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Flash Photolysis of the Serum Albumin–Heme–CO Complex†

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ABSTRACT: Protoheme–CO in aqueous solution does not exhibit a geminate ligand recombination reaction. Addition of a protein, either globin or serum albumin, to which heme binds strongly, leads to an observable geminate reaction in aqueous solution. The bimolecular kinetic data for the albumin–heme–CO complex show two stable components, one heme-like in rate and difference spectrum, and one hemoglobin-like. The geminate reaction correlates spectrally with the hemoglobin-like component.

The binding of heme to globins produces a holoprotein with remarkable properties including in the case of hemoglobin (Hb) the capacity to bind a number of gaseous ligands reversibly and cooperatively (Antonini & Brunori, 1971; Dickerson & Geis, 1983). The effect of the interaction of heme with globin can be seen clearly when the kinetics of the reactions of heme with carbon monoxide (CO) are compared with the corresponding reactions of hemoglobin (Marden et al., 1986). Binding to globin both protects the iron from oxidation and slows the entrance and exit of gaseous ligands from the heme pocket so that the ligands remain correlated with the heme long enough to allow appreciable recombination of the geminate pairs.

Globins, however, are not unique in binding to and modifying the behavior of heme. The protein hemopexin, for example, which is present in plasma, binds free heme in both ferro and ferri forms, and the ferro complex binds oxygen and CO reversibly (Muller-Eberhard & Morgan, 1975). Serum albumin is an abundant constituent of human plasma and has one high-affinity binding site with a dissociation constant of approximately 2×10^{-8} M for hemin as well as additional sites of much lower affinity (Adams & Berman, 1980; Beaven et al., 1974; Moehring et al., 1983).

The static spectrum of heme–albumin is intermediate between that of free heme and that of Hb. In methemalbumin the spectrum suggests that the iron is high spin, and this in turn implies that it may be bound to the protein through one of the many histidines in albumin (Kaminsky et al., 1972). Such axial ligand binding would be analogous both to Hb and to the hemopexin complexes (Brown, 1976; Muller-Eberhard & Morgan, 1975).

The analogy between heme binding to albumin and to globin suggested that an examination of ligand binding kinetics to the heme–albumin complex might help to show how important

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the specific structure of the heme pocket in hemoglobin is in determining the rate of ligand binding, both in the overall reaction and in rapid geminate rebinding following flash photolysis.

Materials and Methods

Hemin (Eastman Hemin 2203, lot A9H) was dissolved in 0.1 M NaOH to give a stock solution of approximately 5 mM. The ferric iron was reduced with sodium dithionite and the ferrous heme suitably diluted in buffers equilibrated with 1 or 0.1 atm of CO. The buffers used were 0.1 M potassium phosphate at pH 7.0 or 0.05 M Bis-Tris also at pH 7.0. Experiments using 75% (v/v) glycerol:buffer to increase the viscosity (from 0.4 P at 20 °C to 100 P at −30 °C) and dimethyl sulfoxide [DMSO, 40% (v/v)] to reduce dimerization of heme in the solutions were also performed. Temperature was controlled by Peltier effect cooling chips (Melcor) with feedback controlled via computer.

Kinetic measurements were made by using a dye laser (Phase-R DL2100B) with 500-ns pulses, which was fired in the cavity dump mode to give 25-ns pulses for the geminate kinetics. The observing light was provided by a xenon arc lamp with appropriate filters, Corning 5–57, or with interference filters (425 or 436 nm with a bandwidth of 5 nm). A Spex monochronometer was interposed between the sample and the photomultiplier. Transmission changes following photolysis were recorded by a Biomation (Cupertino, CA) transient recorder, Model 805, and the data transferred to an IBM PC. Difference spectra were recorded in the wavelength region from 390 to 450 nm. All static spectra were measured with a Cary Model 14 spectrophotometer.

Geminate kinetic data were collected as previously described (Gibson et al., 1985). The xenon arc lamp was pulsed to improve the signal to noise ratio of the data. Signals were recorded by using an RCA 1P28 photomultiplier working into the 50-ohm input of a Hewlett-Packard 1727A storage oscilloscope (275 MHz). The oscilloscope traces were photographed on 35-mm film, enlarged, and digitized for analysis.

Stopped-flow experiments were performed as described by Gibson (1973). The complex of deoxyheme with human serum albumin (HSA) was mixed with oxygen-saturated buffer in the stopped-flow device.

