COMPARISON OF SICKLE CELL HEMOGLOBIN GELATION KINETICS MEASURED BY NMR AND OPTICAL METHODS

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SUMMARY

We describe a technique for monitoring the kinetics of sickle cell hemoglobin gelation by observing the change in the amplitude and linewidth of the water proton magnetic resonance. The resulting kinetic progress curves are very similar to those obtained by optical birefringence and turbidity methods. The curves consist of a delay, followed by a rapidly accelerating signal change which terminates quickly. From a study of the temperature dependence of the delay time, it is shown that all three techniques see the onset of gelation simultaneously. The origin of the change in physical properties upon gelation is briefly discussed in relation to the component steps of the reaction.

Many physical properties of hemoglobin S solutions change upon gelation, a process which consists of the formation of long polymers which spontaneously align to produce an ordered phase. There is a large increase in viscosity (1,2); linear birefringence and dichroism appear (1,3-6); heat is absorbed (7,8); and light scattering increases (9). The time dependence of all of these properties has now been used to study gelation kinetics. We have presented the results of an investigation in which the birefringence and heat absorption were utilized to monitor the kinetics of the thermally-induced gelation reaction. Some measurements on the rate of gelation have also been reported utilizing viscometric (10-12) and turbidimetric (9) techniques. With all of these methods the gelation reaction is characterized by a delay period, during which there is no measurable change in the observed property, followed by the rapid onset of the reaction. The length of the delay period is very temperature-sensitive (7,10), and is reciprocally related to an extraordinarily high power (∿30) of the hemoglobin concentration (7). To explain these results we proposed a model in which the formation of thermodynamically-unstable nuclei for individual polymers is rate-limiting (7).

Another physical property, which is potentially useful for measuring kinetics on red cells as well as hemoglobin S solutions, is the transverse relaxation time (T_2) of water protons. Cottam, Waterman and coworkers (13,14) have shown that there is a large decrease in T_2 upon gelation or cell sickling. Their equilibrium measurements, together with the observations by Cameron and Block (15) that gelation produces a marked broadening of the water proton resonance, prompted us to explore the use of NMR to study the rate of gelation. In this note we present results on the kinetics of gelation measured by the change in water linewidth, and compare them to results obtained with the linear birefringence and turbidimetric techniques. To make continuous measurements of the linewidth with high stability and sensitivity, the amplitude of the water line is monitored with a nuclear sideband oscillator. We find that prior to the increase in linewidth there is a delay period, during which there is a small decrease in linewidth. The delay time is the same as that found for the appearance of birefringence and turbidity for identical samples at the same temperature. This result indicates that the change in all three physical properties is rate-limited by the same process, which we assume to be the nucleation of individual polymers.

MATERIALS AND METHODS

Purified hemoglobin S was prepared as previously described (7). The sample was dialyzed against 0.15 M potassium phosphate buffer, pH = 7.35, prior to concentration by ultrafiltration. Deoxygenation was carried out by the addition of sodium dithionite (final concentration = 0.05 M) under a nitrogen atmosphere. The final concentration of the solution was 26.7 g%, and the final pH between 7.0 and 7.1. One portion of the solution was placed in a 4.20 mm i.d. tube for NMR measurements, another portion in a 1.0 mm pathlength rectangular cuvette for turbidity measurements, and a third between glass coverslips to give about a 0.010 mm layer for birefringence measurements. All samples were sealed against oxygen with dental wax.

Linear birefringence was detected by measuring the light transmitted by the sample placed between crossed linear polarizers as described previously (7,8). Turbidity was measured with a Cary 17 recording spectrophotometer at 1100 nm, the wavelength at which concentrated deoxyhemoglobin S solutions exhibit the least absorption of any wavelength accessible with this instrument (185 - 3000 nm).

For proton magnetic resonance measurements a Varian HR 220 spectrometer was used. A nuclear sideband oscillator circuit (16) was added to the standard instrument to allow long term observation of the resonance peak independent of field fluctuations. The circuit consists of a high gain, automatic gain control amplifier. The input is taken from the signal amplitude switch, and the output feeds the field modulation circuit and the phase detector reference. To increase the recording time up to a maximum of 13.8 hours, a circuit was added that slowed the sweep time of the recorder by a factor of 10.

