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Temperature and domain size dependence of sickle cell hemoglobin polymer melting in high concentration phosphate buffer

J.G. Louderback[a], S.Kh. Aroutiounian[a], W.C. Kerr[a], S.K. Ballas[b], D.B. Kim-Shapiro[a,*]

[a] *Department of Physics, Wake Forest University, Winston-Salem, NC 27109-7507, USA*
[b] *The Cardeza Foundation, Department of Medicine, Jefferson Medical College, Philadelphia, PA 19107, USA*

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Abstract

Deoxygenated sickle cell hemoglobin (Hb S) in 1.8 M phosphate buffer, and carbon monoxide (CO) saturated buffer were rapidly mixed using a stopped-flow apparatus. The binding of the CO to the Hb S polymers and the polymer melting was measured by time resolved optical spectroscopy. Polymer melting was associated with decreased turbidity, and CO binding to deoxy-Hb S was monitored by observation of changes in the absorption profile. The reaction temperature was varied from 20°C to 35°C. Polymer domain size at 20°C was also varied. The data for mixtures involving normal adult hemoglobin (Hb A) fit well to a single exponential process whereas it was necessary to include a second process when fitting data involving Hb S. The overall Hb S–CO reaction rate decreased with increasing temperature from 20°C to 35°C, and increased with decreasing domain size. In comparison, Hb A–CO reaction rates increased uniformly with increasing temperature. Two competing reaction channels in the Hb S–CO reaction are proposed, one involving CO binding directly to the polymer and the other involving CO only binding to Hb molecules in the solution phase. The temperature dependence of the contribution of each pathway is discussed.

Keywords: Sickle cell hemoglobin; Depolymerization; Kinetics; Temperature; Domain size; Melting

* Corresponding author.

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1. Introduction

Sickle cell disease is caused by a single point mutation, β6 glutamate to valine in hemoglobin [1]. The substitution of this hydrophobic residue for the hydrophilic one results in the polymerization of the mutant form of hemoglobin, Hb S, under certain conditions. The polymerization of Hb S causes increased rigidity and deformation of the erythrocyte leading to microvascular occlusion. Sickle cell disease is responsible for a high degree of morbidity and mortality.

Only the low-affinity T-state quaternary form of Hb S is believed to enter the polymer phase [2,3]. Thus oxygenation at the lungs reduces the tendency for polymerization. Due to the kinetics associated with polymerization, many erythrocytes can make it through circulation without becoming rigid or deformed [4]. The double nucleation theory explains most phenomena associated with the kinetics of polymerization [5,6]. According to this theory, polymerization begins with thermodynamically unfavorable aggregation of Hb S to form a critical nucleus. Once the critical nucleus is formed (through homogeneous nucleation), addition of Hb S molecules to the aggregate becomes thermodynamically favorable and the polymer grows. In addition, new polymers can nucleate on the surface of existing ones through heterogeneous nucleation. Thus polymerization is characterized by a long delay time where no polymer can be detected, followed by rapid polymer growth. Since its formation is a relatively rare event, each critical nucleus becomes a cluster of polymers known as a domain.

If a cell containing polymer reaches the lungs, then oxygenation will lead to melting. If polymer melting is not complete by the time the cell enters the tissues where the oxygen pressure is low, then any remaining polymers can act as seeds for relatively rapid polymerization since there will be no delay time. A major goal of our laboratory is to understand the mechanism and determine the kinetics of sickle cell hemoglobin polymer melting. Some early studies have indicated that polymer melting is slow on a time scale compared to the transit time of an erythrocyte [7–9]. Although it has been less well studied than polymerization there has been some important recent work on polymer melting. Based on his observations using differential interference contrast microscopy, Briehl proposed that polymer melting can be seen as the reversal of growth, with hemoglobin molecules coming off the polymer ends [10]. Our laboratory has recently observed polymer melting using stopped flow mixing of carbon monoxide (CO) and Hb S polymers prepared in 1.8 M phosphate buffer [11]. The use of high concentration phosphate buffer enables us to work at lower concentrations of Hb S than is necessary if working in 0.1 M buffer [12]. The structure and kinetics of formation of gels formed in high concentration phosphate buffers are similar to those formed at physiological salt concentrations [12–14]. In this work we examine the effects of temperature and polymer domain size on the kinetics of melting.

