Sickle Hemoglobin Polymer Melting in High Concentration Phosphate Buffer

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ABSTRACT Sickle cell hemoglobin (HbS) prepared in argon-saturated 1.8 M phosphate buffer was rapidly mixed with carbon monoxide (CO)-saturated buffer. The binding of CO to the sickle hemoglobin and the simultaneous melting of the hemoglobin polymers were monitored by transmission spectroscopy (optical absorption and turbidity). Changes in the absorption profile were interpreted as resulting from CO binding to deoxy-HbS while reduced scattering (turbidity) was attributed to melting (depolymerization) of the HbS polymer phase. Analysis of the data provides insight into the mechanism and kinetics of sickle hemoglobin polymer melting. Conversion of normal deoxygenated, adult hemoglobin (HbA) in high concentration phosphate buffer to the HbA-CO adduct was characterized by an average rate of 83 s^{-1}. Under the same conditions, conversion of deoxy-HbS in the polymer phase to the HbS-CO adduct in the solution phase is characterized by an average rate of 5.8 s^{-1} via an intermediate species that grows in with a 36 s^{-1} rate. Spectral analysis of the intermediate species suggests that a significant amount of CO may bind to the polymer phase before the polymer melts.

INTRODUCTION

About one in six hundred African Americans is born with sickle cell disease (Rucknagel, 1975). The disease is characterized by abnormal rigidity and shape of the red blood cells. The abnormal properties of the red blood cells are due to a mutated form of hemoglobin, hemoglobin S (HbS) (Pauling et al., 1949). The mutation is a single point mutation (glu $\rightarrow$ val) at the sixth position of the β globin (Ingram, 1956). This substitution of a hydrophobic residue for a hydrophilic one results in the polymerization of HbS under certain conditions. It is believed that only T-state (low affinity) HbS molecules will polymerize (Sunshine et al., 1982; Padlan and Love, 1985). Thus at high ligand pressures, the hemoglobin S molecules are in the R-state (high affinity) and do not polymerize. Polymerization, under hypoxic conditions, increases cell rigidity and leads to microvascular occlusion. Thus sickle cell anemia is responsible for a high degree of morbidity and mortality.

The kinetics of polymerization has important implications with respect to the pathophysiology of the disease. A red blood cell typically spends $\sim$10 s under hypoxic conditions during the 14 s spent in one round of circulation (Mozzarelli et al., 1987; Altman and Dittmer, 1971). If, after oxygenation at the lungs, the red cell can pass through the microvasculature without becoming rigid due to HbS polymerization, then an occlusion event is avoided. The kinetics of polymerization has been explained in terms of a double nucleation mechanism (Ferrone et al., 1985a, b). Homogeneous nucleation involves the joining of a sufficient number of hemoglobin molecules to form a critical nucleus. After the critical nucleus has formed, the growth of a polymer from this nucleus becomes thermodynamically favorable. The growth of the polymer is governed by the relation $\alpha = k_+(\gamma c_0 - \gamma_s c_s)$, where α is the rate of polymer growth, γ refers to an activity coefficient, c_0 is the total hemoglobin concentration, c_s is the solubility, and k_+ is a rate constant. Heterogeneous nucleation involves the formation of an additional polymer on the surface of an existing one. Homogeneous nucleation is the rate-limiting step in polymer formation. Polymer formation is therefore characterized by a long delay time during which no polymer is detected. Once the polymer begins to grow, more surface area becomes available for heterogeneous nucleation to occur so that polymer growth becomes exponential. Thus, prolonging the delay time for polymer formation can be beneficial in that a cell is more likely to traverse the microcirculation without an occlusion event. Several therapies have been devised to this end.

The mechanism and kinetics of polymer melting have not been as widely studied as those of polymerization, and the pathophysiological importance of melting kinetics is controversial. However, it is certain that if all the polymers do not melt before a cell enters hypoxic conditions, then the remaining polymers could promote relatively rapid growth of other polymers (through heterogeneous nucleation) and cause occlusion. The absence of a delay time observed during melting (Mozzarelli et al., 1987) has led to the prediction that melting upon re-oxygenation at the lungs is fast (Ferrone, 1994). Other early studies indicate that melting is slow: on the order of tens of seconds (Hofrichter et al., 1974; Moffat and Gibson, 1974; Messer et al., 1976). More recently, Briehl and co-workers have used differential interference contrast microscopy coupled with ligation control through the use of photolytic epi-illumination to observe the growth and melting of HbS polymers (Samuel et al., 1990;

Received for publication 28 September 1998 and in final form 21 December 1998.