The samples for birefringence and turbidity measurements were thermostatted to within 0.1°C by circulating water from a constant temperature bath through metal sample holders. The temperature of the NMR samples was held constant by circulating nitrogen gas that had passed through a copper coil immersed in a constant temperature bath. The temperature was determined by measuring the difference in chemical shift between the hydroxyl and methyl protons of methanol before and after each kinetic run. The temperature stability was found to be as good as the reproducibility of the chemical shift difference measurements, equivalent to about ± 0.2°K. The calibration supplied by Varian Associates (17), $T(°K) = 480.9 - 0.5342\Delta\nu$, was used to convert the observed chemical shift difference to temperature. An independent temperature calibration by Van Geet (18) yields temperatures which are about 1.7°K higher for the same shift difference, suggesting that the absolute accuracy of our temperature determination is only about ± 1°K.

RESULTS

Deoxyhemoglobin S in concentrated solution exhibits the characteristic property that gelation can be induced by heating, and degelation by cooling (2,8). The kinetics of gelation can then be studied by changing the temperature in a time which is short compared with that of the gelation process. The typical experiment consists of heating a sample from 0°C, where it is a non-birefrigent, weakly-scattering liquid, to some elevated temperature where gelation will eventually take place. Figure 1 shows the water proton magnetic resonance spectrum of a 26.7 g% deoxyhemoglobin S solution at various times after the temperature of the sample had been changed from 0°C to 16.4°C. The spectrum is unchanged from 9 min., the time of the first spectrum, to about 40 min. At 40 min. the broadening of the line, with a corresponding decrease in amplitude, is first measurable. About 90% of the total change in the spectrum is observed between 60 and 100 min. Several of these spectra are shown in Figure 1. The 143 min. spectrum is the same as that observed for the next 60 min. The insert in Figure 1 shows a plot of the reciprocal peak amplitude

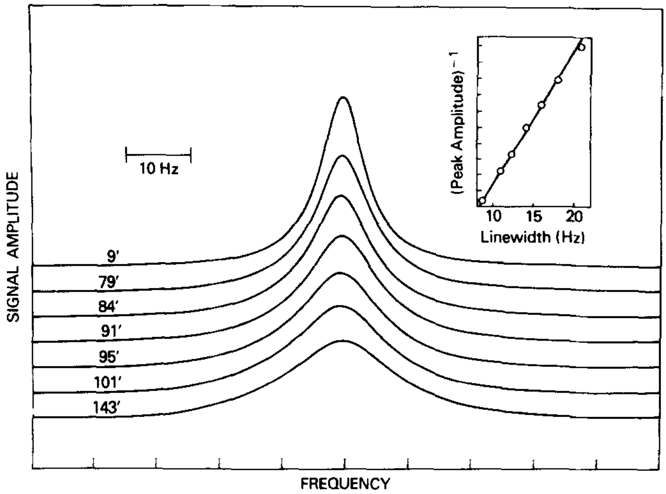

Figure 1: Water proton magnetic resonance spectra as a function of time. A sample containing a 26.7 g% deoxyhemoglobin S solution was heated from 0°C at time zero to 16.4°C. The spectra were recorded with a Varian HR 220 spectrometer at the indicated time intervals after the initiation of the temperature jump. Small shifts in the line position have been corrected in displaying the spectra. The inset contains the reciprocal peak amplitude plotted as a function of the linewidth at half the peak height for these spectra.

versus the linewidth at half the peak height. The linearity of this plot indicates that the linewidth can be monitored by the amplitude. We therefore turned to amplitude measurements using a nuclear sideband oscillator, which provides greater sensitivity and stability, and also has the virtue of allowing continuous recording of measurements.

