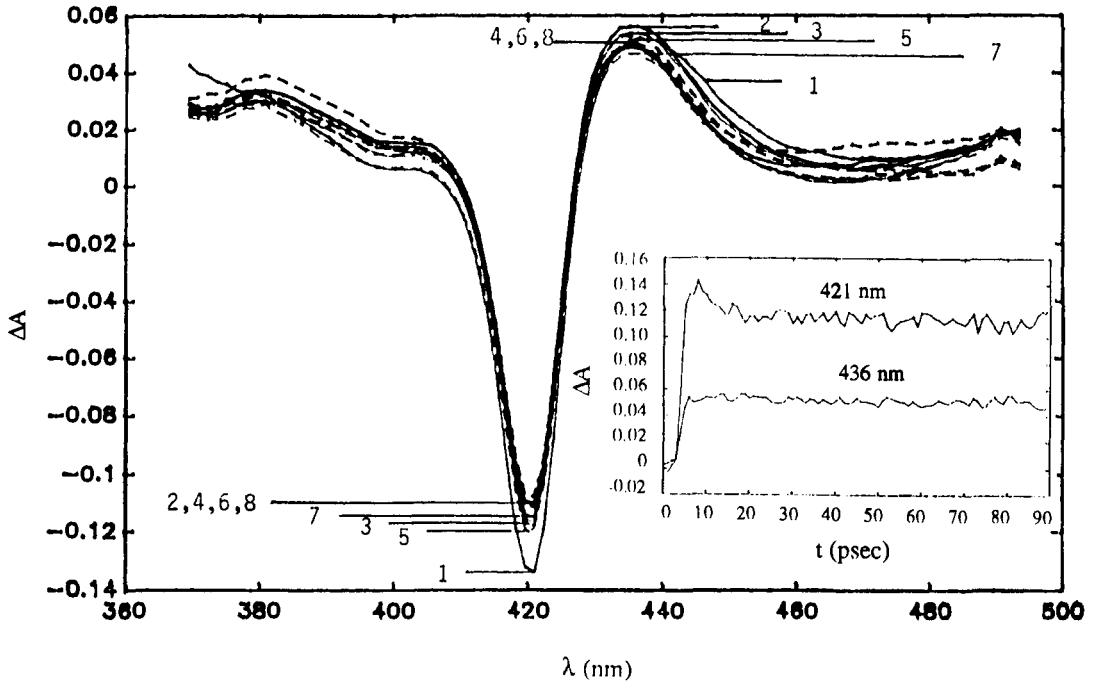


Figure 7. Picosecond transient absorption difference spectra for chelated protoheme–CO in mineral oil, [heme] = 1×10^{-5} M and [CO] = 7.8×10^{-3} M. The numbers 1–8 label the curves recorded at 0, 10, 20, 30, 40, 50, 60, and 70 ps after photolysis. The insets show the absorbance changes at 421 and 436 nm vs time. The absorbances remained constant to 200 ps, the longest time in this experiment.

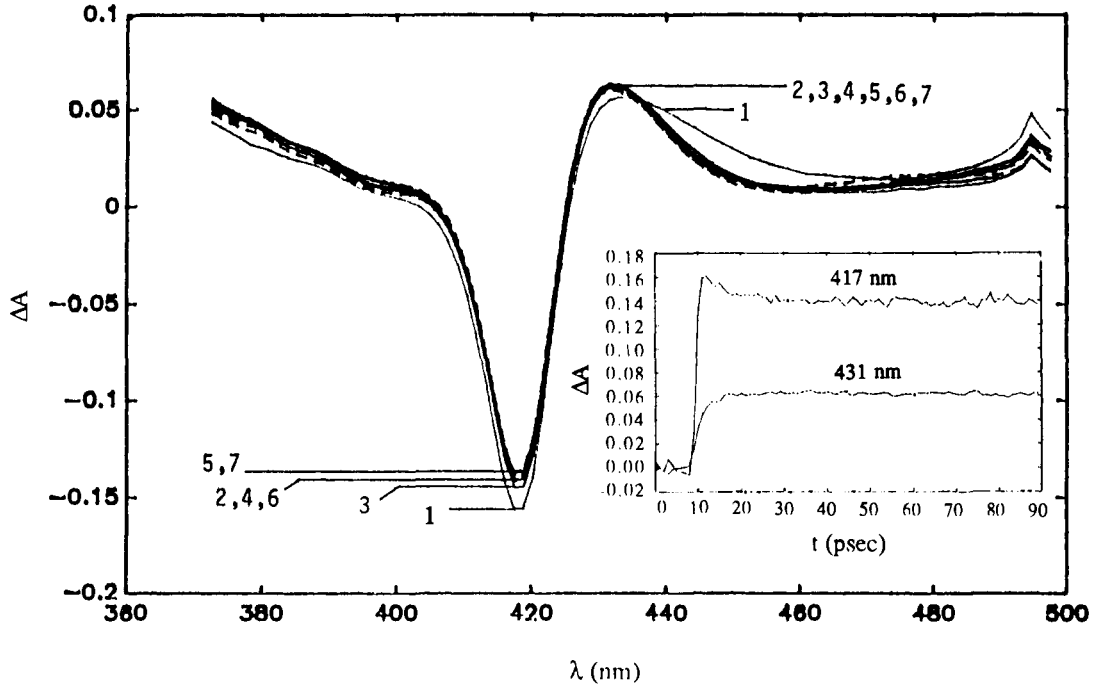


Figure 8. Picosecond transient absorption difference spectra for chelated protoheme–CO in dimethyl formamide, [heme] = 1×10^{-5} M and [CO] = 2.5×10^{-3} M. The numbers 1–7 label the curves recorded at 0, 10, 20, 30, 40, 50, 60, and 70 ps after photolysis. The insets show the absorbance changes at 417 and 431 nm vs time. The absorbances remained constant to 200 ps, the longest time in this experiment.

To be certain of the chemistry of the fast reaction, we must show that none of the indicated processes interfere with the one of interest, either as a result of photolysis of a mixture of species or interference of other fast processes in Scheme I. Knowledge of all the rate constants and equilibria in Scheme I allows us to separate out and study only the photolysis process k_B^{CO} with no interference from any other species. For example, the equilibrium constant between B + Hm + CO and BHmCO is about 4×10^{12} M^{-2}, whereas that between CO + Hm and $Hm(CO)_2$ is around 2×10^6 M^{-2} and that of B + Hm and B_2Hm around 10^9 M^{-2}. This derives from the synergistic binding of an imidazole and carbon monoxide, each increasing the binding of the other by $>10^4$ fold.[23b,24] Additionally, each of these species has a different and well-characterized UV–vis spectrum (Figure 1). Therefore, by comparing transient spectra with our previously reported spectra, or with those in Figures 1 and 2, we can easily identify the species being photolyzed as well as transient species (see below).