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0006-3495/99/04/2216/07 $2.00

Briehl, 1995). Melting was observed to take seconds to tens of seconds for completion. It was suggested that the same equation that governs the rate of polymer growth governs that of melting with $\gamma c_0 < \gamma_s c_s$ (Briehl, 1995). The solubility, c_s, depends on the degree of ligation of hemoglobin molecules. Therefore, one important factor in determining the kinetics of melting is the kinetics of ligand binding, both of the monomer and polymer phases.

The equilibrium bindings as well as the kinetics associated with binding of CO or oxygen to solution phase HbS are the same as to normal adult hemoglobin, HbA (Allen and Wyman, 1954; Penneley and Noble, 1978; DeYoung and Noble, 1981; Shapiro et al., 1994). The oxygen binding curve of HbS polymers was measured using linear dichroism spectroscopy, which is able to distinguish between absorption due to polymer HbS from that in the solution phase (Sunshine et al., 1982). A similar study was conducted using CO (Hofrichter, 1979). By using spectral decomposition of HbS linear dichroism spectra, it was found that HbS polymer has ~⅓ the oxygen affinity of T-state HbA. The ligation kinetics of sickle red blood cells was studied by Harrington et al. using a stopped-flow apparatus (1977). It was found that the presence of HbS polymers caused the rate of oxygen uptake to be about twice as slow as when polymers were absent. A question that was not addressed in the work of Harrington et al. was how much oxygen binding was occurring directly to the polymers as opposed to solution phase molecules. Harrington et al. observed pseudo-first-order kinetics in oxygen binding to sickle cells occurring on a time scale that is faster than some preliminary studies showed melting to be completed. This leads one to believe that the oxygen uptake measured in the work by Harrington et al. was dominated by solution phase rebinding and that a slower phase corresponding to direct rebinding to the polymer and/or rebinding to molecules that come off the polymer upon melting went unnoticed. Slow rebinding of the polymer phase has been measured following laser photolysis of a partially CO-saturated gel (Shapiro et al., 1995). Using time-resolved linear dichroism to selectively probe the ligation state of the polymer phase, it was found that the rate of CO rebinding to HbS polymers is ~1000 times slower than to solution phase molecules (Shapiro et al., 1995, 1996).

The solubility of HbS decreases exponentially with increasing potassium phosphate concentration (Adachi and Asakura, 1979). The possibility of working with large volumes of relatively dilute solutions containing HbS polymers makes the use of high concentration phosphate buffers potentially useful in stopped-flow measurements. The structure and kinetics of formation of gels formed in high concentration phosphate buffers are similar to those formed at physiological salt concentrations (Adachi and Asakura, 1979, 1981; Wang et al., 1996; personal communication from R. Josephs, 1998, Dept. of Molecular Genetics and Cell Biology, University of Chicago).

We have measured the kinetics of polymer melting and binding of CO to HbS prepared in 1.8 M phosphate buffer. Suspensions of HbS in argon-saturated buffer were rapidly mixed with CO-saturated buffer. Changes in the turbidity of the mixture were attributed to polymer melting, while changes in the absorption profile were used to follow CO-HbS combination. The data obtained in these studies are used not only to determine the kinetics of polymer melting and HbS-CO combination, but also to further understand the mechanism of polymer melting. The slow rebinding of CO to HbS polymers measured in photolysis experiments implies that melting would occur mainly by CO binding to HbS molecules as they come off the polymer, rather than through direct CO ligation to HbS molecules in the polymer phase. However, the present study suggests that, at least in high concentration phosphate buffer, a significant amount of polymer phase HbS molecules might bind CO during the process of polymer melting.

