Carbon Monoxide Religation Kinetics to Hemoglobin S Polymers following Ligand Photolysis*

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The re-equilibration rate of carbon monoxide binding to hemoglobin S polymers is determined by time-resolved measurements of linear dichroism spectra. Linear dichroism is used to detect religation to hemoglobin in the polymer in the presence of rebinding to free hemoglobin S tetramers. Measurement of the linear dichroism resulting from photolysis of the small percentage of ligand bound to the polymer is accomplished through the use of an ultrasensitive, ellipsometric linear dichroism technique developed for this purpose. The major finding is that the return of the polymer phase to its equilibrium ligation state is much slower than that of the solution phase hemoglobin tetramers. Assuming all of the hemes in the polymer are equally likely to participate in rebinding, the re-equilibration rate for carbon monoxide to hemoglobin S polymers is found to be 0.14 ± 0.07 (s⁻¹ mm⁻¹), about 1000 times slower than the re-equilibration rate of carbon monoxide to T-state monomer hemoglobin. Several interpretations of this result are discussed. An understanding of the ligand binding kinetics to hemoglobin S polymers could have pathophysiological significance in its relevance to polymer formation and melting during red blood cell circulation.

Hemoglobin S (HbS)¹ differs from normal adult hemoglobin, hemoglobin A (HbA), in the substitution of a single amino acid, β6 Glu → Val (1). The presence of this mutation, when homozygous, results in sickle cell anemia. This disease, which affects 1 out of 600 people of African descent born in the United States (2), is characterized by microvascular occlusion that results in painful episodes or "crises" and acute organ damage. The polymerization of HbS, which occurs under conditions of hypoxia, deforms the red blood cell and makes them rigid, contributing to the microvascular occlusion.

The kinetics of polymer formation and melting have important consequences in the pathogenesis of the disease. If HbS polymerization is slow enough so that during circulation in areas of hypoxia insufficient polymer is formed to alter the red blood cell shape and rigidity, and if the HbS that has begun to polymerize during circulation is melted immediately upon oxygenation at the lungs, then microvascular occlusion would be avoided. If, on the other hand, complete melting does not occur before the cell reaches an area of hypoxia, further polymerization would be facilitated. In fact, the kinetics of gellation (for review, see Ref. 3) and the double nucleation theory that describes these kinetics (4, 5) would predict that this secondary polymerization could be extensive. Thus the kinetics of polymerization and that of melting are important factors in determining whether or not vascular sequestration of sickle cells will occur. Whereas the kinetics of polymerization have been extensively studied, relatively few reports involving the kinetics of melting have been made (6–12).

Polymerization of HbS depends on several factors including pH, temperature, the presence of polyphosphates, hemoglobin concentration, and ligand pressure. Under physiological conditions, the most important of these factors is the ligation state. Thus religation kinetics could be a significant factor in polymer melting. When studied under conditions where HbS does not polymerize (below a critical concentration necessary for polymerization) it follows the same equilibrium binding curve as HbA (13). Moreover, the kinetics associated with ligand rebinding following laser photolysis are the same for HbA and unpolymerized HbS (14–16). However, polymerized HbS has a lower ligand affinity than HbA (17–22).

Due to partial alignment of the HbS polymers, a static linear dichroism (LD) is observed in heme absorption bands upon HbS gellation. This LD is a result of the heme groups being nearly parallel to each other and nearly perpendicular to the HbS polymer (23). The hemes are near perfect planar absorbers of linearly polarized light (24, 25). Thus linearly polarized light is more strongly absorbed when the polarization axis is perpendicular to the long axis of the polymers. A spectral decomposition of measured static LD into oxygen bound and deoxy-hemoglobin components has been used to measure the equilibrium binding curve of HbS polymers (22). It was found that the oxygen affinity of HbS polymers is about one-third that of T-state HbA. A similar study using carbon monoxide (CO) had been performed previously (26). Although this study found no evidence of CO bound to the HbS polymer in equilibrium, it was later suggested that if temperature affects are accounted for, the relative affinity of the polymer for CO would be the same as for oxygen (22).