We have previously determined the rate constants k_B^{CO}, k_B^{-CO}, k_B^B, k_B^{-B}, k^{-B}, k^B, k^{CO}, k_{CO}^{-B}, k_{CO}^B, and k^{-CO}.[23,26] At low CO pressures $Hm(CO)_2$ can be kept to negligible concentration. The rate of dissociation of the five-coordinated 1-methylimidazole–heme complex, $k^{-B} < 10^4$ s^{-1}, and the fact that $k_B^{CO}[CO]$ can be made to exceed this value as well as the value $k_B^B[B]$ means that we can select from all the processes in Scheme I only the step leading directly to BHmCO after photolysis (outlined with a dashed line in Scheme I).[23b] All of these studies were necessary to make the

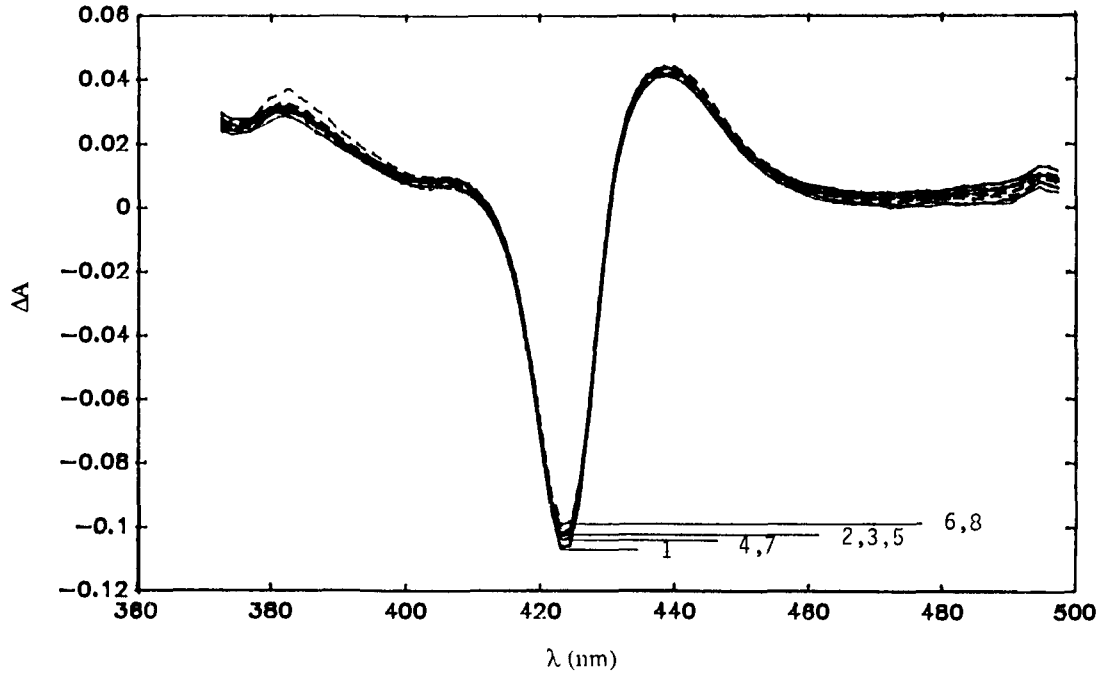


Figure 9. Nanosecond transient absorption difference spectra for chelated protoheme–CO in mineral oil, [heme] = 1×10^{-5} M and [CO] = 7.8×10^{-3} M. The numbers 1–8 label the curves recorded at 200 ps, 2, 3, 4, 5, 6, 7, and 8 ns after photolysis. The absorbances at 421 nm remained constant to 8 ns.

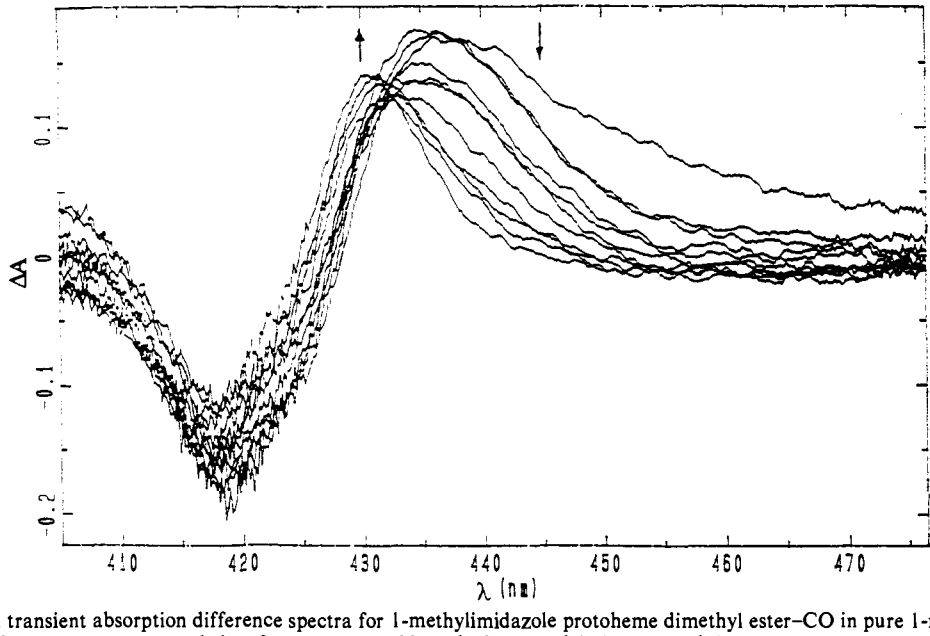


Figure 10. Picosecond transient absorption difference spectra for 1-methylimidazole protoheme dimethyl ester–CO in pure 1-methylimidazole under 400 psi of CO. Transient spectra are recorded at 0, 10, 20, 40, 100, and 300 ps and 1, 2, 3, 4, and 6 ns.

system we are examining an accurately biomimetic model of myoglobin and to identify the photolysis process. Secondly we measure the actual fast processes themselves at the temperature and in the solvents where all species have been characterized. Furthermore, the dynamics of the model system have already been shown to duplicate those of R state hemoglobin on a slow time scale.

Many of the previous studies of cage recombination of CO with model systems have involved the observation of kinetic processes at low temperatures and usually on slower time scales.[12] Furthermore, the kinetics were usually determined on protoheme in glycerol/water or ethylene glycol/water mixtures,[4,5,12,22] both at high pH. Neither the rate constants corresponding to Scheme I nor the structure of the complex photolyzed are known in these systems. Thus the LHmCO complex in the protoheme systems could have glycerol, primary or secondary ROH, water, or the conjugate bases as L. The effect of having attached carboxylate ions is also unknown. Furthermore, mixtures of these species are likely. Just as important is the fact that k_B^S and k_B^S are not known. Since we have found that weak ligands can dissociate with rate constants approaching 10^8 s^{-1},[27] we cannot be sure that such processes with weak alcohol ligands are not interfering. It is possible that previously reported nonexponential binding in protoheme–alcohol–water mixtures[12] could be due to a mixture of chemical reactions rather than a mixture of diffusion processes in a single chemical process. When such systems are cooled to very low temperatures, the equilibria among the several species LHm, L_2Hm, LHmCO, and HmCO shift drastically.[27] Therefore the populations of chemical species are likely to change and the temperature dependence of kinetics will be confusing. This problem also arises in the reported low-temperature study of the cytochrome *c* peptide (a kind of "chelated heme").[12b,26] Myer and Harbury[28] have shown that the ligation of this species changes with pH and have shown that gross ligation changes accompany