MATERIALS AND METHODS

Hemoglobin S was obtained from excess blood originally donated by patients homozygous in HbS with <6% fetal hemoglobin, following federal regulations and guidelines outlined by the National Institutes of Health. The hemolysate was prepared as described previously (Geraci et al., 1969). Cells were washed in 0.95% NaCl and lysed by incubation in distilled water. Samples were then pelleted in liquid nitrogen for storage. Hemoglobin A was prepared similarly. To assess the contribution of fetal hemoglobin (and other minor components) some measurements were also taken with HbS purified by ion-exchange chromatography using a DE-52 matrix (Whatman) developed with 0.05 M Tris-HCl, pH 8.3. The results obtained from the purified HbS did not differ significantly from results using the hemolysate. The hemoglobin was added to an argon-saturated, 1.8 M, pH 7.3, potassium phosphate buffer solution yielding a final Hb concentration of 0.85 mM. After further exposure to an argon atmosphere, sodium dithionite (Sigma Chemical Company, St. Louis, MO) in a final concentration of 3 mM was added to scavenge residual oxygen. Increased turbidity indicated the presence of HbS polymers. HbS solubility was measured by centrifugation of the HbS and determination of the concentration remaining in the supernatant. Extinction measurements were performed on an OLIS RSM 1000 Spectrophotometer (Bogart, GA) coupled with the OLIS-U.S.A. stopped-flow apparatus. The two solutions were mixed in ratio of 1 part Hb buffer to 2.5 parts CO buffer (giving a final concentration of 0.24 mM Hb and ~0.63 mM CO). This ratio was chosen to obtain an excess of CO for the reaction. The spectrometer measured 1000 spectral scans per second. All measurements were made at room temperature. The kinetic data were analyzed using Specfit (Spectrum Software Associates, Chapel Hill, NC) for singular value decomposition (SVD) and global analysis (Golub and Reinsch, 1970; Henry and Hofrichter, 1992). The percentage of pure species in the intermediate spectra was determined by a least-squares fit to known species normalized by their extinction coefficients (Di Iorio, 1981) and to a scattering function that varied as $1/\lambda^a$, where λ is the wavelength and a is an adjustable parameter computed to be 1.5 ± 0.4.

RESULTS

The absorption spectra obtained after rapid mixing of deoxy-HbA and CO-saturated (1.8 M phosphate) buffer are shown in Fig. 1 *A*. Each scan was obtained in 1 ms. The scans are shown from 29 to 69 ms after mixing in steps of 5 ms. The disappearance of the deoxy-HbA species is evidenced by the decrease in absorbance at 555 nm. The broadening of the peak at 555 nm is probably due to some CO binding within the dead time of the instrument. The