2. Materials and methods

Hemoglobin S was obtained from excess blood originally donated by patients homozygous in Hb S with $< 6\%$ fetal hemoglobin, following federal regulations and guidelines outlined by the National Institutes of Health. The hemolysate was prepared as described previously [15]. 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 in the same manner. Previous work [11] has indicated that the presence of $< 6\%$ fetal Hb in our samples does not have a significant effect on experimental results. For most of the experiments, the hemoglobin was added to enough argon-saturated 1.8 M potassium phosphate buffer at pH 7.3 to produce a final Hb concentration of 0.60 ± 0.13 mM. The experimental results proved independent of the hemoglobin concentration over this range. After further exposure to argon, sodium dithionite (Sigma Chemical Company, St Louis, MO) was added at a 1.7-mM concentration to scavenge oxygen. The Hb solution was then placed in a temperature-controlled water bath, and allowed to reach equilibrium. To study the effects of the size of the Hb S aggregates on

melting kinetics, polymerization was rapidly induced by adding Hb S to an argon-saturated buffer which already contained sodium dithionite. This latter method resulted in smaller polymer domain sizes, as evidenced by the reduced scattering. All of the experiments on small polymer domains were conducted at room temperature.

An OLIS RSM-1000 Spectrophotometer (Bogart, GA) with the stopped-flow option was used for extinction (absorption and scattering) measurements. The hemoglobin solution was mixed with a CO-saturated 1.8-M buffer in a ratio of 1:2.5. This ratio ensured an excess of CO for the reaction Hb + CO → Hb–CO.

Temperature regulation during the reaction was achieved via a water bath surrounding the deoxy-Hb and the CO buffer solutions while they were in the RSM-1000. The stopped-flow reaction chamber extended below the bath. Consequently, before each data run a clearing shot was fired to remove any products which may have cooled during the previous reaction. Immediately after this clearing shot was fired, an actual data run was started.

3. Results

Typical time-resolved absorption spectra for the Hb A–CO reaction are shown in Fig. 1. Fig. 1a was taken at 20°C, Fig. 1b at 30°C, and Fig. 1c at 35°C. These spectra are qualitatively alike, differing only in the speed of the reaction (summarized in Table 1). The Hb A goes from deoxygenated to fully ligated, as shown by the change in absorption profile from a single peak to two peaks. There are no signs of turbidity in these spectra. This contrasts with the spectra for reactions involving Hb S at 20°C, 30°C, and 35°C presented in Fig. 2a–c, respectively. Here the initial, deoxygenated, traces are displaced upwards relative to the final, ligated, traces. In order to show the complete reaction, in Fig. 2b,c the time between traces doubles with each trace. There are two processes going on in these Hb S–CO reactions. First, CO is binding to the Hb S molecules, as evidenced by the same change in profile as seen with the Hb A. In addition, there is a scattering