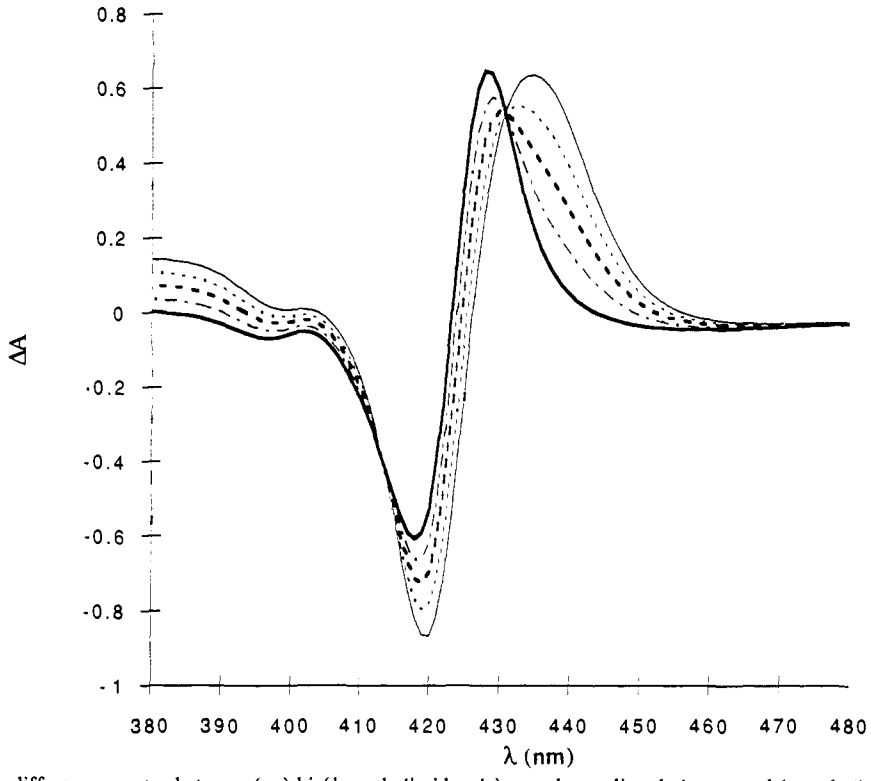


Figure 11. The Soret equilibrium difference spectra between (—) bis(1-methylimidazole) protoheme dimethyl ester and 1-methylimidazole protoheme dimethyl ester–CO (A); (- - -) 80% of A + 20% of the soret equilibrium difference spectrum between 1,2-dimethylimidazole protoheme dimethyl ester and (1,2-dimethylimidazole) protoheme dimethyl ester–CO (B); (- -) 60% of A + 40% of B; (- -) 40% of A + 60% of B; (—) 20% of A + 80% of B.

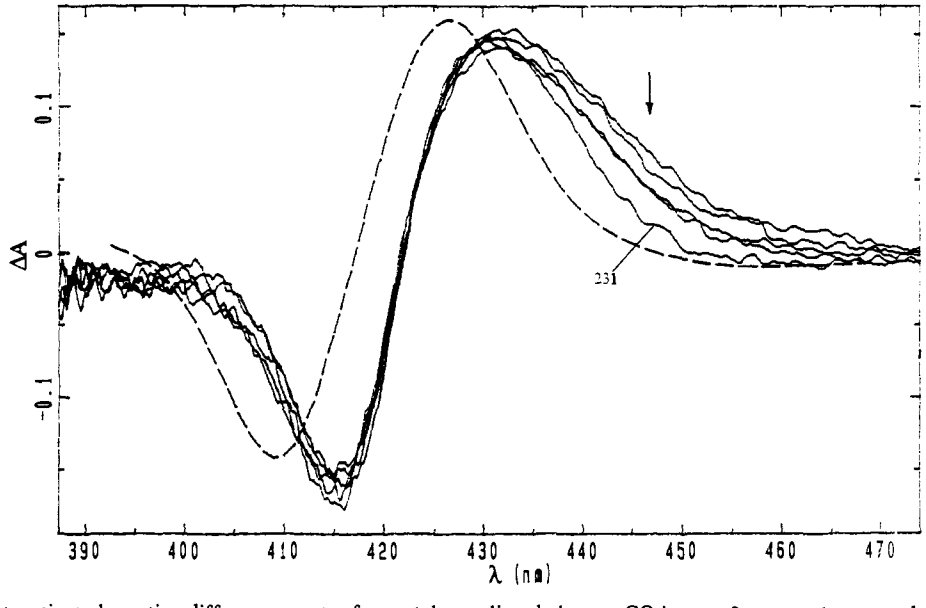
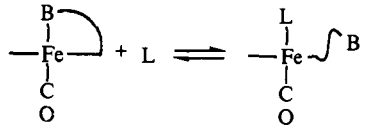


Figure 12. Picosecond transient absorption difference spectra for protoheme dimethyl ester–CO in pure 2-propanol saturated with CO at 1 atm (—), and the equilibrium difference spectrum of protoheme dimethyl ester and protoheme dimethyl ester–CO in 2-propanol (- -). Transient spectra are recorded at 1, 2, 3, 6, and 231 ps and are red shifted by 4–5 nm due to calibration error and are presented uncorrected.

the lowering of temperature in hydroxylic solvents. In addition to the chelated protoheme–CO complex shows a spectrum in pure methanol which suggests that the base is partially displaced by a methanol.[27b] Whether L is a solvent molecule or a side chain



(7)

these processes result in changes of kinetics and their temperature dependence. This means that knowledge of the rate constants of Scheme I at each temperature is necessary to be sure that the actual chemical process being observed is the same at every temperature. The presence of 1-methylimidazole, the use of nonligating solvents, and careful attention to Scheme I helps to remove these uncertainties while providing an accurate biomimetic model system. It is possible that mixtures of complexes occur in some of our experiments as well, but a knowledge of the various equilibrium constants reduces this possibility.

Identification of the Picosecond Photolysis Products. Having characterized the species undergoing photolysis, we now address the photolysis itself. It has been proposed that some exicted state is first formed and decays in about 3–4 ps in proteins and in

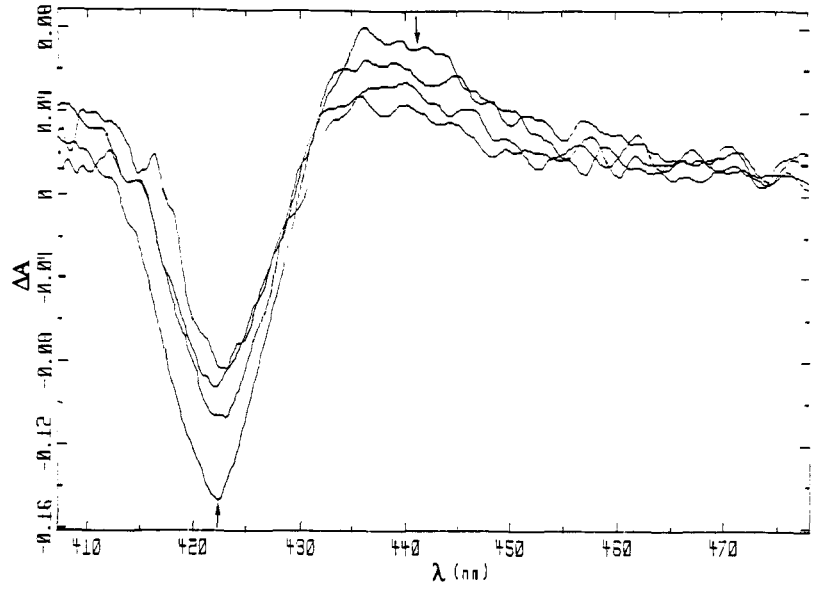


Figure 13. Picosecond transient absorption difference spectra of (1-MeIm)PP(IX)Fe^{II}–CO in 98% glycerol containing 0.4 M 1-methylimidazole (2%, v/v). Transient spectra are recorded at 0, 250, 750, and 1250 ps.