Laser photolysis and time-resolved absorption studies have made important contributions to understanding kinetics associated with ligand rebinding to HbA (for reviews, see Refs. 27
and 28). Since most of the ligand bound in a partially saturated HbS solution will be bound to the monomer (a single HbS tetramer) phase, it would be difficult or impossible to detect religation to polymer in a time-resolved absorption experiment. We have thus implemented time-resolved LD (TRLD) measurements to study religation to HbS polymers. After ligand photolysis, a transient LD is observed between directions parallel and perpendicular to the polarization of the actinic light. The polarized actinic light beam photoselects hemoglobin sites that are parallel to its polarization. Thus, soon after photolysis, there will be more deoxy-hemes parallel to the actinic polarization and more ligand bound hemoglobin in the direction perpendicular to this polarization. The photoinduced LD due to HbS in the monomer phase disappears upon orientational randomization resulting from rotational diffusion. On the other hand, the LD due to photoselected deligation of HbS polymer hemes decays with kinetics dominated by polymer religation because the polymers rotate slowly or not at all. The difference of the linear dichroism (defined as absorption parallel to the laser polarization minus that perpendicular), measured on a HbS gel taken at a time following photolysis when the monomers have rotated into an isotropic distribution, minus the static linear dichroism gives a deoxy-carboxy difference spectrum. This transient-difference linear dichroism signal may also arise due to the presence of partial order in the polymer gel, with the transient polymer state being primarily deoxy. Thus the transient-difference linear dichroism will be in the form of a deoxy-carboxy difference spectrum, whether due to photoselection or initial polymer alignment. The photoinduced LD of the polymer is very small because the polymer is mostly ligand free, so in this work, we apply an ultrasensitive time-resolved LD technique (29) to determine the partial ligation and total concentration of the solution phase saturation was then related to the solubility, \( c_s \), and concentration of hemoglobin. The concentration within the polymer, \( c_{t} \), is the total concentration of hemoglobin. The concentration within the polymer phase, \( c_p \), was taken as 0.69 g/ml (34). The above equation was solved using Mathematica (Wolfram Research); the single positive real root of the equation was taken as the solubility. The mole fraction of polymer was calculated from the equation (see Ref. 3).

\[
x_p = (1 - c_s/c_p) / (1 - c_s/c_o)
\]

Time-resolved absorption (TROD) measurements were performed as described previously (35, 36). A xenon flashlamp produced probe pulses at 1 Hz which are focused on the sample to a diameter of about 8 mm. Actinic pulses of 15 mJ pulse were produced by a Quanta Ray DCR-2A Nd:YAG laser, frequency doubled to 532 nm. The excitation pulses made an angle of about 30° with respect to the probe beam. The diameter of the laser beam was about 10 mm. A clean-up Glan-Taylor polarizer was used to insure the polarization purity (vertical) of the actinic beam. The probe beam was passed through a polarizer with its polarization axis oriented along the magic angle (the angle where photoselection effects are minimal, 54.7°) to reduce artifacts from reorientation kinetics of the photolyzed hemoglobin molecules. The probe beam was focused through a 100-μm slit into a 1 cm long Ash spectrograph (150 grooves/mm, 800 nm blaze) and detected with an EG&G OMA II detector. A Stanford Instruments DG535 delay/pulse generator was used to control the timing of the detector gate (10 ns) and the firing of the flashlamp with respect to the laser.

The ultrasensitive TRLD method used in this study has been described in detail previously (29). To implement the TRLD measurement, the sample is probed at a given time following photolysis for two separate rotations by a small angle, \( \beta \), of the initial polarization. The LD signal is calculated as the detected intensity when the initial polarization is rotated by \( \beta \) (parallel) minus the detected intensity when the polarization is rotated by \( -\beta \) (perpendicular), divided by the sum of these intensities. As shown previously (29) this gives:

\[
LD = \frac{I(\beta) - I(-\beta)}{I(\beta) + I(-\beta)}
\]

where we have assumed that LD is much smaller than \( \beta \), which is a reasonable assumption for our measurements. A value of 0.011 radians was chosen for \( \beta \) in this work so that a signal to noise advantage of about 100 was obtained with respect to standard LD measurements probing vertical and horizontal direction separately. The sign (parallel vs. perpendicular) of the LD was determined by measuring the linear dichroism at early times, before significant rotational diffusion of the solution phase hemoglobin. Before each measurement, sufficient time was allowed between laser pulses to allow the return of the system to its pre-photolysis state.