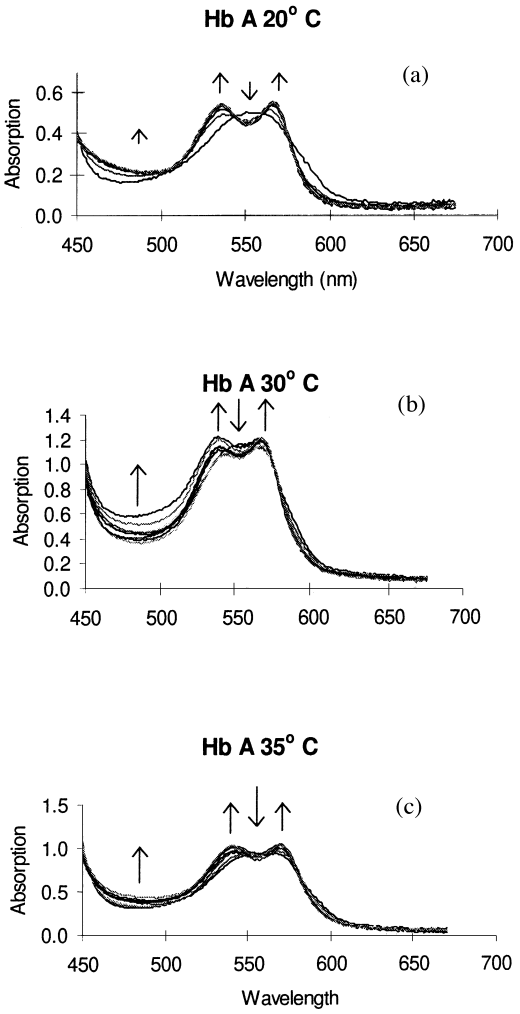

Fig. 1. Absorption spectra of deoxygenated normal hemoglobin (Hb A) rapidly mixed with CO saturated buffer. The arrows show the conversion of deoxy-Hb A, which has a single peak, into Hb A–CO, which has two peaks. (a) 20°C, the spectra begin 17 ms after initial mixing, and are spaced 5-ms apart. (b) 30°C, the spectra begin 24 ms after initial mixing, and are spaced 3-ms apart. (c) 35°C, the spectra begin 21 ms after mixing, and are spaced 2-ms apart.

component which decreases over time. We attribute this change to a decrease in polymer concentration in the sample.

SVD and global analysis [16,17], as implemented by Specfit (Spectrum Software Associates, Chapel Hill, NC) was used to determine the kinetics of the Hb–CO reaction. The Hb A + CO

Table 1
Average exponential rate constants for Hb A reacting with CO in 1.8 M phosphate buffer

Temperature	Initial → final (s^{-1})
20°C[a]	80 ± 20
30°C[b]	130 ± 20
35°C[c]	150 ± 40

[a] 22 mixtures.
[b] 50 mixtures.
[c] 47 mixtures.

reaction data reproducibly fit to a single exponential process. Table 1 shows how this rate varies with temperature. Representative examples of the two species for this process are shown in Fig. 3. These species closely resemble the deoxy- and carboxy-forms of Hb A, although the peak in the initial species is slightly broader than a pure deoxy-Hb peak would be. This is due to some CO binding to the hemoglobin during the dead time of the stopped-flow apparatus.

The Hb S data could not be reproducibly fit to a single exponential process; they could, however, be fit to a two-step process with an initial, an intermediate, and a final species. Typical examples of these three species, as determined by SVD analysis of Hb S are presented in Fig. 4a–c. The initial species resembles a displaced deoxygenated hemoglobin peak. This displacement is due to scattering from the polymers. Again, the initial peak at each temperature is broadened for the same reasons the initial Hb A peak is. The intermediate species includes both a partially ligated component, and a scattering component. The final species resembles fully ligated Hb–CO. At 35°C, and to a lesser extent at 30°C, the separation between the initial and intermediate species increases. In addition at 35°C, the initial species shows more ligation than the initial species at either of the lower temperatures. As would be expected from these differences, the associated reaction rates also change with temperature. Table 2 presents these two rates which characterize the Hb S–CO reaction as a function of temperature.

Fig. 5 shows a typical Hb S–CO reaction where rapid polymerization has been induced in the sample, resulting in smaller domain sizes. While