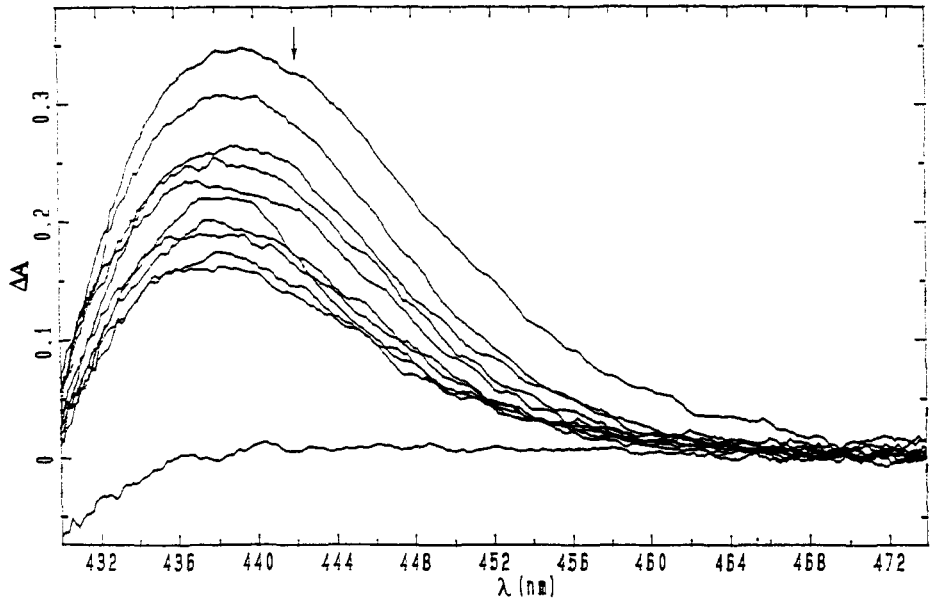


Figure 14. Picosecond transient absorption difference spectra of (1-MeIm)PP(IX)Fe^{II}–CO in 98% glycerol containing 0.4 M 1-methylimidazole (2%, v/v). Transient spectra are recorded at 0, 56, 140, 223, 306, 400, 600, 1000, 2000, 3000 ps and before 0 from top to bottom, respectively.

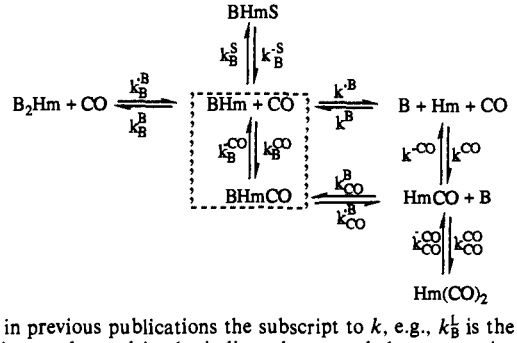
imidazole complexes of hemes.[16] The product of this relaxation could be either four-, five-, or six-coordinated heme. We have

$$\text{BHmL} \overset{h\nu}{\rightleftharpoons} [\text{excited state}] \begin{cases} \rightarrow \text{BHm} + \text{L} \\ \rightarrow \text{HmL} + \text{B} \\ \rightarrow \text{Hm} + \text{B} + \text{L} \end{cases} \quad (8)$$

taken static difference spectra corresponding to these possibilities (Figure 2). The four-coordinate, six-coordinate difference spectrum has essentially no absorbance on the long wavelength side of the Soret bleaching. Since all of the picosecond difference spectra reported here have such absorbance, there is no production of four-coordinated heme in any of these experiments.

The photolysis of 1,2-dimethylimidazole–Hm–CO shown in Figure 5 can be identified with the production of the BHm species since the static and picosecond difference spectra are essentially superimposable. Although all spectra after 5 ps in Figure 5 are identical within our signal-to-noise ratio, the decay at long wavelengths with a 2–3-ps half-life requires some comment. This decay is present in most of our picosecond studies and was previously observed by others.[16] It is often attributed to some excited-state decay in the five-coordinate heme,[16c] although it may

Scheme I. Kinetics of Heme Ligations[a]



[a] As in previous publications the subscript to k, e.g., k_B^L is the ligand which is not changed in the indicated step and the superscript represents addition or loss (– sign) of the ligand. The appropriate equilibria are indicated similarly using, e.g., K_B^L in the direction of addition of L to HmB. S represents binding solvent such as water, alcohols, etc.

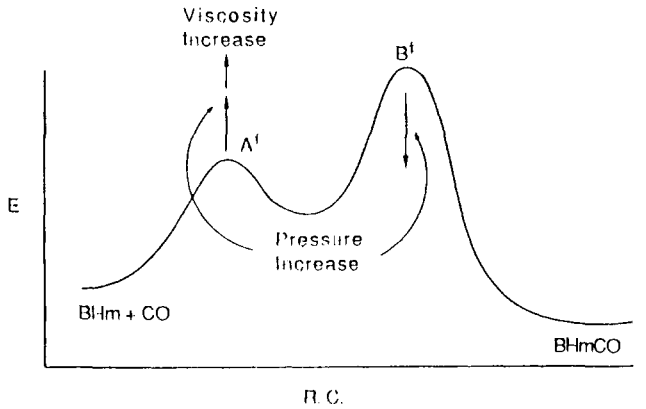


Figure 15. Reaction coordinate diagram for carbon monoxide reaction with the complex protoheme dimethyl ester–1,2-dimethylimidazole or chelated protoheme. Effects of alternatively increased viscosity or pressure on activation energies are indicated by the arrows.

also reflect vibrational cooling.[29a] Its presence makes our measurements in the first ~6 ps difficult to interpret, but we can assert that no recombination of CO occurs in the time period of 10–2000 ps. Either the small amount of CO returned in <6 ps or it occurs after 2000 ps. Because all of the picosecond returns we have observed (1-methylimidazole, various isocyanides, ethers, etc.) show rates of diffusion from the first intermediate of around 3×10^{10} s^{-1} we can estimate that small amounts of return would give rate constants approaching this ($k_{obs} = k_2 + k_{-1} \cong k_2$ when $k_2 \gg k_{-1}$). This rate is expected to have a half-life of 20–40 ps. We do not see this. If $k_2/k_{-1} > 20$, less than 5% return would occur, and we would have difficulty in seeing this amount of return.