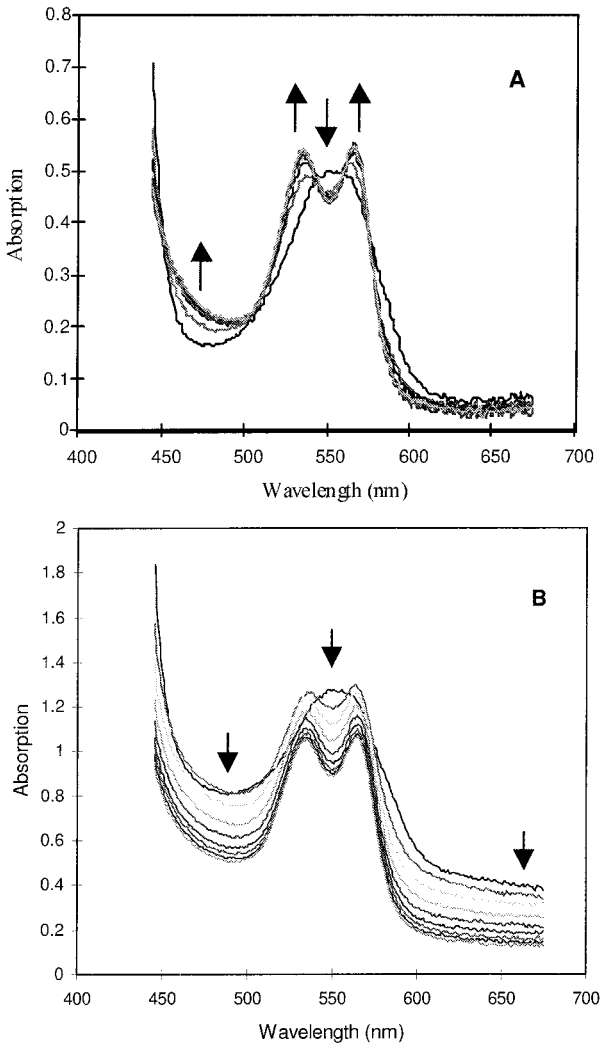

FIGURE 1 Absorption spectra of deoxygenated hemoglobin rapidly mixed with CO-saturated buffer. (*A*) HbA spectra spaced 5 ms apart, beginning 29 ms after initial mixing. The arrows indicate the growing of absorption peaks of HbCO and the disappearance of deoxy-HbA. (*B*) HbS spectra are spaced 30 ms apart, beginning 25 ms after initial mixing. The growing in and disappearance of the absorption peaks are similar to those of HbA (the initial species has a single broad peak at 555 nm and the final has two peaks at 540 and 570 nm). However, as the arrows indicate, the decreasing turbidity results in overall decreased apparent absorption as a function of time. The absorption at 570 nm first increases due to CO binding and then decreases due to diminished scattering. The *y* axis label in (*B*), "Absorption," is actually absorption and scattering; the apparatus does not distinguish between the two effects. The peak at 555 nm for the deoxy species appears broadened for both HbS and HbA due to binding of CO that occurs during mixing.

conversion to the CO-HbA adduct results in the increase in absorption at 540 and 570 nm. Spectra obtained after rapid mixing of deoxy-HbS and CO buffer under the same conditions as those used for HbA are shown in Fig. 1 *B*. The spectra shown were taken from 25 to 265 ms after mixing in steps of 30 ms. The shape of the curves resemble those of the mixtures containing HbA, indicating the conversion of deoxy-HbS to HbS-CO. The displacement of the spectra is due to increased turbidity resulting from the scattering of the HbS polymers. As CO binding continues, the turbidity decreases.

The kinetics after rapid mixing of deoxy-HbA or deoxy-HbS with CO was analyzed by SVD and global analysis. The HbA data collected consistently fit well to a single exponential process, whereas the HbS data could not be consistently fit to a single exponential process but could be fit consistently to two exponential processes. The species involved in a typical fit to the HbA mixture are shown in Fig. 2 *A*. The data are summarized by the deoxy-HbA species (having a single absorption peak at 555 nm) converting to the carboxy-HbA species (having absorption peaks at 540 and 570 nm) with a rate constant of 101 s^{-1} in this instance. The three species involved in a typical fit to the HbS mixture are shown in Fig. 2 *B*. The initial species can be described by a deoxy-HbS absorption spectrum displaced due to scattering. Under these conditions, almost all of the HbS is in the polymer phase (Adachi and Asakura, 1979). The intermediate species, which grows in with a rate of 44.2 s^{-1}, can be described by displaced partially CO-

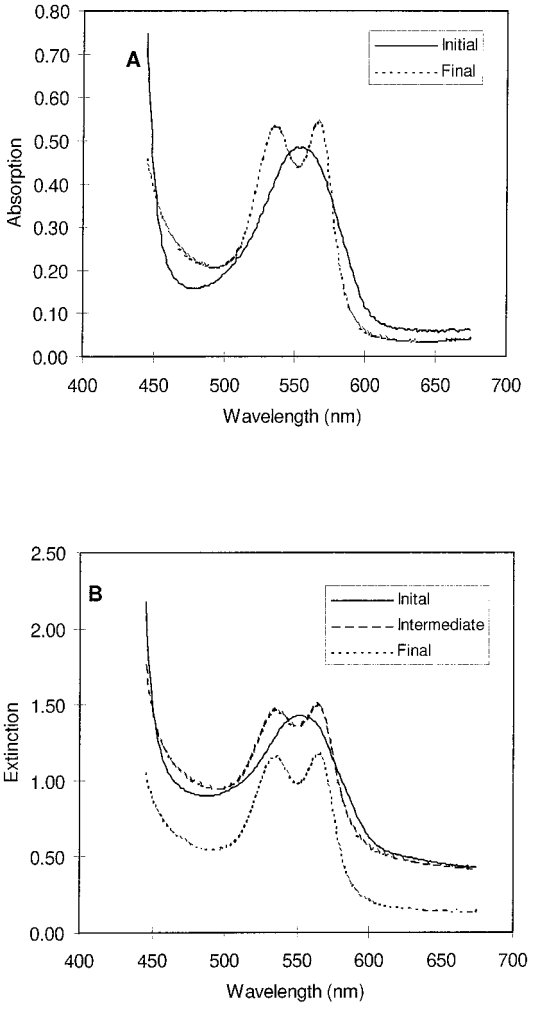

FIGURE 2 Species determined by SVD and global analysis of the Hb mixtures. (*A*) HbA. (*B*) HbS.

ligated HbS. The nature of this intermediate is examined further below. The final species is the fully ligated HbS-CO in the solution phase, which grows in with a rate of 9.9 s^{-1}. Table 1 shows the average rates found in the analysis of 125 reactions of HbS and 22 reactions of HbA.