The serum albumins were defatted protein (Sigma A3782, human, or A6003, bovine); however, some experiments were performed with fraction V powder (Sigma A4503). The latter compound was not homogeneous as seen by standard polyacrylamide electrophoresis (Cooper, 1977). Additional experimental work was performed on HSA (Kabi, Stockholm). The defatted proteins also contained minor (<1%) quantities of globins. The albumins were dissolved in pH 7.0 buffers to give 3.0 mM stock solutions. The concentrations were confirmed by spectrophotometry using an absorbance of 0.55 at 280 nm for a 0.1% solution (Beaven et al., 1974). The hemin and albumin stock solutions were diluted as required to give a final heme concentration of 40 μM at heme–protein ratios of 1:1 or 1:10.

Sodium oleate (Sigma O7501) in ratios of 1:1 and 10:1 (fatty acid–protein) was added as a competing albumin ligand for some experiments. Human globin was prepared by the method of Geraci et al. (1969). The lyophilized globin was dissolved in buffer, and heme was then added to yield molar ratios of 1:1 with the globin.

Results

Figure 1 shows static spectra of free heme–CO with a peak Soret absorbance at 407 nm, Hb–CO with its peak at 419 nm,

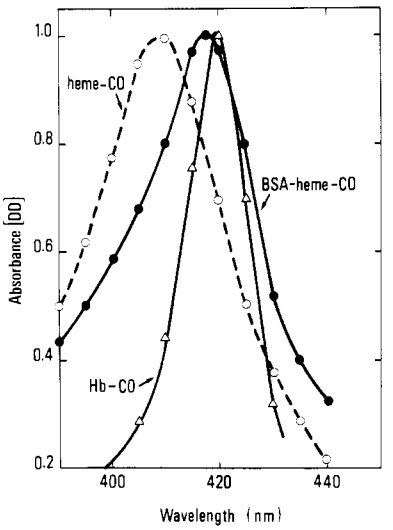

FIGURE 1: Static absorption spectra of protoheme–CO (○), human hemoglobin–CO (△), and bovine serum albumin–heme–CO (●) at a protein to heme ratio of 3:1. The spectra were scaled to give the same maximum absorbance. The spectra were recorded in 50 mM Bis-Tris, pH 7.0 at 20 °C, in the presence of sodium dithionite.

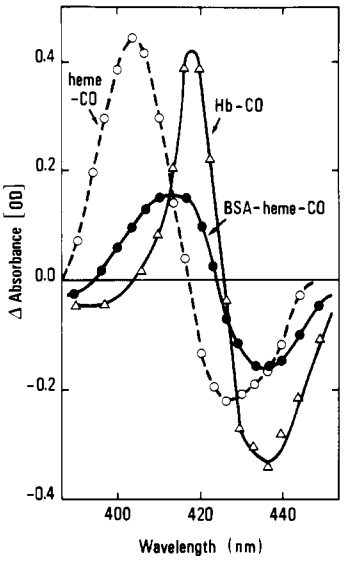

FIGURE 2: Kinetic difference spectra of heme–CO (○), human Hb–CO (△), and heme–CO–human serum albumin (●) with a protein to heme ratio of 3:1 at pH 7, 50 mM Bis-Tris, 20 °C.

and the spectra of human serum albumin–heme–CO with a peak absorbance at 415 nm. The static spectrum of the bovine serum albumin–heme–CO also has its maximum absorbance at 415 nm. The Soret spectrum of the heme–albumin complex is intermediate between that of Hb and that of free heme.

Photochemical difference spectra are presented in Figure 2. As with the static spectra, the kinetic difference spectrum of BSA–heme–CO is intermediate between the difference spectra of free heme–CO and Hb–CO. The observed amplitudes for BSA–heme–CO are about half those of either heme or Hb–CO, although the BSA–heme has the same Soret amplitude as the reference compounds. Similar results were obtained with HSA.

The geminate kinetics of heme compounds in various environments are shown in Figure 3. Protoheme–CO in aqueous solution (a) shows no measurable geminate reaction under these conditions, although heme in a high-viscosity solvent such as 75% glycerol shows appreciable geminate recombination (b). In the presence of globin either in the reconstituted protein (d) or in the native Hb (e) there is sufficient constraint of the

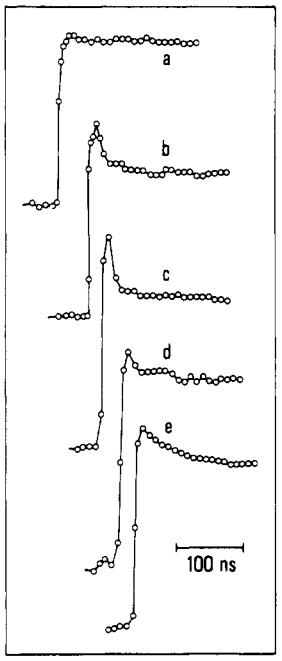

FIGURE 3: Geminate kinetics of heme–CO in various environments, 20 °C: (a) free heme–CO in aqueous buffer (50 mM Bis-Tris at pH 7); (b) heme–CO in 75% (v/v) glycerol–water; (c) BSA–heme–CO in aqueous buffer; (d) reconstituted Hb produced by mixing heme–CO with human globin; (e) native Hb–CO.