The recombination of CO with the photolyzed hemoglobin sample can be described by the equation,

\[
\frac{d[C_O]}{dt} = -k[HbS][CO] - k[Hb][CO] - k[Hb][CO]
\]

where \( k \) refers to the rate constants and [Hb] to concentration of free hemes with the subscripts R, T, and p indicating solution phase R-state, solution phase T-state, and polymer phase, respectively. The TRLD measurements are only sensitive to changes in [Hb] and can thus be used to study CO religation kinetics to the polymer.

Data was analyzed using singular value decomposition (SVD) (37-39). SVD rewrites the data matrix of signals at each wavelength and time as the product of three matrices.

\[
A = USV^T
\]

U is an m x n matrix containing the optical density for n orthonormal basis spectra at m wavelengths. \( V^T \) denotes the transpose of \( V \), a n x n
matrix giving the amplitude of each basis spectrum at n time delays. S
is an n × n matrix containing the singular values of A, a determinant
of the contribution of each basis spectrum to the measured spectrum at
a given time. In practice, only the largest singular values and time
amplitudes are retained. The smaller values and amplitudes are dis-
carded as noise. Thus SVD provides a concise, noise-filtered representation
of the data. This truncated representation was used to fit the
TROD data to an exponential lifetime using a (non-linear) least square
global analysis fitting technique (36). This lifetime, τ, yields the ob-
served re-equilibration rate for CO binding to the HbS polymer, kᵢ₊, by

\[ kᵢ₊ = \frac{1}{τ} \]  

(Eq. 6)

If re-equilibration of the polymer with CO is achieved through the
binding of CO by hemes in the polymer as described in Equation 4 then
the rate constant for CO binding to the polymer is given by,

\[ kᵢ = kᵢ(Hbp) = \frac{1}{τ[Hbp]} \]  

(Eq. 7)

Alternative mechanisms of polymer reigation, to be discussed below,
are not consistent with the assumption of kᵢ(Hbp) with kᵢ thus refer-
hereafter to a re-equilibration rate operationally defined as kᵢ(Hbp).

RESULTS

An absorption spectrum of a typical sample of HbS before
gelation is shown in Fig. 1a. Also shown is the fit of this
spectrum to pure components of carboxy and deoxy spectra
(and light scattering). The basis curves to which the absorption
spectra were fit are shown in Fig. 1b. These fits were used to
determine the initial saturation of the sample, γ₀, and calculate
the molar fraction of polymer. The results of these analysis for
three samples studied are shown in Table I (concentrations are
given in terms of heme concentration, [Hbp] = \( \gamma_0 \cdot c_0 \)).

Results of a typical TROD measurement performed on a
gelled sample are shown in Fig. 2. Each curve represents the
difference in absorption of the sample at given delay time
following ligand photolysis minus the absorption of the sample
before photolysis. Data is shown for 50 delay times (10 per
decade) from about 50 ns to 1 ms. The data show that over 90%
of CO recombination is complete by 1 ms. The kinetics of CO
recombination to the monomer phase are complicated. Before
photolysis most of the ligation will be to R-state (solution phase)
hemes. The R-state recombination rate is about 60
times faster than that to T-state (40) and if the T-state rebind-
ing were to be ignored the CO recombination kinetics could be
explained by hyperbolic recombination to R-state hemes. How-
ever, since this recombination is hyperbolic rather than expo-
nential in nature, and since (especially as R-state recombina-
tion proceeds) T-state hemes will be in excess of free CO, the
presence of the T-state hemes should be accounted for. One
relatively simple model (and not necessarily the only one) that
is consistent with the data is one which assumes that the
concentration of free CO is equal to the concentration of ligand-
free R-state hemes and that the concentration of ligand-free
T-state hemes is in excess of free CO,

\[ [CO] = [Hbp]_0 \]  