Examination of the intermediate species in Fig. 2 *B* indicates that a large degree of CO binding occurs without a large reduction in scattering. In fact, when the larger extinction coefficient for deoxy-Hb at 650 nm is compared to that of Hb-CO, it appears that the scattering from the intermediate is greater than that of the initial species. It is unlikely that any more polymer would form after mixing. How then can the intermediate species scatter more than the initial species? We believe that this is due to the increased polarizability of HbCO compared to deoxy-Hb. Ignoring interaction effects within the macromolecules, the scattering from the polymer phase will be proportional to $|\alpha|^2/\lambda^4$. The real and imaginary components of the polarizability can be found from the electronic absorption spectrum using the relations (Devoe, 1964, 1965),

$$\mathrm{Im}(\alpha) = A(\nu)\frac{C_1\epsilon(\nu)}{\nu} \tag{1}$$

and

$$\mathrm{Re}(\alpha) = A(\nu)\frac{2C_1}{\pi}P\int_0^\infty \frac{\epsilon(\nu)\mathrm{d}x}{x^2-\nu^2}$$

where ν is the frequency, P refers to the principal part, x is a variable of integration, ϵ is the measured Molar extinction coefficient, $C_1 = 6909c/8\pi^2N_0$, with c representing the speed of light, N_0 is Avogadro's number, and $A(\nu)$ is a function of ν that depends on the index of refraction of the solvent that is nearly unity for water (Devoe, 1964, 1965). A plot of $(|\alpha|^2)/(\lambda^4)$ for HbCO and deoxy-HbCO, obtained from absorption spectra taken from 188–850 nm, is shown in Fig. 3. The larger polarizability of HbCO compared to deoxy-Hb is probably due to the larger absorption in the Soret.

To further interpret the nature of the intermediate species formed during CO binding and polymer melting, we have determined the contributions to the spectrum from scattering and solution phase deoxy-Hb and HbCO using a least-squares analysis. As the aggregates of polymers were significantly >1/10 the wavelength of light, their scattering did not have a $1/\lambda^4$ dependence. The scattering is quite complicated, as the particles are large and have large polarizabilities. We used a scattering function A/λ^x where both A and x were variable parameters. Fig. 4 shows the components used (A) and the result (B) of a typical fit of the intermediate species. The results of fitting 125 spectra are shown in Table 2. Fits of the initial species included very little of the CO-bound species, as the final species included very little of the ligand-free species. The intermediate species was found to be ~80% ligated.

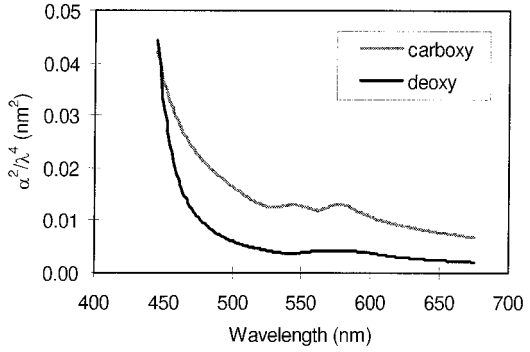

FIGURE 3 Predicted wavelength-dependence of scattering for deoxy- and carboxy-Hb. The polarizability, α, was calculated from absorption spectra taken from 188 nm to 850 nm of HbA, and using the Kramer-Kronig relation.

In addition to the above analysis of the intermediate spectra, the mechanism of polymer melting was analyzed by another method. Each individual spectrum obtained after mixing was broken into its deoxy, carboxy, and scattering components. The kinetics of the changes in the contribution of each component was then analyzed. An example of this analysis is shown in Fig. 5. From the analysis of 17 mixtures we found that deoxy-HbS decayed with an exponential rate of (23 ± 6) s^{-1} and the HbCO grew in with a rate of (23 ± 6) s^{-1}, whereas the scattering decayed with a rate of (7 ± 3) s^{-1}. Note that the lower limit of this latter rate (4 s^{-1}) corresponds to relatively slow disappearance of scattering.