The photolysis of the 1-methylimidazole–heme–CO complex in 1-methylimidazole is at once complicated and clarified by the spectral evolution. Except for the usual long wavelength decay in the first few picoseconds, no change occurs in the first 40 ps, indicating that CO does not return in this time scale (Figure 10). The spectral evolution which follows has a half-life around 0.5 ns and corresponds to the development of a [B_2Hm–BHmCO] difference spectrum (see Figure 11). The rate constant is consistent with the observed bimolecular rate constant for BHm + B of 2×10^8 M^{-1} s^{-1}. Therefore, the five-coordinated heme binds the solvent outside of the contact pair instead of carbon monoxide.

$$\text{BHmCO} \xrightarrow[\text{(1-MeIm solvent)}]{h\nu} [\text{BHm CO}] \xrightarrow{\sim 3 \times 10^{10}\ \text{s}^{-1}} \text{BHm} + \text{CO} \xrightarrow[\sim 3 \times 10^{9}\ \text{s}^{-1}]{\text{B}} \text{BHmB} \quad (9)$$

We have deliberately chosen the solvent 1-methylimidazole because this ligand reacts faster than others within its own contact pair and it would certainly replace CO if CO and 1-methylimidazole became diffusionally equivalent. Yet no spectroscopic change occurs in 40 ps, in which time 1-methylimidazole in the contact pair would have completely returned.[18,30]

$$\text{BHmB} \underset{8 \times 10^{10}\ \text{s}^{-1}}{\overset{h\nu}{\rightleftharpoons}} [\text{BHm B}] \rightleftharpoons \text{BHm} + \text{B} \quad (10)$$

Petrich et al.[16a] have shown that the picosecond difference spectra for the photolysis of protoheme–CO in ethylene glycol–water is identical to the static difference spectrum in that solvent. They also report that MbCO photolysis produces a difference spectrum that is essentially identical to the static difference spectra. They conclude that excited-state five-coordinated species form and decay predominantly to the ground-state five-coordinate species. We have previously shown that the chelated protoheme–CO complex, upon picosecond photolysis, shows a difference spectrum which matches that of the static difference spectrum.[30]

(30) Hutchinson, J. A.; Traylor, T. G.; Nöe, L. J. *J. Am. Chem. Soc.* **1982**, *104*, 3221–3223.

The combination of difference spectra and devolution of picosecond photolyzed solutions into those previously characterized by NMR and other methods can be taken as strong evidence that all the picosecond photolysis studied here are simple CO dissociation from BHmCO complexes. If the base were to photolyze to the extent of less than 10%, we could not detect that small perturbation even though the spectra are different. This question can be investigated with picosecond infrared measurements.

While Petrich et al. and others observe the fast decay (~2 ps half-life) in the long wavelength region, we show in Figures 3 and 6 that myoglobin and the model system show no spectral change from 2–50 and 4–200 ps, respectively. Since these are the times of interest, we agree with Petrich et al. that there is no *observable* CO return on these time scales. Subsequent discussions of our statements concern observations occurring after the long wavelength (450–470 nm) decay is finished. We do not interpret what happens during this decay. We have some evidence for an excited-state five-coordinated species which will be reported later.

Picosecond Kinetic Processes in Low Viscosity Solvents. Having characterized the model compound in solution as a biomimetic imidazole–heme–CO complex and shown that photolysis produces a five-coordinate heme–imidazole complex we can address the ligation processes. Knowing the rate of dissociation of imidazoles,

$$\text{BHmCO} \overset{h\nu}{\rightleftharpoons} [\text{BHm CO}] \quad (11)$$

we can be certain that dissociation of B-Hm does not occur during these reactions. However, there are several other rapid processes which can occur and could easily be mistaken for geminate return of CO. But we can first exclude such processes in the photolysis of 1,2-dimethylimidazole–heme–CO (Figure 5) and of the complexes 2-propanol–heme–CO (Figure 12) and chelated protoheme (Figures 6–9). In these cases no recovery is seen, and therefore no measurable geminate recombination of CO or solvent occurs. Further proof of the absence of CO geminate recombination of up to ~100 ps is seen in Figure 10 in which the solvent is 1-methylimidazole. In this case the loss of absorbance at the "deoxy" wavelength occurs with a half-life of about 400 ps. This is very close to the calculated rate based upon the bimolecular rate constant for the reaction of 1-methylimidazole. Furthermore, the Hm(1-MeIm)$_2$ peak at ~430 nm is seen to replace the deoxy spectrum. Therefore, under these conditions the CO does not return appreciably either from the contact pair or from solution, whereas the highly concentrated 1-methylimidazole does react to some extent in a bimolecular process.

$$\text{BHmCO} \xrightarrow{h\nu} [\text{BHm CO}] \rightarrow \text{BHm} + \text{CO} \xrightarrow[\text{slow}]{\text{B}} \text{B}_2\text{Hm} \quad (12)$$

The photolysis in pure 1-methylimidazole was carried out to show that solvent recombination can compete with CO rebinding if CO were to diffuse as suggested by Miers et al.[22] The bimolecular rate constant for 1-methylimidazole binding to Hm–1-methylimidazole is 2×10^8 M^{-1} s^{-1}. Therefore, in pure 1-methylimidazole (~13 M), if solvent effects are neglected, the pseudo-first-order rate constant would be $\sim 3 \times 10^9$ s^{-1} which was observed at 430 nm. Since this peak does not appear at lower concentrations of 1-methylimidazole or with chelated protoheme it can be identified with base binding (eq 9).

This experiment demonstrates that solvents which bind to iron(II) can replace CO at very fast time scales and can easily be confused with cage recombination. As an example, to be discussed in detail in a future publication, the photolysis of protoheme in pure methanol shows rapid geminate recombination (10^{11} s^{-1}), and photolysis of (MeOH)HmCO in methanol reveals spectroscopic changes similar to those after photolysis of Hm(MeOH)$_2$.

The case of MbCO photodissociation is well-studied. Not only is CO dissociation the sole photoreaction, it occurs with near unity quantum yield. Several lines of evidence support this conclusion. First, it is widely agreed that even at long times, the quantum yield for CO dissociation exceeds 90%. This statement was

documented again recently in an extensive study that showed it is true not only at ambient temperature but also even at very low temperatures.[17] Second, even subpicosecond photolysis shows that a deoxy-like spectrum appears rapidly and does not decay at later times, as replicated in Figure 3. Two intermediate states were postulated to lie between the species MbL and [Mb L] in eq 3.[16] One of these was a direct precursor to [Mb L], which was formed in 50 fs and decayed to what we call the contact pair with a time constant of 300 fs. The second intermediate, also formed in 50 fs, is supposed to decay to MbL with a characteristic time of 2.5 ps without passsing through [Mb L]. This is interpreted as a dissociation and subsequent recombination not involving the thermalized deoxy form [Mb L]. It is not pertinent to the rate k_{-1} we are interested in here, and in any case it is a very small amplitude process in Mb.

We conclude that none of the time-resolved spectra in these low viscosity solvents show any spectroscopic evidence for observable carbon monoxide return over the time period studied. The only spectroscopic changes were due to bimolecular solvent binding. This corroborates our previous and present (Figures 6–9) observations that no measurable CO return occurs after picosecond photolysis of chelated protoheme–CO within the observed time scale (10–8000 ps).[30] Hochstrasser et al.[5b] have recently shown by picosecond infrared spectroscopy that there is no measurable (less than 4%) CO recombination in hemoglobin in the 1–1000 ps time scale. This accords with previous observations of Henry et al.,[29] in nanosecond studies, and with Figures 3 and 4 in the present study. Therefore in neither model systems nor myoglobin or hemoglobin is there more than a 4% geminate return of carbon monoxide up to 1 ns. We have also photolyzed chelated protoheme–CO complexes with a nanosecond laser and found no observable return until the bimolecular process begins ($k_B^{CO} = 10^7$ M^{-1} s^{-1}).