and \([CO]_0\) is the initial concentration of free CO at the begin-
ing of the recombination process. Most of the measured CO
religation kinetics is given by the time dependence of the first
basis vector (the primary spectral component) obtained by ap-
plying SVD to the TROD data. This time dependence is shown
for a typical TROD measurement in Fig. 3. Also shown in this
figure is a theoretical fit to these data using Equation 10. The
theoretical fit is not a "best fit" but rather that obtained using
The time course of the principal component of TROD data is shown in Fig. 1 with [HbT] taken from Table I with [CO] = 0.6 mM, [Hbp] = 1 mM, and [HbT] = 8.4 mM. Each TRLD curve is decomposed into approximately a 50% deoxy-hemoglobin – 50% carboxy-hemoglobin absorption curve. This decomposition fits the TRLD data reasonably well, however, there is about a 3-nm blue shift of the experimental data relative to the theoretical fit. A similar shift is observed when a fit is made to our transient absorption difference spectra (Fig. 2). An examination of the basis vectors for the TRLD data obtained by SVD, shown in Fig. 6a, also demonstrates the similarity of the TRLD data to a deoxy-carboxy difference spectrum. The time course of the first basis spectrum is shown in Fig. 6b. That the time dependence of this basis spectrum fits a straight line when plotted as log (intensity) versus time demonstrates that the recombination to the polymer follows pseudo-first order kinetics. The TRLD data were fit to exponential processes the lifetimes of which are shown in Table I. These lifetimes were used to calculate the CO re-equilibration rate, as described above (Equation 7), giving an average value of 0.14 ± 0.07 (s⁻¹ mm⁻¹).

The static (ground state) linear dichroism for a gelled sample is shown in Fig. 4. This linear dichroism signal is due to the partial alignment of the HbS polymers. As expected, it resembles a mostly deoxy-hemoglobin absorption spectrum. Time-resolved linear dichroism difference spectra (transient – ground state) are shown for a typical experiment in Fig. 5a for 0.65, 0.95, 1.25, 1.55, 1.75, and 1.85 s following laser photolysis. The spectral signature of these measurements resembles a deoxy-carboxy difference spectrum. Fig. 5b shows the transient-ground state linear dichroism difference spectra for 0.65 and 1.55 s following laser photolysis, as well as a fit to these spectra using the static absorption curves of deoxy- and carboxy-hemoglobin shown in Fig. 1b. Each TRLD curve is decomposed into approximately a 50% deoxy-hemoglobin – 50% carboxy-hemoglobin absorption curve. This decomposition fits the TRLD data reasonably well, however, there is about a 3-nm blue shift of the experimental data relative to the theoretical fit. A similar shift is observed when a fit is made to our transient absorption difference spectra (Fig. 2). An examination of the basis vectors for the TRLD data obtained by SVD, shown in Fig. 6a, also demonstrates the similarity of the TRLD data to a deoxy-carboxy difference spectrum. The time course of the first basis spectrum is shown in Fig. 6b. That the time dependence of this basis spectrum fits a straight line when plotted as log (intensity) versus time demonstrates that the recombination to the polymer follows pseudo-first order kinetics. The TRLD data were fit to exponential processes the lifetimes of which are shown in Table I. These lifetimes were used to calculate the CO re-equilibration rate, as described above (Equation 7), giving an average value of 0.14 ± 0.07 (s⁻¹ mm⁻¹).

DISCUSSION

Our TRLD measurements on recombination to HbS polymer reveal a slow process that takes about 1–2 s for completion. In contrast, photoinduced TRLD performed on concentrated HbA in a control experiment decayed with about a 100-ns lifetime. The TRLD difference spectra (linear dichroism measured at a given delay time after photolysis minus the static linear dichroism) resemble a deoxy-carboxy difference spectrum. The simplest interpretation of these data is that the difference linear dichroism spectra are due to deligation of HbS polymer upon photolysis followed by slow recombination. From this finding, we conclude that the CO re-equilibration rate of the polymer is 1000 times slower than for free monomers. Before considering the implications of a reduced CO re-equilibration rate, we discuss several caveats to this result.

First we should point that our ultrasonic linear dichroism technique yields linear dichroism convoluted with optical rotary dispersion, but that this optical rotary dispersion contribution is expected to be negligible in measurements of photoselection-induced LD (29, 41). Furthermore, the shape of the measured transient difference spectra also shows that any contribution to the measured spectra from transient optical rotary dispersion is insignificant.

The transient linear dichroism will also be affected by polymerization and melting of the polymer that will occur due to
changes in the ligation state of the sample. One would expect that following photolysis there would be a transient increase in the fraction of polymerized HbS and that after ligand rebinding (to the monomer phase in particular) the polymer would partially melt. The addition of a hemoglobin molecule to the polymer would appear in the linear dichroism spectrum as a carboxy- or deoxy-hemoglobin absorption spectrum or some combination of these spectra, depending on the ligation state of the added hemoglobin. Such a change in the linear dichroism could be positive or negative depending on whether the hemoglobin molecule binds so that its hemes were parallel or perpendicular to the laser polarization or whether the molecule is coming on or off the polymer. The second basis vector, shown in Fig. 6a, is suggestive of a change in the amount of polymer due to increased polymerization or depolymerization. Unfortunately, the signal to noise in our current measurements is insufficient to describe the kinetics of any net changes in the concentration of polymer suggested by this basis vector.