TABLE 1 Exponential rates of processes following CO-Hb mixing

Species	Rate (s^{-1})
HbA*	83 ± 20
HbS$^{\#}$ intermediate	36 ± 14
HbS$^{\#}$ final species	5.8 ± 5.4

Values are given ± one standard deviation.
*Average of 22 mixtures.
$^{-}$Average of 125 mixtures.

DISCUSSION

We have found that CO binding-induced HbS polymer melting occurs via an intermediate species that grows in with an average rate of 36 s^{-1} followed by conversion of the intermediate to solution phase HbCO characterized by a 5.8 s^{-1} rate. These results should be compared to CO binding to HbA under the same conditions characterized by a 83 s^{-1} rate. Analysis of the intermediate species indicates that a large amount of CO may bind to the HbS polymers during the process of melting in 1.8 M phosphate buffer.

The bimolecular rate of CO binding to HbA is between 100 and 300 (s^{-1} mM^{-1}) (Bertini et al., 1994; Hassinoff, 1981). We have found that the solubility of CO in 1.8 M phosphate buffer is ~0.88 mM in one atmosphere CO at room temperature. Thus the concentration of CO was not in extreme excess compared to that of Hb in our mixtures and

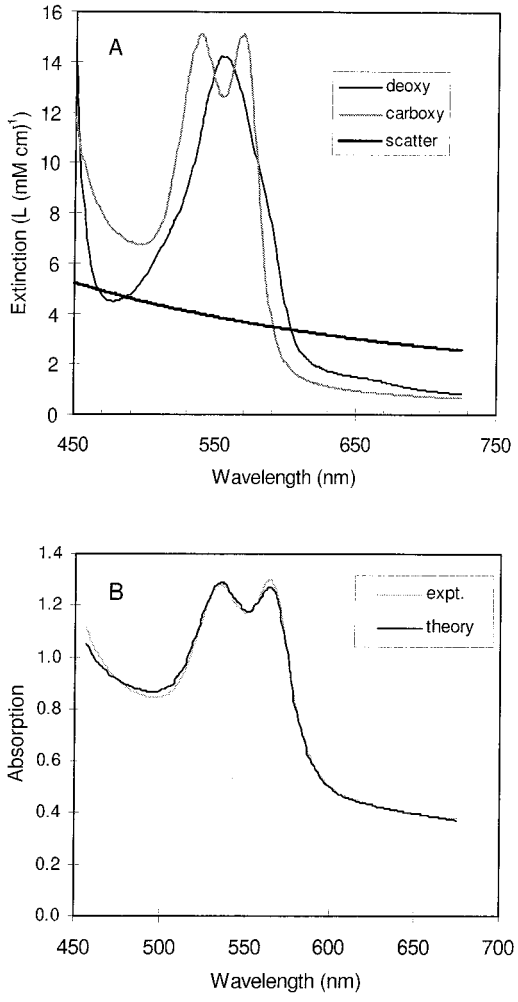

FIGURE 4 Determination of the contributions of deoxy- and carboxy-HbS, and $1610/\lambda^{1.3}$ scattering to the intermediate species (see Fig. 2 and Results). (*A*) The three species used. The coefficient for the scattering is arbitrary. (*B*) A typical fit to the intermediate species, using the components from (*A*).