These results show that the geminate recombination after photolysis of various Hm–CO complexes either does not occur or else its magnitude is too small to be observed with present instrumentation. If the latter possibility is the case and the rate of bond formation in the cage is around 10^9 s^{-1} as we have suggested, then we should be able to observe this return by slowing the contact pair dissociation rate ($\sim 3 \times 10^{10}$ s^{-1}) with high viscosity solvents.

Picosecond Processes in Viscous Solvents. The spectroscopic evolution in Figure 13, when compared with those in Figure 10, shows that base rebinding does not occur in this time period. At 0.4 M 1-methylimidazole the rate of base binding should be around 10^8 s^{-1} and is not occurring. There is no absorbance increase at 430 nm. The spectroscopic change is consistent with CO rebinding. In fact, Figure 13 is almost identical to the spectroscopic evolution observed upon photolysis of horseradish peroxidase–CO by Berinstain et al.,[31] where a rate constant of 2×10^9 s^{-1} was observed. Increasing the viscosity of the solution in which the photolysis of BHmCO is carried out therefore results in the appearance of carbon monoxide return. The geminate rate constant is about 3×10^9 s^{-1}, very close to rates reported for protoheme–CO complex in glycerol.[4,5] This suggests that the rate of return to an imidazole–heme complex is similar to that for an alcohol heme or water–heme complex. In the latter cases it is possible that some of the heme–CO complex is five-coordinated and this might explain the small amount of faster processes sometimes observed by other workers.[5]

In this highest viscosity case, the quantum yield for cage escape [$\Delta A(t = 3000$ ps$)/\Delta A(t = 0)$] is approximately 0.6. This can be seen in Figure 13 where a 40% recovery of the deoxy heme absorbance occurs. Using this value and the observed average rate constant (log $(A_t - A_\infty)$ vs time) for the spectral evolution, taken at four different wavelengths ($k_{gem} = 3 \times 10^9$ s^{-1}),[32] we calculate the individual rate constants for the system of eq 3.

$$k_{gem} = k_{-1} + k_2 \quad (13)$$

$$k_{-1} = k_{gem}\left(\frac{k_{-1}}{k_{-1} + k_2}\right) = 3 \times 10^9 s^{-1}(0.4) = 1.2 \times 10^9 \text{ s}^{-1} \quad (14)$$

$$k_2 = k_{gem}\left(\frac{k_2}{k_{-1} + k_2}\right) = 3 \times 10^9 \text{ s}^{-1}(0.6) = 1.8 \times 10^9 \text{ s}^{-1} \quad (15)$$

While the data are not as accurately determined as those for isocyanide return, they clearly demonstrate a viscosity dependent cage return of CO after photolysis from the CO complex of the 1-methylimidazole–protoheme dimethyl ester species and corroborate our estimate of 10^9 s^{-1} for this process.[19] Our failure to observe any appreciable carbon monoxide return at low viscosities is now understandable in terms of eq 16. The amount

$$\text{BHmCO} \underset{10^9\ s^{-1}}{\overset{h\nu}{\rightleftharpoons}} [\text{BHm CO}] \underset{3 \times 10^8\ M^{-1}\ s^{-1}}{\overset{3 \times 10^{10}\ s^{-1}}{\rightleftharpoons}} \text{BHm} + \text{CO} \quad (16)$$

of return in toluene is given by $k_{-1}/k_{-1} + k_2 = 0.03$. At the present signal-to-noise ratio we are not able to observe this amount of return with confidence.

Our observations accord with those of Martin, Petrich et al.,[16] but they stand in rather strong contrast with the results of Moore et al.[5a] on photolysis of protoheme–CO complexes in glycerol at room temperature and those of Postlewaite et al.[22] taken at 100 K. In both cases, CO return on picosecond time scales was observed. We find that in solvents such as toluene and dimethyl formamide, CO does not rebind to the five-coordinated heme–imidazole complex on the picosecond time scale. Others have reported and we have confirmed that little or no CO return occurs in myoglobin on this time scale. In this regard as well as in picosecond isocyanide rebinding our five-coordinated model compounds strongly resemble myoglobins. Since we observe picosecond recombination with other ligands we could easily detect >10% return of CO if it occurred, but we see no picosecond return of CO to BHm. Moore et al.[5a] find large differences between myoglobin and a solution of protoheme–CO in basic glycerol–water, the latter system displaying 25% CO return in 60 ps, while myoglobin showed no return. Postlewaite et al.[22] observed a large fraction of CO rebinding occurring in less than 100 ps at 100 K. These measurements were also carried out in glycerol–water. These authors discuss CO binding to five-coordinated heme–B (B = Im, ROH) as occurring with rate constants in the picosecond range at 100 K. This relaxation disappeared at 300 K. Our results taken with those of others[4,5b] suggest that CO rebinds in the cage with a maximum rate of $\sim 2 \times 10^9$ s^{-1} at ambient temperature. Referring to eq 16 it is clear that cage rebinding would be absent in low viscosity solvents where $k_2/k_{-1} \geq 30$ and that geminate rates around 10^9 s^{-1} would be observed in high viscosity solvents where k_2 is retarded by perhaps a factor of 30. However, geminate rates of $>10^{10}$ at high viscosity cannot be accommodated by reaction 16 unless k_{-1} is accelerated. Reaction of CO with four-coordinated heme is diffusion-controlled ($k_{obsd} = 4 \times 10^8$ M^{-1} s^{-1}) and thus a planar heme might rebind with CO with rate constants in excess of 10^{10} s^{-1}. Postlewaite et al.[22] suggest that their results are best explained by rebinding to the five-coordinated heme before it relaxes to the domed configuration in the viscous solvent at low temperature. This might also explain the results of Moore et al.[5a] In heme proteins and in model systems at room temperature the rate of doming has been estimated at greater than 10^{12} s^{-1}.[16a] Thus this fast ($>10^{10}$ s^{-1}) rebinding process in viscous solvents at low temperature as well as the excited state relaxation at long wavelengths[16a] are not directly relevant to the thermal behavior of hemes or heme proteins at room temperature.

We have observed fast binding of methanol to heme after CO photolysis, and this process could also complicate reactions in hydroxylic solvents or other solvents which bind to heme. Studies of picosecond kinetics in viscous hydrocarbon solvents should help

(31) Berinstain, A. B.; English, A. M.; Hill, B. C. *J. Am. Chem. Soc.* **1990**, *112*, 9649–9651.

(32) Although there is considerable scatter at each wavelength, the rate constants are the same within ±20%.

to further clarify the nature of these very fast processes.

Because the object of our fast kinetic studies is an understanding of the thermal processes involved in oxygen transport and related reactions and these $>10^{10}$ s^{-1} relaxations observed in heme–CO photolysis have been assigned to short-lived photoprocesses involving electronically or vibrationally excited states we will not include these observations in our discussions. But the 10^9 s^{-1} collapse of the relaxed heme with carbon monoxide is relevant to the thermal reaction of chelated protheme and heme proteins with carbon monoxide. This is the rate of the iron–carbon monoxide bond formation in chelated protoheme, and we conclude that heme proteins have very nearly the same rate constant.