Analysis of TRLD measurements lead to the calculation of a re-equilibration rate of CO to the HbS polymer of $0.14 \pm 0.07$ (s$^{-1}$ mM$^{-1}$). Carbon monoxide binding rates to T-state hemoglobin have been found to be independent of hemoglobin concentration (42) with reported values ranging from 100 to 300 (s$^{-1}$ mM$^{-1}$) for T-state CO recombination (40, 43). Thus our measured value for CO recombination to the polymer is about 1000 times slower than to T-state hemoglobin. It is unlikely that the presence of small amounts of Hbf and HbA$\alpha_2$ affected our measurements as their sparring effect would not make a large difference in our calculated value of the polymer recom-

Fig. 5. a, time-resolved linear dichroism difference spectra of the HbS gel. Each curve represents the linear dichroism signal of the sample measured at a given delay time after laser photolysis minus the ground state linear dichroism. The signal is shown magnified 92 times; a linear dichroism signal shown in the figure of 0.01 corresponds to a linear dichroism of $1.1 \times 10^{-4}$ optical density units. Each curve represents the average of 128 measurements. Delay times were 0.65, 0.95, 1.25, 1.55, 1.75, and 1.85 s. The spectra were offset to zero at 650 nm. b, fit to time-resolved linear dichroism difference spectra. A fit (dotted curves) using the static deoxy- and carboxy-hemoglobin absorption curves (Fig. 1b) is shown for TRLD difference spectra (solid curves) at 0.65 and 1.55 s following photolysis. The fit to the 0.65-s time point is composed of 49% deoxy-hemoglobin – 51% carboxy-hemoglobin absorption curves and the 1.55-s time point is composed of 55% deoxy – 45% carboxy curves. These values are at least as good a fit to a deoxy-carboxy difference spectrum as the values obtained in a similar fitting to TROD data.

Fig. 6. Singular value decomposition of HbS TRLD. The three most significant singular values normalized to the largest singular value were 1, 0.35 $\pm$ 0.05, and 0.20 $\pm$ 0.05 for three TRLD measurements. a, two primary basis vectors (normalized by the square root of the corresponding singular value of $S$) obtained by applying SVD to TRLD data taken on HbS gel. The larger (primary) spectrum resembles a deoxy-carboxy difference spectrum. The secondary spectrum loosely resembles a deoxy spectrum. The third basis spectrum (not shown) resembles random noise. b, the time course (column of $V$) for the first basis vector. The log of the value of $V$ for each measured time point (normalized by the square root of the corresponding singular value of $S$) is plotted ($\circ$) as well as a fit of these points to a single exponential function with a lifetime of 1.4 s. The second basis vector follows the same time course as the first basis spectrum but with an opposite slope and the third basis vector had a random time course.
bination rate. A small error in the calculation of \( c_p \) would have led to an overestimation of \([ \text{Hb}_p] \). In an extreme example, if our calculations of \( c_p \) were overestimated by 10% (for which the third case in Table I would have no polymer formed), an average value for the polymer re-equilibration rate of 0.3 \( \text{s}^{-1} \text{m}^{-1} \) would be obtained for the first two cases from Table I. Thus, even if we allow for a significant sparring effect, we obtain a value for the CO re-equilibration rate of the HbS polymer that is at least 300 times slower than that for T-state HbA.

In our calculations of the HbS solubility we used a value of \( c_p \) = 0.69 g/ml. Recently, a value of 0.547 ± 0.007 g/ml was measured (44). If we had used this value of \( c_p \) then we would have obtained a larger value for the concentration of HbS polymer and hence a smaller value for the polymer re-ligation rate. It could be argued that the relevant value for the concentration of polymer is not the moles of hemes in the polymer form divided by the total volume, \([ \text{Hb}_p] \) (as we have used), but rather the concentration of hemes in the polymer divided by the volume taken up by the polymer itself (given by \( c_p \)). Such a scheme might make sense if only CO initially trapped within the polymer bundles would rebind to the polymer. In any case, this would also yield a slower value for the polymer re-equilibration rate than the one that we have calculated.