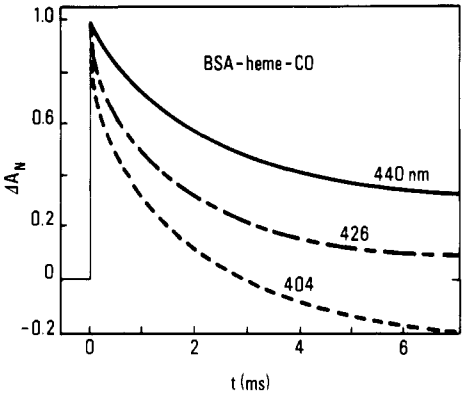

FIGURE 4: Bimolecular recombination kinetics of BSA–heme–CO complex at the detection wavelengths indicated, pH 7, 0.1 atm of CO, 20 °C. The curves were normalized to compare the relative amplitude of the fast and slow phases.

ligand diffusion to give an observable geminate reaction. The complex of heme with BSA likewise shows a geminate reaction (c). The geminate reaction in the heme–albumins is apparently faster than that in the hemoglobins. For protoheme–CO in aqueous media, escape via diffusion to the solvent is much faster than recombination, which implies a low probability for the geminate reaction. High-viscosity solvents decrease the diffusion rate and therefore increase the relative amplitude of the geminate phase, since the iron ligand barrier rate is little affected by solvent viscosity.

Bimolecular kinetics (rate proportional to CO concentration) at three detection wavelengths are shown in Figure 4. The three recombination curves were normalized to compare the relative proportions of the fast (1.5×10^6 M^{-1} s^{-1}) and slow (10^5 M^{-1} s^{-1}) components. The fast rate is intermediate to those of protoheme–CO and the rapid component (R state) of hemoglobin; the slow rate is more similar to those of myoglobin or the slow component (T state) of hemoglobin. At 440 nm the fast and slow bimolecular components are roughly equal in amplitude. The slow component has an isosbestic point near 426 nm. At 404 nm the two kinetic components

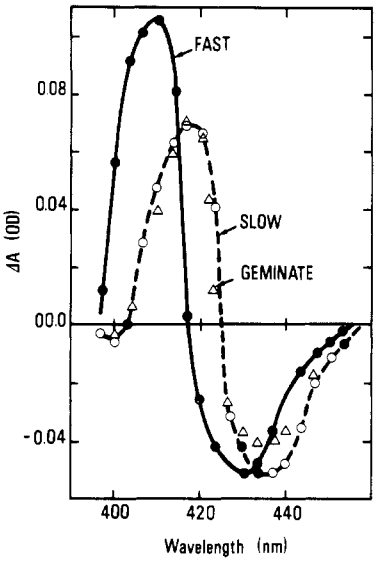

FIGURE 5: Correlation of the geminate reaction (△) of bovine hemalbumin with the fast (●) and slow (○) components of the bimolecular reaction of the same material. The solvent was 75% glycerol and 25% Bis-Tris (50 mM) pH 7, 20 °C.

Table I

condition	% slow	condition	% slow
aqueous		40% DMSO	
heme–BSA (1:3)	56	heme–HSA	59
heme–HSA (1:1)	69	heme–BSA	65
heme–HSA (1:10)	66	75% glycerol	
heme–HSA + sodium oleate (protein–oleate 1:1)	54	heme–HSA	87
		heme–BSA	58
heme–HSA + sodium oleate (protein–oleate 1:10)	61		
heme–HSA + pMB	28		

are opposite in sign (the curve at 404 nm is reversed in sign relative to the other two wavelengths).