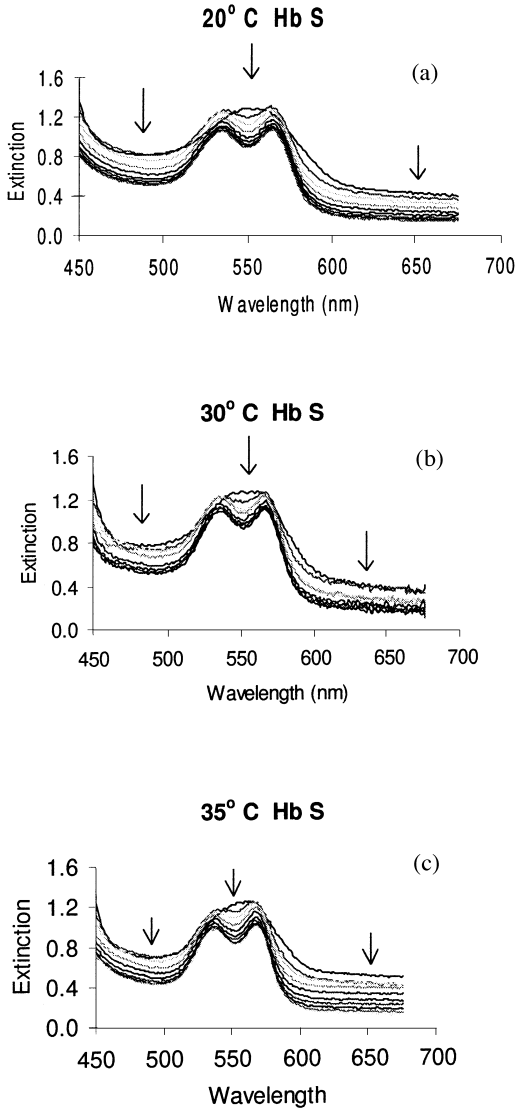

Fig. 2. Extinction spectra of deoxygenated sickle cell hemoglobin rapidly mixed with CO saturated buffer. As with Hb A, the deoxy-form has one peak, while Hb S–CO has two. The delay between the onset of mixing and the initial spectrum is (a) 28 ms at 20°C, (b) 31 ms at 30°C, and (c) 30 ms at 35°C. The time between the initial spectrum and the second one is 20 ms and the delay between each pair of consecutive spectra doubles afterwards. The change in peak profile from a single broad peak at 555 to two peaks at 540 and 570 nm resembles the Hb A spectra of Fig. 1. In addition, the turbidity of the sample is decreasing, as shown by the arrows. Consequently, the apparent absorption decreases at 540 nm and 570 nm, as compared to the increase in absorption at these wavelengths for the Hb A + CO reaction. Both absorption and scattering (resulting in turbidity) contribute to extinction, measured by our instrument.

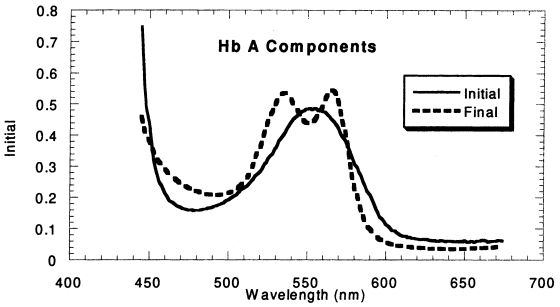

Fig. 3. Hb A reaction components at 30°C as determined by SVD and global analysis. There was no significant variation in these components with changing temperature. The final component grows in at a rate of 137 s^{-1}.

there is some scattering, the reaction spectra more closely resemble the Hb A spectra than the Hb S, as the displacement of the initial, deoxy, species is slight. However the reaction rates for the rapidly polymerized hemoglobin, are closer to the other Hb S reaction rates than to those of the Hb A reactions, as may be seen in Table 3. This is not an artifact of the analysis, as attempts to fit the Hb A reaction to a two step process resulted in widely varying rates, and no species resembling deoxygenated hemoglobin. Fig. 6 shows the species obtained by fitting a rapidly polymerized sample to two exponential processes. As would be expected from the original spectra, the intermediate species is only slightly displaced from the final species, Hb S–CO.

4. Discussion

We have found that Hb A binds CO more rapidly as the temperature is increased from 20°C to 35°C, the more physiological temperature, as would be expected. On the other hand, the initial → intermediate rate for Hb S + CO increases only slightly when the temperature is raised from 20°C to 30°C, and then decreases significantly as the temperature goes from 30°C to 35°C. The intermediate → final rate remains fairly constant as the temperature is raised from 20°C to 30°C, but again decreases noticeably when the temperature is increased to 35°C.