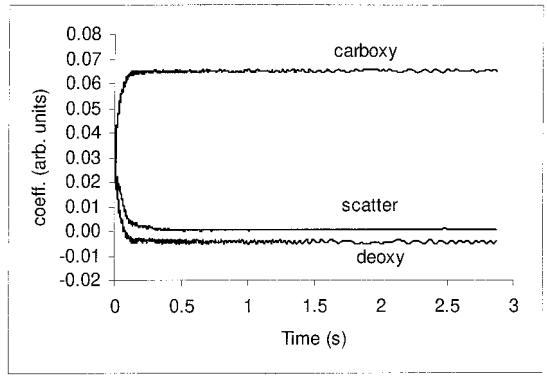

FIGURE 5 Time series of deoxy-hemoglobin, carboxy-hemoglobin, and scattering contributions for a complete set of spectra. These components (shown in Fig. 4) are fit to the raw data. The coefficients for the deoxy and carboxy components are proportional to the concentrations of those species, relative to each other. The scattering coefficient is normalized to an arbitrary value. The analysis showed that the deoxy component decayed with a rate of 29 s^{-1}, the carboxy component decayed with a rate of 31 s^{-1}, while the scattering decayed with a rate of 14 s^{-1}. The deoxy component becomes slightly negative due to small variations in wavelength calibration, which results in a small difference between the time-resolved data and the components shown in Fig. 4. The scattering component does not go completely to zero due to baseline offset.

the early part of CO-Hb ligation may have digressed slightly from pseudo-first-order exponential kinetics. However, if we calculate the bimolecular rate constant based on a CO concentration of 0.88 mM we find a bimolecular rate constant of 94 ± 23 $(s \cdot mM)^{-1}$. This is in reasonable agreement with previous studies with normal salt concentrations. The lack of a large excess in the concentration of CO could explain the variations observed in rate constants for CO combining with both HbA and HbS.

TABLE 2 Relative concentrations of carboxy- and deoxy-hemoglobin for the three species in the HbS-CO reaction

Parameter	Initial	Intermediate	Final
Deoxy spectrum	4.7 ± 1.3	1.3 ± 0.7	0.5 ± 0.5
Carboxy spectrum	1.2 ± 0.7	4.7 ± 1.1	5.7 ± 1.3

The coefficients were determined by fitting to the model A *[deoxy-HbS] + B*[carboxy-HbS] + C/λ^D, with A, B, C, and D varied. Values are given ± one standard deviation.

There are at least four processes that may occur after mixing CO with HbS in the polymer phase: melting of deoxy-polymer, melting of ligated polymer, binding of CO to the polymer phase molecules, and binding of CO to the monomer phase molecules. Thus the kinetics can be complicated. We have tried to analyze the data using SVD and global analysis (Fig. 2) and by directly fitting the data to pure species (Fig. 5). Whereas the latter method looks at the total amount of hemoglobin ligation (both polymer and monomer phase) separately from the amount of polymer present, the former looks at both processes at once and reveals an intermediate that has both ligation and polymer content information. Analysis of the intermediate species shows that it is ~80% ligated (Table 2). Further analysis demonstrates that the decay in scattering observes slower kinetics than the CO saturation (Fig. 5). There are at least three models that could explain these phenomena. One entails the CO binding to monomer phase HbS rapidly, and binding to the polymer phase much more slowly. A second model consists of CO binding to monomers that result from rapid melting of polymers (such as those at the edges of domains or small loosely packed domains). Finally, the intermediate state could be due to CO binding directly to the polymers.

The first model, while certainly true, cannot be the sole explanation because there aren't enough monomers. The solubility of HbS in 1.8 M phosphate at 30°C is ~15 μM (Adachi and Asakura, 1979). In our experiments at room temperature we measured the solubility of deoxy-HbS to be (at most) 120 μM (14% of the HbS before mixing). [The presence of small aggregates left in the supernatant could explain the surprising increase in our determination of the

solubility at room temperature compared to that reported for 30°C. If the solubility is in fact lower, our argument against significant initial binding to the monomer phase is strengthened.] Even at room temperature, the percentage of solution phase molecules is so low that they could not account for the large amount of ligation that occurs before there is a reduction in scattering. This is evidenced by comparison of the 14% of HbS in the solution phase to the percentage ligation of the intermediate species (80%). Analysis of the kinetics of disappearance of scattering and ligation to HbS (Fig. 5) also do not support a model where there is rapid binding to the solution phase molecules followed by binding molecules that come off the polymers. Such a model predicts that scattering and binding follow the same kinetics (after the initial binding to the polymer phase), and that is not observed. In addition, if there were a significant amount of binding to solution phase molecules, then we would expect that the formation of the intermediate species would be governed by the same rate constant as that governing CO binding to HbA; but that is not the case (see Table 1).