Quite separate from the issue of ultrafast, picosecond recombination is the matter of the best description of geminate recombination of CO with hemes in glycerol, the one solvent in which CO clearly does recombine in a geminate process. On the basis of measurements over a wide temperature range, Miers et al.[22b] propose that geminate rebinding in glycerol is nonexponential and well-described by a Smoluchowski-like diffusion treatment. The diffusion model is attractive, since it incorporates in an explicit manner a feature—translational diffusion—that must be pertinent, so one hopes to find a good example. However, as currently developed, the model includes unrealistic assumptions that must fail at short times, such as a continuum solvent and a ligand, in this case CO, that is assumed to be completely spherical in its reactivity. Consequently, it is not entirely clear how accurately the diffusion model should account for geminate recombination. Given our concern about the chemical homogeneity of glycerol–heme solutions, particularly at low temperatures, we carefully tested both the diffusion model and simple exponentials for their ability to fit geminate recombination of ImHm with CO or other ligands at room temperature. The absorbance vs time for 1-MeIm protoheme reacting with CO in a glycerol solution can be fitted approximately to either an exponential or a diffusion model although neither appears to fit exactly. For purposes of the present discussions we will use the exponential fit and the first-order rate constant 3×10^9 s^{-1} even though the diffusion model might be applicable in very viscous solutions. The question of the kinetic description of the geminate processes in heme reactions with ligands will be discussed in detail elsewhere.

Mechanisms of Ligation. We have interpreted the binding of isocyanides and other ligands to chelated protoheme (or other similar five-coordinated hemes) in terms of eq 4 because we observe in these cases two apparently exponential processes[19] with no changes occurring from 100 ps to ms time scales (at low ligand concentrations). The fast process is independent of ligand concentration, and the slow process is first order in ligand concentration.

We have called the intermediate a contact pair with some justification. The spectral evolution after photolysis of bis(1-methylimidazole) protoheme dimethyl ester, half-life ~8 ps,[19] is very similar to that for ferrous cytochrome b_5, having the same bis(imidazole) structure, and has a similar rate constant.[3c]

$$\text{BHmB} \overset{h\nu}{\rightleftharpoons} [\text{BHm B}] \rightleftharpoons \text{BHm} + \text{B} \qquad (17)$$

Additionally, photolysis of ferrous cytochrome *c* shows return with a similar rate.[3c] The crystal structure of cytochrome *c* shows that the bound imidazole can be moved away from the iron far enough to break the bond without encountering steric hindrance. But it is still practically in "contact" with the heme. There is no solvent in this region and no diffusion. Return is quantitative. We can therefore call this species a "contact pair". Because the model system shows the same rate of collapse (within a factor of 2) we feel justified in proposing that the model also returns from a similar contact pair.

We can now represent the photolysis of base–heme–ligand complexes by eq 4 in which k_2 and k_{-2} are rather similar for small ligands if diffusion rates are similar, and the differences in geminate yields varies strongly with k_{-1}. We can predict changes in geminate behavior with changes in structure, viscosity, and pressure, by examining the reaction coordinate diagrams of Figures 15 and 16 along with previous results concerning structure-reactivity.

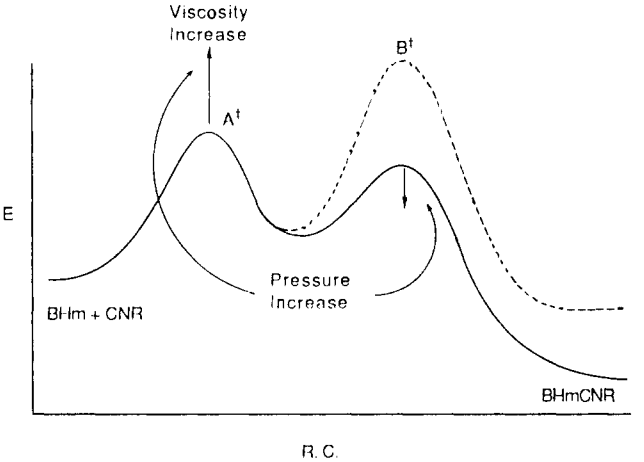


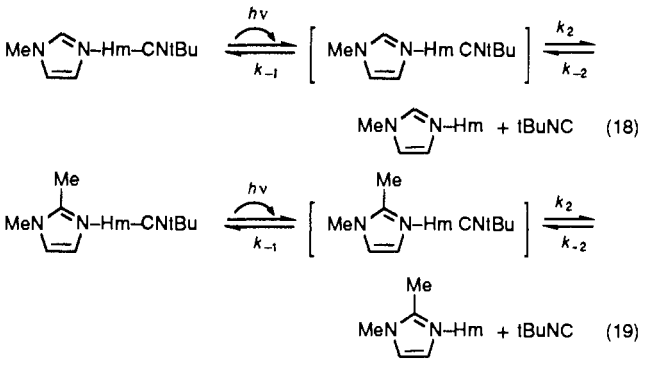
Figure 16. Reaction coordinate diagram for isocyanide reactions with the complexes protoheme dimethyl ester–1-methylimidazole (—) and protoheme dimethyl ester–1,2-dimethylimidazole (--).

Figure 15 represents the binding of carbon monoxide as an activation-controlled process in which the contact pair [BHm CO] is formed from reactants many times for each successful reaction. Increasing hydrostatic pressure increases $A^‡$ energy but decreases $B^‡$ energy since there is a volume decrease at $B^‡$. The result is that the rate of reaction should increase with pressure as we have recently observed.[3b] By contrast, an increase in pressure increases the energy of the rate limiting $A^‡$ in the reaction of isocyanides (Figure 16), with a resulting decrease in rate. This too has been observed.[3b]

Increasing the viscosity of the solvent raises the energy of $A^‡$ by retarding diffusion but has very little effect on $B^‡$. Therefore by sufficient increase in viscosity to slow diffusion to near 10^9 s^{-1}, geminate recombination of CO becomes observable and the reaction changes to diffusion control. Caldin and Hasinoff[21] reported a positive activation volume for CO return in glycerol in contrast to our negative activation volume for this return in the nonviscous solvent toluene. This delicate balance between activation and diffusion control is in very good agreement with the assumption that the rate of bond formation is only 30 times slower for carbon monoxide than for isocyanides or nitrogen bases. Interestingly, Hasinoff[33] also reports a negative activation volume in the reaction of CO with hemoglobin and myoglobin. This is understandable in terms of the rather small effect of the protein on the overall rate of CO binding[23] and our finding that picosecond reactions of our models and myoglobins are similar.[18,19] It also indicates that hemes in glycerol solution do not model myoglobin, whereas chelated protoheme in toluene does model myoglobin in this and other respects.