There are several mechanisms by which the polymer phase can be re-ligated. The most obvious of these is through a bimolecular recombination of CO with the polymer. If all the polymer hemes are equally likely to recombine in this manner then the re-equilibration rate that we have defined as \( k_p^p/\text{[Hb}_p] \) is the bimolecular rate constant, \( k_p \), defined in Equation 4. Our finding that this rate constant is about 1000 times smaller than for T-state hemoglobin is surprising given the results of equilibrium binding studies to the HbS polymer. The equilibrium binding affinity to oxygen of HbS in the polymer phase was found to be about one-third that of T-state hemoglobin (22). Although another study indicated a much lower CO affinity for the polymer phase (26), this result has been attributed to either conditions of gel formation or temperature and it has been suggested that the relative affinity of the polymer to that of T-state hemoglobin is the same for carbon monoxide as it is for oxygen (22). Using a value of one-third for the relative CO affinities our results predict that the off-rate of CO from the polymer phase is about 300 times smaller than it is for T-state hemoglobin.

Another possible mechanism for religation of the polymer involves a bimolecular recombination of CO with polymer hemes with the condition that only hemoglobin molecules at the ends of the polymer can bind CO. In this case, the bimolecular recombination of CO with the polymer is slow not because the CO-polymer on-rate constant is small but because the effective concentration of available polymer hemes is small. This mechanism is consistent with the rate constant, \( k_p \), being comparable in size to \( k_p \), the rate constant for CO binding to T-state hemoglobin.

The two mechanisms of polymer religation involving bimolecular recombination outlined above are difficult to reconcile with the fact that by the time re-ligation to the polymer begins, the monomer phase should have already bound virtually all the free CO molecules. Fig. 7 shows the TRLD difference spectra of a HbS gel taken 10 μs and 10 ms following photolysis. No polymer re-ligation is observed before 10 ms. However, the TROD data shown in Fig. 3 demonstrate that monomer rebinding is practically complete by 10 ms. Furthermore, based on the concentration of free T-state molecules and the value of \( k_p \), one would expect that by 100 ms or so there should be almost no free CO left to rebind the polymer. A possible resolution of this paradox is that the diffusion of CO through hemoglobin molecules in the polymer is very slow, so that some CO molecules would be "trapped" in the polymer. The diffusion of CO through the globin to the heme iron depends partially on the ease of internal protein movements that allow the CO access to the heme (45, 46). It is possible that Hb molecules in the polymer are restricted in these internal motions.

One might propose that re-equilibration of the polymer phase is achieved after monomer rebinding of free CO through release of CO by hemoglobin monomers and recapture by the polymer, but the off-rate of T-state hemoglobin (about 10 s lifetime (40)) is inconsistent with the observed lifetimes reported here (Table I). However, there is another mechanism that avoids the paradox of the polymer re-binding CO molecules that should have been already bound by the monomer phase. In this mechanism, after the initial monomer rebinding phase the distribution of ligated T-state molecules achieves its equilibrium distribution with ligated polymer molecules by exchange, a ligated T-state hemoglobin replaces a delayed one on the polymer. There is a dynamic equilibrium between T-state molecules in the polymer and monomer phases; they are constantly exchanging but, in our experiments, the amount in each phase is constant. There is also an equilibrium distribution of ligated T-state molecules between the polymer and the monomer phase that is governed by the relative CO equilibrium binding constants of each phase. Using a value of one-third for the polymer equilibrium binding constant relative to that of T-state hemoglobin,

\[
\frac{k_p^p}{k_p^T} = \frac{k_p^p}{k_p^T} = \frac{3k_p^T}{k_p^T},
\]

where the subscripts p and T refer to the polymer and T-state hemoglobin respectively, \( K^p \) is the equilibrium binding constant and \( K^p \) and \( K^T \) are on- and off-rates (k_p^p and k_p^T are identical to the bimolecular re-binding constants, k_p and k_p, but we have added the superscripts here to avoid confusion with the off-rates). If the off-rates were the same for polymer bound hemoglobin molecules and T-state hemoglobin molecules, then re-equilibration of the distribution of CO between these two species would occur in a parallel process with an observed rate constant equal to the sum of the apparent rate constants for
each process. Each phase would approach equilibrium simultaneously much faster than is evidenced by the TRLD measurements reported here. However, if $k_{p}\text{r} < k_{p}\text{f}$ then $k_{p}\text{r} < k_{p}/3$ and CO rebinding would not lead directly to equilibrium. Thus as long as the on-rate for polymer binding is sufficiently diminished (not necessarily by several orders of magnitude) the rebinding process may occur such that the monomer phase binds an excess of CO relative to the equilibrium distribution. The equilibrium distribution of ligated hemoglobin molecules can then be achieved by exchange between the phases.