The second model is a viable explanation of the data provided that the melting of the small or loosely packed polymers (to which binding occurs) proceeds without a significant reduction in scattering. Examination of the intermediate species (Fig. 2 *B*) taken together with the fact that deoxy-HbS has a higher extinction coefficient at 650 nm suggests there is no significant decrease in scattering upon formation of the intermediate. The fact that light scattering decreases as size decreases then suggests there is no melting upon formation of the intermediate. However, it is possible that some melting occurs but that the decrease in scattering accompanying the melting is not detectable. Light scattering and turbidity have often been used to detect polymer content (Eaton and Hofrichter, 1990). However, an exact theory of scattering from sickle cell hemoglobin gels has not been made (partly due to a lack of structural information concerning higher order aggregates). The use of a realistic model for the polarizability (Fig. 3) is an improvement, but far more work needs to be done. It is quite possible that some melting occurs from smaller domains or the edges of domains without a detectable change in scattering. This group has been working on improving both light scattering theory and detection capability (Kim-Shapiro and Hull, 1997). However, at this time, we cannot exclude the second model where some undetectable melting occurs as an explanation of our observations. Nevertheless, we do suggest that if scattering is accepted as a measure of polymer content, then the third model (involving CO binding to the polymers) is most likely. Further work is underway to clarify this point.

The amount of CO binding to the polymer phase during melting could seem surprising given that the on-rate for CO to polymer phase HbS was measured to be 1000 times slower than to HbA (Shapiro et al., 1995, 1996). If binding of CO to the polymer phase in 1.8 M phosphate buffer were 1000 times slower to HbS than to HbA, then one would not expect to have the observed CO binding to the polymer phase growing in with a rate of 36 s^{-1}. As the rate for binding to HbA was observed, here, to be 83 s^{-1}, then a rate that is 1000 times slower would be 0.083 s^{-1}. Such slow binding of CO would probably lead to a mechanism of melting where no direct binding to the polymer phase occurred, but rather binding occurred to molecules that came off the polymer into solution.

There are several differences between the present study and the earlier photolysis study measuring a slow CO on-rate to the polymer phase that might explain the discrepancy. The earlier study involved CO photolysis of a partially saturated HbS gel prepared in 0.1 M sodium phosphate buffer (Shapiro et al., 1995, 1996). Time-resolved linear dichroism was used to monitor the kinetics of CO rebinding to the polymer phase. An immediately apparent difference between the photolysis experiment and the present, stopped-flow study is that the former was performed on a highly concentrated solution (~20 mM in heme) whereas the latter involved dilute solutions. It is possible that the increase in exposed surface area in the dilute solutions can account for the faster kinetics observed here. Binding to molecules that are exposed to the solvent may be easier than to molecules that are surrounded by other protein molecules. An earlier study did not show any dependence of CO on domain size (Shapiro et al., 1996). Larger domains have less exposed surface area. However, the range in difference of domain sizes achievable in the studies in low phosphate is far less than that compared to studies in high phosphate. In addition, there is a significant difference between the photolysis studies and the stopped-flow studies. An interpretation put forth by Shapiro et al. (1996) was that following photolysis of the partially saturated gel, most CO rebinding occurred quickly to the monomer phase after which a small amount of CO rebinding occurred to the polymer phase. The rebinding to the polymer phase was said to be due to CO molecules that were trapped in the polymer matrix; that is, their diffusion was limited by inhibited protein internal motions or blocked entry and exit sites for the CO. In the stopped-flow experiments presented here, there are very few solution phase molecules around for the CO to bind to after mixing. Thus, in the mixing experiment the polymer phase HbS molecules do not have to compete with solution phase HbS molecules and the CO can bind to polymer phase HbS molecules exposed on the surface of the polymer. Finally, it is possible that the CO binding properties of the HbS polymer formed in 1.8 M phosphate buffer are fundamentally different from those of HbS polymers formed in 0.1 M phosphate buffer. Indeed, the equilibrium binding to the HbS in the polymer phase in high phosphate was reported to be greater than that in low phosphate buffer (Adachi and Asakura, 1982). The differences between ligand binding properties of HbS polymers prepared in low and high phosphate buffer are among the future work that will be done in order to assess the pathophysiological implications of the mechanism and kinetics of polymer melting.

The authors thank Dr. Frank A. Ferrone for helpful discussion.

This work was supported in part by National Institutes of Health Grants HL58091 (to D.B.K-S.) and HL38632 (to S.K.B.).

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