The amplitude of the two major components was calculated at each wavelength by a two-exponential simulation to determine the kinetic difference spectrum for the two components. Figure 5 shows two well-defined components of nearly equal amplitude for the bimolecular kinetics of CO recombination with heme–albumin. Table I shows that this equality of amplitude is maintained under a variety of conditions: presence of oleate; heme–albumin ratio (1:1 or 1:10); 75% glycerol concentration; and the addition of 40% DMSO (v/v to the buffer). In all these conditions the results showed similar two-component kinetic profiles. This is particularly noteworthy for the heme–albumin ratios of 1:1 and 1:10 since it implies that essentially all of the heme in the reaction mixture was protein bound and that the two kinetic components cannot be ascribed to a mixture of free and protein-bound heme–CO.

The ratio of the amplitude of the two kinetic components is, however, significantly altered by reacting the albumin with *p*-(hydroxymercuri)benzoate (pMB), which increases the proportion of the rapid component to 70% (Table I). The pMB reacts with free sulfhydryl groups of proteins, and there is only one such residue on HSA (Peters, 1985).

Difference spectra for the two components in 75% glycerol are plotted in Figure 5. The spectrum of the fast component is similar to but not identical with that of protoheme–CO, while the spectrum of the slow component appears more like that of Hb. Also shown is the difference spectrum of the geminate reaction which is correlated in isosbestic wavelength and maximum amplitudes with the slow rather than with the fast component of the bimolecular reaction. At high viscosity,

albumin–heme–CO did not show the greatly nonexponential kinetics that extend to the microsecond time region, as observed with Hb and Mb.

The slow rebinding and the appearance of a geminate phase for CO after the addition of BSA or HSA to heme–CO suggested that for some of the hemes the environment was like that in Hb. To see if albumin also afforded protection against oxidation, stopped-flow experiments were performed in which deoxyalbumin–heme was mixed with oxygenated buffer. No evidence of a transient population of oxygenated intermediates was found. The time course of the oxidation reaction was independent of wavelength and of the concentration of added albumin. This suggested that little or no protection from oxidation was afforded by the albumin.

Throughout the experiments a slowly reacting species was observed (>10 ms) as a small percentage (<5%) of the total signal produced by both albumins and free heme. Addition of 40% DMSO to the buffers eliminated this slowest component. The presence of free heme dimers and polymers in aqueous solutions has been reported by Falk (1964). We suggest that the slowest component derives from polymeric heme compounds and will not be discussed further.

For similar amplitudes of the Soret peak, the heme–albumin complex shows weaker flash signals than free heme or Hb. This suggests that not all the ligands were photodissociated or some very fast component exists. Variation of the laser energy showed that, unlike free heme–CO, the fast bimolecular component has a much weaker yield than the slower phase. This yield was found to depend on the age of the sample and incubation time at higher temperatures. This could be due to protein denaturation that might expose residues like histidine; solutions of histidine with protoheme–CO also show a red shift in the Soret absorption spectra and heme-like bimolecular rebinding rates.

Discussion

The data presented here show three kinetic processes in the heme–albumins: a geminate process, a bimolecular heme-like process, and a bimolecular Hb-like process that is correlated spectrally with the geminate reaction. All of the heme is bound to a high-affinity site on the albumin in what are apparently two modes. This conclusion follows from the observation that the dissociation constant for the complex is too small to allow significant free heme to be present in the solutions [(2×10^{-8} M) Adams & Berman, 1980; (1.1×10^{-8} M) Beaven et al., 1974]. Experimental observation of nearly equal amounts of fast and slow components independent of the concentration of albumin supports this conclusion and also suggests that minor sites do not contribute to the observed kinetics.

Adams and Berman (1980) report that the binding of metheme to the single high-affinity site of albumin occurs as a two-stage process. They suggest that an initial external binding is followed by an enfolding of the heme by the albumin, with rate constants of 1.7×10^5 M^{-1} s^{-1} for initial binding about 6 s^{-1} for refolding. These rates are too slow to contribute to the kinetics described here.

The proportions of the two kinetics components did not change after the heme–HSA ratios were raised 10-fold. This result was reproduced with both conventionally prepared HSA (Kabi, Sweden) and with defatted HSA (Sigma). We are forced to suggest, therefore, that the spectral components which we observe, from both static and kinetic data, represent the binding of CO by heme bound in two different conformations to a single site on albumin molecules. It appears that these conformations may be influenced by the free SH group (Cys 34) in the albumin since the mercaptide derivative of this residue favors the heme-like rather than the Hb-like conformation (Table I).

The difference spectrum of the heme-like component is similar to, but distinct from, that of free heme. The recombination rates for this component are about 20% slower than those for free heme–CO. Given the high affinity of albumin for heme, the altered spectra, and CO combination rates, the data imply that the heme is bound and experiences a modified environment compared to free heme.