An alternative demonstration of this delicate balance between diffusion and activation and the value of $k_{-1} \cong 10^9$ s^{-1} for carbon monoxide return comes from the picosecond studies of isocyanides. In the two reactions below the ligand is the same, and the size of both dissociated parts are essentially the same. Therefore, k_2 and k_{-2} can be assumed to be identical in the two cases. Additionally, the dissociation rates of ligands opposite 1,2-dimethylimidazole in these complexes are larger than those opposite 1-methylimidazole, demonstrating that the difference is in the bond-making and bond-breaking steps. Therefore, the differences in these two systems is in k_{-1}. Since the overall association rates differ by about 20, this means that $k_{-1} \cong 2 \times 10^9$ s^{-1} for eq 4. The introduction of the 2-methyl group in the imidazole, adding steric strain, thus converts *tert*-butyl isocyanide binding from the diffusion-controlled process of Figure 16 (—) to the activation-controlled process (--). We have recently reported that, like CO

(33) (a) Hasinoff, B. B. *Can. J. Chem.* **1977**, *55*, 3955–3960. (b) Hasinoff, B. B. *Archives Biochem. Biophys.* **1981**, *211*, 396–402. (c) Hasinoff, B. B. *Biochemistry* **1974**, *13*, 3116–3117.

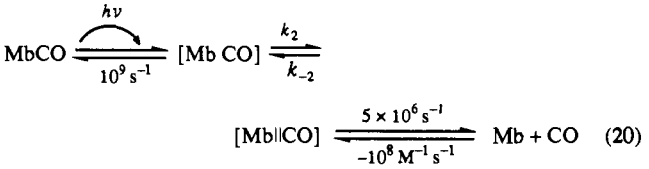


binding, the binding of *tert*-butyl isocyanide to the five-coordinated 1,2-dimethylimidazole–heme complex shows *no measurable geminate recombination.*[3a] This observation provides strong confirmation of our description of carbon monoxide and other ligand binding to biomimetic five-coordinated heme compounds.

Relevance to Heme Proteins. This work and other studies[4,5] firmly establishes the value of $k_{-1}^{CO} \cong 10^9\ s^{-1}$ for unhindered model systems (eq 4). It is quite clear that a photolyzed diatomic ligand remains in myoglobin for more than 100 ns.[29] If during that time the CO can combine at a rate of $10^9\ s^{-1}$, why is it that no more than 5% geminate recombination occurs? This could be explained by steric reduction of k_{-1}, as has been suggested by several researchers.[4,5,12] Alternatively, we have suggested[18] that there is a protein-separated intermediate, which has the ligand migrating far enough from the iron that bond formation cannot take place until there has been a diffusional return to the "contact pair," as defined in eq 3. Such migration need not be very distant. In fact, our "cage wall" effects suggest that a single intervening molecule (or residue in the protein) would suffice to prevent contact pair return.

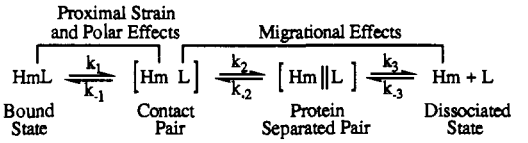
There are several observations which provide strong evidence against the retardation of k_{-1}. First, where this process has been observed (various RNC) there is little difference in k_{-1} among RNC of various sizes.[11a] This argued against a major role for distal steric effects influencing k_{-1}. More importantly, changing the heme from an unhindered chelated protoheme to the hindered myoglobin or to a hindered model heme adamantane–Hm–6,6 cyclophane does not appreciably change the value of k_{-1} for *tert*-butyl isocyanide, despite the fact that in this series the overall rate of isocyanide binding changes by a factor of about 10^4.[3a] It is difficult to explain why k_{-1} would be retarded for CO and not for the larger ligand.[35]

While the evidence is not yet definitive in the absence of direct observation we suggest that the data for myoglobin are qualitatively summarized with the four state scheme of eq 20.



In this scheme, we require $k_2 \gg k_{-2}$ to account for the small amount of geminate recombination observed in myoglobin.[29b]

Scheme II. Factors Controlling Ligand Dynamics in Heme Proteins



Factors Controlling Ligand Dynamics in Heme Proteins

Minor adjustments, then, will accommodate the observation that measurable geminate recombination does occur in hemoglobin. We do not suggest that eq 20 models all features of the actual reaction. However, since eq 3 seems to be required for isocyanide binding, it seems logical to use eq 20 for carbon monoxide without, at this time, excluding a more complex nature of what we show as [Mb||CO].

Control of Ligation in Heme Proteins. Upon the basis of these and previous studies of model systems we propose that kinetics and equilibria of CO, O_2, and other ligands binding to heme proteins are controlled by polar and proximal pull effects at the contact pair stage and by migrational effects upon moving out of the contact pair as shown in Scheme II. The proximal strain effect retards k_{-1} and accelerates k_1 for all ligands. Migrational effects, involving entry into (k_{-3}) or escape from the protein (k_3) or movement to (k_{-2}) or from (k_2) the contact pair, operate as steric effects retarding diffusion (migration) and reducing k_2, k_{-2}, k_3, and k_{-3} to an extent which depends upon the size of the ligand. Ligands of similar size and shape, such as O_2, CO, and NO, should have similar diffusion coefficients and, therefore, should be equally affected by changes in migrational effects. Since we find little or no steric blocking affect in k_{-1} or k_1, this means that distal steric effects, which we now call migrational effects, should not differentiate O_2 and CO with regard to association rates or equilibrium constants unless some complex forms with the proteins. As we have previously shown,[11,23,34] the dominant effect in the variation of the relative CO and O_2 affinities is the change in dioxygen dissociation rates resulting from alteration in polar environments.

Scheme II seems to be a minimum for heme proteins. Since proteins are not isotropic, the migration of ligands inside the proteins could be more complicated than we show by the single rate constants k_2 and k_{-2}. Although we generally find exponential decay in the nanosecond range for return of, e.g., isocyanides to myoglobin at ambient temperatures, more complex decays have been reported, even by ourselves.[36] At present the amount of geminate recombination after CO photolysis is too small to establish whether its rate is represented by a single exponential. We will address this issue for other ligands in a future paper.

We therefore conclude that carbon monoxide binding to myoglobin is very similar to that of isocyanides. The major difference is in k_{-1} which is in the $2–7 \times 10^{10}\ s^{-1}$ range for isocyanides and near $10^9\ s^{-1}$ for CO. For this reason we have modified the scheme originally proposed by Frauenfelder et al. in which k_{-1} was variable to that in Scheme II. In this view, heme proteins such as myoglobin, hemoglobin, horseradish peroxidase, cytochrome *c* peroxidase, etc. should all display a CO return in the range of $10^9\ s^{-1}$. Values very near to this have been reported for horseradish peroxidase,[7,35] hemoglobin Zürich,[12c] and cytochrome *c* peroxidase.[37]

Acknowledgment. We are grateful to the National Institutes of Health (Grants DK 07233-14 and HL-13581) and to the National Science Foundation (Grant CHE-8715561 [D.M.]) for support.

(34) Traylor, T. G.; Koga, N.; Deardurff, L. A. *J. Am. Chem. Soc.* **1985**, *107*, 6504–6510.

(35) Interestingly, Doster et al.[7] report that horseradish peroxidase, which has almost the slowest ligation rates and is thus one of the most sterically hindered proteins, shows the fastest rate of the CO bond-making step. We take this as further evidence that the intrinsic bond-making rate constants for all heme proteins (which bind CO) are in the range of $10^9\ s^{-1}$ and that all slower processes (e.g., in myoglobin) are "protein separated"[19] or matrix[12] processes.

(36) Chatfield, M. D.; Walda, K. N.; Magde, D. *J. Am. Chem. Soc.* **1990**, *112*, 4680–4687.

(37) Miller, M.; Taube, D. Unpublished results.