It is not clear that the CO re-equilibration kinetics observed here are consistent with the kinetics of exchange between the polymer and monomer phase. Growth and melting of the polymer has been measured at about one hemoglobin molecule per 4 ms and rarely approaching one hemoglobin molecule per 400 $\mu$s (11, 47). In our measurements, however, there is no growth or melting, only a dynamic exchange between the phases. One might expect for the exchange rate to be faster than the measured growth rates. The rate of re-equilibration of CO between the monomer and polymer phases would depend on the exchange rate, the number of ends (assuming exchange can only occur at the ends), and how far from equilibrium the system is after monomer re-binding is complete.

The ligation of the polymer, and hence the kinetics of ligand binding to it, could be important in the kinetics of polymer melting. Recently it has been suggested that an equation that governs the rate of polymer growth (the rate is proportional to the difference of the products of the activity coefficients and solution concentrations of hemoglobin at a given time minus that product at equilibrium (5)) can also be applied to polymer melting (11). It was also suggested that these equations would be modified to account for partially ligated species (11). One can think of melting in terms of the dynamic equilibrium between the monomer and polymer phase where there is a dynamic exchange between T-state hemoglobin molecules in the two phases but, in equilibrium, the total number of molecules in each phase is constant. R-state hemoglobins are excluded from the polymer and ligated T-state hemoglobins are less likely to polymerize than unligated ones (22). As the monomer phase becomes religated it becomes less likely to polymerize and the polymer melts. Thus hemoglobin molecules that come off the polymer could be prevented from re-entering the polymer faster if they are already partially ligated. Although the polymer generally melts at the ends (11), it is possible that a conformational change (T to R) in a molecule within the polymer could cause a break and thus speed up melting.

The rate of polymer melting could contribute to persistence of the polymer in sickle red cells in the arterial circulation which in turn can act as a nucleation seed to greatly accelerate polymerization in the venous circulation. The red blood cell will normally spend about 1 s in the alveoli, about 3 s in the arterial circulation, about 1 s in the microcirculation of the tissues, and about 10 s in the venous system (12, 48). Thus the cell is exposed to a relatively high oxygen pressure for about 1–4 s. However, the oxygen diffusion into the cell will slow down the rate of oxygen uptake relative to that of hemoglobin in solution (9). Although no thorough study of polymer melting kinetics has been made, it has been established that polymer melting does not encompass a delay time which characteristically slows down the overall kinetics of polymerization (12). However, under certain conditions, polymer has been found to persist up to 25 s after reoxygenation and thus polymer melting may not be complete during oxygenation at the lungs (8).

The main finding of this work is that the religation of the polymer with CO is very slow. We have discussed several mechanisms that could account for this slow religation. If the mechanism involves bimolecular rebinding to any of the polymer hemies, the difference in these kinetics could be due to altered affinity of the hemies in the polymer or due to changes in the entry and exit rates of the ligand into the polymer (especially in the heme pocket). Examination of the geminate yields of R versus T-state hemoglobin showed that the heme affinity alone could account for differences in overall binding affinity of these hemoglobin (49). It would be possible to set up a TRLD experiment to measure geminate yield from the polymer where the geometry of the apparatus precluded any contribution from the monomer phase and thus weigh the importance of heme affinity in ligand recombination kinetics to the polymer. However, the small geminate yield in T-state hemoglobin (49) would indicate that such a measurement for the polymer would probably be very difficult. If religation is accomplished through rebinding only to the polymer ends then the kinetics of religation would be dependent on the number of ends. This is also true if religation is achieved by exchange between the monomer and polymer phases and exchange only occurs at the ends. These models can be tested by preparing gels in different manners so that the number of ends is varied. Such studies are currently being pursued in our laboratory.

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