In order to better understand the significance

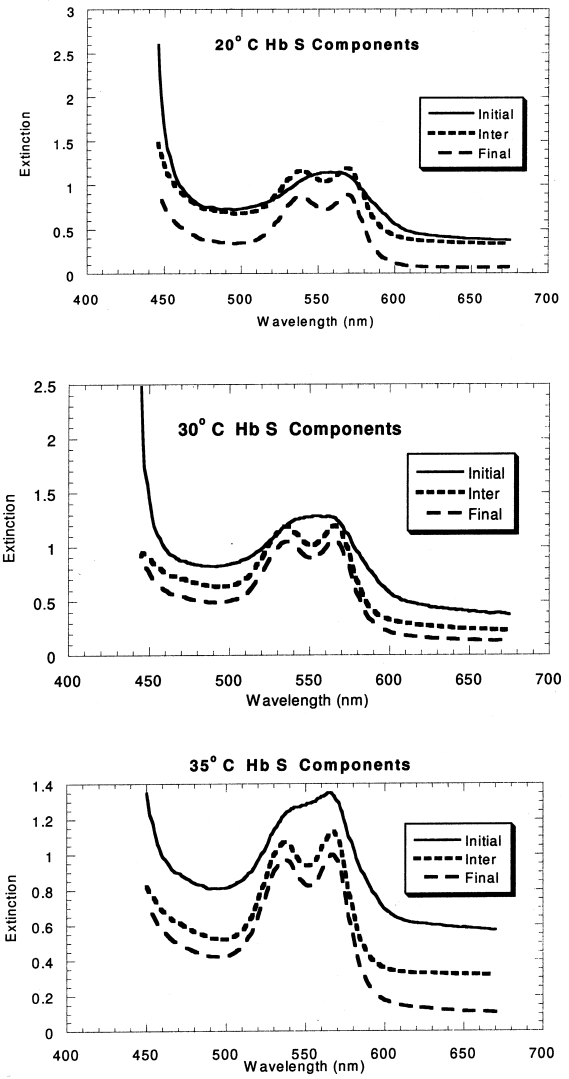

Fig. 4. Hb S reaction components as determined by SVD and global analysis. (a) 20°C, the intermediate species grows in with a rate of 30 s^{-1}, while the final grows in with a rate of 3.2 s^{-1}. (b) 30°C, the intermediate species grows in with a rate of 35 s^{-1}, while the final grows in with a rate of 4.9 s^{-1}. (c) 35°C, the intermediate species grows in with a rate of 21 s^{-1}, while the final grows in with a rate of 1.2 s^{-1}.

of the reaction species determined by SVD and global analysis (Fig. 4), we have decomposed these species into three components: a deoxygenated Hb spectrum, an Hb–CO spectrum, and a scattering component, A/λ^b. Because the polymers are not small compared to the wavelengths of light

Table 2
Average exponential rate constants for Hb S reacting with CO in 1.8 M phosphate buffer as a function of temperature

Temperature	Deoxy → intermediate (s^{-1})	Intermediate → final (s^{-1})
20°C[a]	33 ± 13	4.1 ± 2.4
30°C[b]	40 ± 14	4.7 ± 4.5
35°C[c]	18 ± 8	1.0 ± 0.3

[a]125 mixtures.
[b]54 mixtures.
[c]30 mixtures.

used in the experiment, the scattering exponent was 1.2 ± 0.8, rather than 4. A least squares fit determined the contribution of each of these components to the initial, intermediate, and final species of the CO + Hb S reactions. At 20°C, the intermediate species was approximately 80% ligated. The scattering of the intermediate species has not decreased substantially, as evidenced by the extinction at 680 nm, so polymers are still present. It appears CO may be binding to the polymers. At the higher temperatures, the intermediate species' ligation was 83% at 30°C, and 86% at 35°C. However, the intermediate state is not scattering nearly as much as the initial state at these higher temperatures.