Since the addition of a fatty acid salt (sodium oleate) to the reaction mixture also failed to alter the combination rates or the proportion of the heme-like component, it seems probable that both components are strongly bound to the albumin. It was shown long ago (Smith, 1959) that binding of heme to a nitrogenous base, such as pyridine, slows CO binding to rates like those of hemoglobin. The implication is that there is no appropriate axial ligand within reach in the conformation with heme-like behavior. This is not inconsistent with the strong binding we observe, since photoporphyrin has been shown to bind strongly to albumin (Beaven et al., 1974; Lamola et al., 1981).

A geminate reaction of protoheme–CO is observable only in high-viscosity environments (Marden et al., 1986). The lack of a geminate reaction associated with the heme-like component of the kinetics suggests that ligands released from the heme bound in this configuration immediately experience the solvent viscosity, unshielded by structures analogous to the heme pocket of natural hemoproteins. Myoglobin also shows a weak geminate recombination of CO in aqueous solution; in this case the reaction rate is greatly reduced, which favors the ligand escaping to the solvent despite appreciable hindrance by the globin.

The slow bimolecular reaction of the heme–albumins is correlated spectrally with the geminate reactions (Figure 5). Since binding through an axial ligand is a general feature of porphyrin binding to HSA (Parr & Pasternack, 1977), the slow bimolecular component may arise as a consequence of the binding of heme with a nitrogenous base as an axial ligand. In addition, the geminate reaction indicates that the ligand released from these hemes does not at once reach the solvent. The heme–albumin complex shows several properties of native Hb, suggesting that the details of the structure of the heme pocket may be less significant than generally assumed. The albumin–heme–CO complex did show us geminate kinetics, but the fraction geminate was less sensitive to temperature and viscosity than for Hb or Mb. This might indicate a rigid protection of the ligand binding site, rather than the more flexible globin of Hb or Mb. The main differences between Hb and the heme–albumin complex are no protection to oxidation and a weaker solvent effect.

So many theories have been advanced to explain the variation in heme reactivity and the mechanism of ligand binding as to make full discussion impossible. Collman et al. (1983) suggest that distal hindrance may be important for model heme in organic solvents. Traylor et al. (1980), on the basis of a study of model hemes, selected proximal tension on the heme as the most significant protein influence. Hoffman et al. (1980) also argue that proximal effects are the most important in hybrid Hbs. Crystallographic work of Baldwin and Chothia (1979), theoretical considerations due to Gelin et al. (1983), and experimental resonance Raman work of Desbois et al. (1981), Friedman et al. (1985), and Scott et al. (1985) lead to the same conclusion.

The fraction of ligand recombination occurring as geminate depends on the competition between direct rebinding to the

iron atom and escape of the ligand from the vicinity of the heme. The rate of escape may be controlled (Marden et al., 1986) by varying the external viscosity. The presence of a globin as in native Hb or, as shown in the present work, by the addition of an albumin may provide the high-viscosity environment around the heme that will enhance the geminate phase. The mechanism of protein influence on the geminate reaction is a matter of serious debate.

Since the two kinetic components appear to be two conformations of the same binding site, one possible explanation is that the porphyrin ring is bound to the albumin, having two orientations of the heme group: with the ligand exposed to the solvent or with the ligand trapped between the heme and the albumin. This would explain the roughly equal populations of the two components and the fact that only one shows a geminate phase.

Additional experiments were performed to try to determine whether the iron atom was bound to the protein. The deoxy spectrum of heme–albumin shows a doublet for the Soret, suggesting the bindings of the two components are quite different. This supports the idea that only the Hb-like component has the iron bound, although it is then surprising that the two kinetic components exist is nearly equal proportions.

The spectrum of ferrous albumin–heme at high concentrations of KCN shows a Soret near 434 nm, characteristic of heme binding two CN molecules. This result supports the model with only the porphyrin bound, leaving the iron free to bind two CN molecules. However, both the deoxy and CN experiments probably perturb the system. They introduce new competition for binding of the heme groups; heme dimers or Fe–CN could displace the iron–protein interaction. The static deoxy spectrum is clearly different from that of the species generated by photolysis of CO in the albumin–heme–CO complex. Thus, no firm conclusions can be made from these modified systems. In conclusion, these experiments show that the detailed structure and arrangement of the heme pocket are not necessary to give the behavior associated with specialized hemoproteins in respect of the overall rate of reaction with CO and the geminate phenomenon; however, the protection against oxidation apparently requires a specific structure.

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