To further probe this behavior, each individual spectrum obtained by the OLIS machine after mixing was decomposed into deoxy-, carboxy- and scattering-components. An example of this analysis for Hb + CO reactions at 20°C is shown in Fig. 7a. The time series for the concentration of each component was then fit to an exponential process. It was found that in this instance Hb–CO grew in with a rate of 20 s^{-1}, deoxy-Hb S decayed at a rate of 19 s^{-1}, and the scattering decayed with a rate of 7 s^{-1}. One explanation for this persistence of scattering well into Hb ligation is that the gel may be inhomogeneous on the scale of our probe beam cross-section. If this is the case, the scattering could be caused by a few large polymers. The turbidity caused by these scattering centers may dominate the signal, even though the hemoglobin contained in them is not a large fraction of the total Hb. In this case, our apparatus would be more sensitive to scattering than to ligation. Another interpretation of the scattering persisting after the deoxy-Hb has gone away, is that some CO is binding to the scattering centers, the polymers.

Fig. 7b shows the results of this analysis for a 30°C reaction. The deoxy- and carboxy-components still resemble exponentials, but the scattering component is beginning to deviate from this form. Here the deoxy-Hb S decreases at a rate of 33 s^{-1}, while the scattering decreases at a rate of 14 s^{-1}. The Hb–CO increases at a rate of 51 s^{-1}. The figure still suggests binding to polymers (or the presence of a few dominant scatterers), although not nearly so strongly as Fig. 7a does. There is a long tail to the scattering component. Fig. 7c shows the results of this analysis on a 35°C reaction. The time series for these three compo-

Table 3
Average exponential rate constants for Hb S reacting with CO in 1.8 M phosphate buffer as a function of polymer domain size

Preparation method	Deoxy → intermediate (s^{-1})	Intermediate → final (s^{-1})
Slow (large domains) 20°C[a]	33 ± 13	4.1 ± 2.4
Fast (small domains) 20°C[b]	45.9 ± 6.5	28.7 ± 3.2

[a]125 mixtures.
[b]18 mixtures.

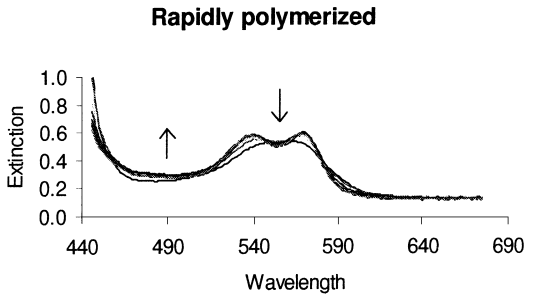

Fig. 5. Absorption spectra of rapidly polymerized Hb S mixed with CO saturated buffer at 20°C. The first spectrum is 26 ms after initial mixing, and the spectra are spaced 8-ms apart. There is very little turbidity in these samples.

nents do not fit well to exponentials. The scattering and deoxy components more closely match here, in contrast to Fig. 7a and to a lesser extent Fig. 7b.

We propose four processes, diagrammed in Fig. 8, to consider in these Hb S + CO mixtures. Carbon monoxide binds to monomers at a rate k_m^{CO}. Carbon monoxide binds to the Hb S polymers with a rate k_p^{CO}. Polymerized deoxy-Hb S molecules enter the solution phase at with a rate k_-^D. Finally, polymerized Hb–CO melts at a rate of k_-^{CO}. In principle deoxy monomers can also bind to the polymer, creating growth with a rate k_+^D. However, under the non-equilibrium conditions present after mixing CO buffer with Hb S polymers, there is no growth. Likewise, we assume the CO stays bound to the hemoglobin molecules once it attaches.

The main results of our temperature-dependent measurements are summarized in Table 2. We see that the overall melting is dramatically slower at 35°C than at 20°C. We cannot definitively explain all of our observations of the temperature dependence of melting in terms of the microscopic rate constants depicted in Fig. 8 at this time. However, we propose here some reasonable ideas that are consistent with the data.

According to Ferrone et al. [6] melting of the polymer can be described by the equation $(dc/dt) = k_- c_p(1 - c/c_s)$, where c is the concentration of, in this case deoxy, monomers (Hb tetramers) in solution, c_s is the solubility, c_p is the concentration of polymers and k_- is the rate constant for dissociation of monomers from polymers. We have taken the activity coefficient to be one due to the low concentration of Hb S in these experiments. Thus there are two factors in this equation that could vary with temperature, k_- and the equilibrium factor, $1 - c/c_s$. The solubility, $c_s = k_-/k_+$ [6] decreases as the temperature is raised from 20°C to 35°C [18,19], where k_+ is the rate constant describing addition of monomers to polymers. This is true in 1.8 M phosphate as well as in 0.1 M phosphate concen-

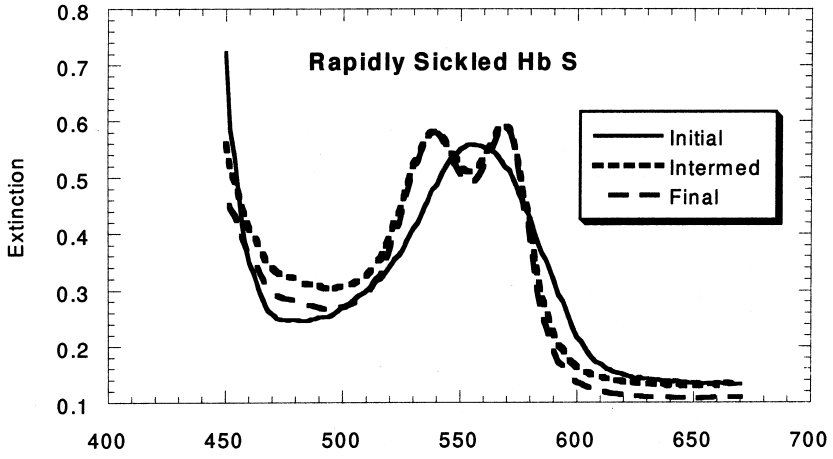

Fig. 6. Hb S reaction components for the rapidly polymerized hemoglobin, as determined by SVD and global analysis. The intermediate species grows in at a rate of 48 s^{-1}, while the final grows in with a rate of 24 s^{-1}.

tration buffers [12,20]. Thus as the temperature increases from 20°C to 35°C, k_- decreases with respect to k_+.

We note that the Hb A–CO rate constant increases as the temperature is raised (Table 1). As the solution phase CO binding properties of Hb S are the same as those of Hb A [21], it is reasonable to conclude that k_m^{CO} (Fig. 8) increases as the temperature increases. We also note that as the temperature is raised that the amount of scattering of the intermediate species decreases compared to the scattering of the initial species (Fig. 4). So whereas at 20°C the intermediate scatters as much as the initial species (indicating that CO is binding to the polymer-pathway 1 in Fig. 8) pathway 2 makes more of a contribution at the higher temperatures. The increase in k_m^{CO} as the temperature is raised in our experiments would tend to deplete the concentration of monomers in solution, c. Thus as the temperature is increased the equilibrium factor becomes larger and could thus account for the increased role of pathway 2 (Fig. 8).[1] In addition, this could explain the increase in rates (Table 2) as the temperature is raised from 20°C to 30°C. As the temperature is raised from 30°C to 35°C, k_-^D must decrease since the overall kinetics decrease. In addition, since pathway 1 competes with pathway 2, it is probable that k_p^{CO} decreases as the temperature is raised from 30°C to 35°C.

It may be surprising that k_m^{CO} decreases as the temperature is raised from 30°C to 35°C since k_m^{CO} increases over this temperature range. Per-

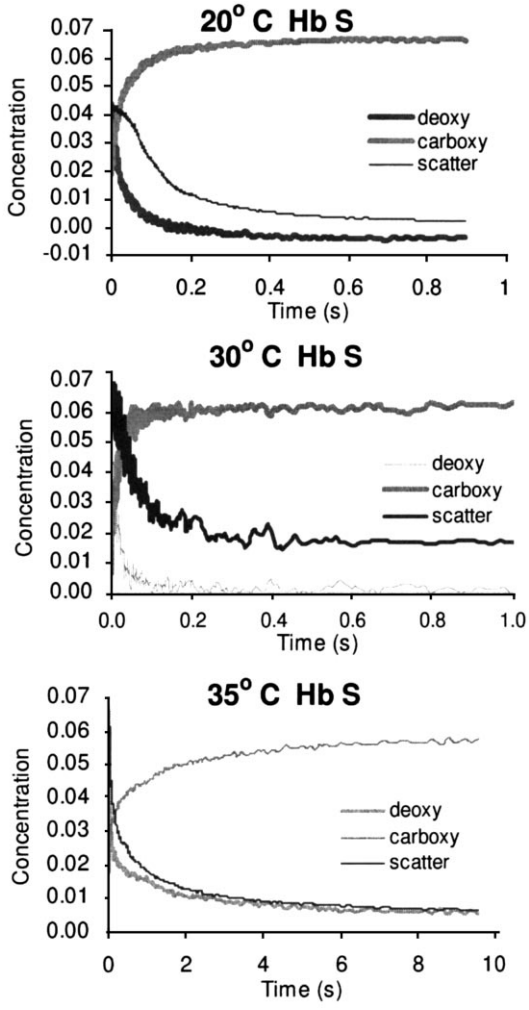

Fig. 7. Time series of deoxy-Hb, carboxy-Hb and scattering proportional to $1/\lambda^{1.2}$ for a complete set of Hb S reaction spectra. The deoxy- and carboxy- components used were taken from Hb A spectra to restrict scattering to a single independent term. The deoxy- and carboxy-components are proportional to the concentrations of those two species relative to one another. The scattering term is normalized by an arbitrary constant: (a) 20°C; (b) 30°C; (c) 35°C.

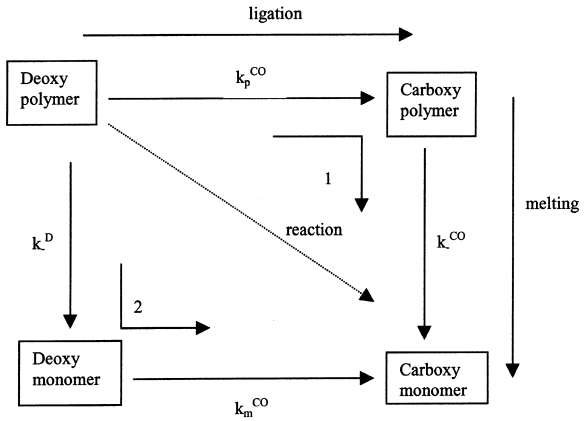

Fig. 8. Diagram showing possible reaction paths. Details are given in Section 4.

[1] Note that the decrease in c_s as the temperature is raised is not as potentially important as the rate of decrease in c since c can essentially go to zero whereas the variance in c_s is not more than a factor of four.

haps since the polymer is stabilized over this temperature increase the binding of CO may become more difficult. Diffusion of CO may be hindered in the stabilized polymer or entry sites into hemoglobin may be closed a larger proportion of the time. It is possible that the movement of CO into and within hemoglobin molecules at the edges of the polymer domains is easier than in and within hemoglobin molecules at the center of polymer domains. This appears to be supported by our domain size studies. The rapidly polymerized samples have smaller domains than the other samples, as indicated by the low turbidity. Table 3 shows the small domain samples react more rapidly than the other samples, although the reaction is still slower than the Hb A + CO reaction. The faster reaction rate for the rapidly polymerized Hb S may be due to the higher surface area to volume ratio of its small domain sizes.

This study begins to address two factors which affect sickle hemoglobin polymer melting, temperature and domain size. It is seen that at the most physiologically relevant temperature, 35°C, melting was the slowest of all the temperatures studied here. However, as the domain size decreased, the rate of polymer melting was found to increase. In vivo domain size can vary, but it is always limited by the size of the erythrocyte. Further work, particularly on polymer melting within erythrocytes, is needed to fully assess the physiological relevance of sickle hemoglobin polymer melting.

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