The results of single kinetic runs at 6 different temperatures between 15.0 and 21.3°C are shown in Figure 2. The data are plotted as the fraction of the total decrease in amplitude versus time on a logarithmic scale. For each curve we define a delay time as the intersection of the time axis with an extrapolated straight line having the maximum slope of the progress curve. This definition provides a rapid graphical procedure for extracting a characteristic time from progress curves. The major features of the progress curves in Figure 2 are: (i) prior to the decrease in amplitude (corresponding to an increase in linewidth) there is a delay that is very temperature-sensitive; (ii) during the delay there is a small, but reproducible, increase in ampli-

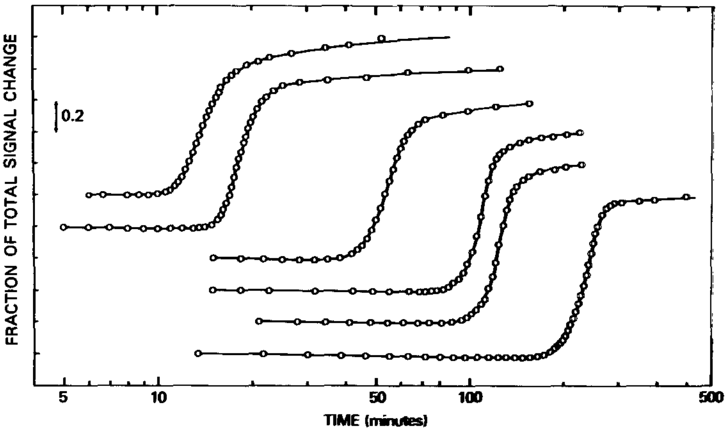

Figure 2: Gelation progress curves as a function of temperature. The reciprocal of the peak amplitude measured with a nuclear sideband oscillator is plotted as a function of time for a sample having a deoxyhemoglobin S concentration of 26.7 g%. Using the temperature calibration for methanol supplied by Varian Associates (17), the temperatures for the individual curves were found to be, from left to right, 21.5, 20.2, 18.4, 16.4, 16.1, and 15.0°C. The curves have been normalized by plotting the fractional change in the reciprocal amplitude, and have been offset for clarity. The linewidths of the ungelled samples were 9.9 ± 0.2 Hz, and those of the gelled samples were 18 ± 3 Hz. There was an indication that the gelled linewidths increase with increasing temperature. Prior to each kinetic run, the sample was thoroughly mixed by continuously rotating the NMR tube in a 1°C refrigerator for at least 24 hours.

tude; (iii) about 90% of the observed decrease in amplitude following the delay takes place in a time equal to or less than the delay time; and (iv) the curves have very similar shapes, although those with longer delay times appear to have somewhat larger maximum slopes.

The progress curves measured by turbidity and birefringence are compared with the NMR curves in Figure 3. The turbidity progress curves are very similar to those measured by NMR except that there is no measurable change in optical density during the delay period. The birefringence progress curves are very similar to those found previously under slightly different conditions (7). The reciprocal delay time, which we identify as the rate of the reaction, is plotted versus the reciprocal absolute temperature in Figure 4 for all 3 techniques. Within the accuracy of the temperature measurement of the NMR sample, and the reproducibility of the delay times, all of the data fall

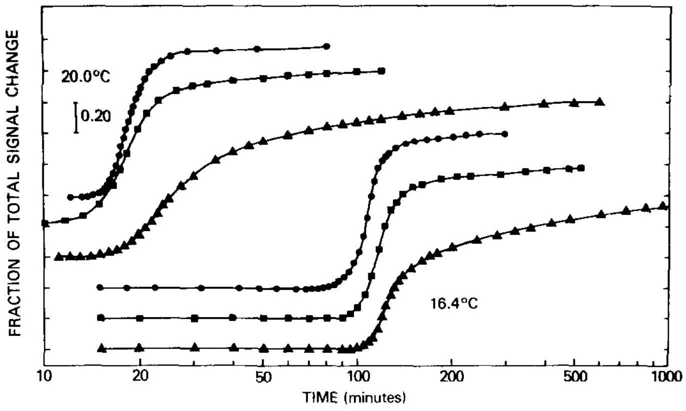

Figure 3: Comparison of NMR (closed squares), birefringence (closed triangles), and turbidity (closed circles) progress curves at 20.0 and 16.4°C.

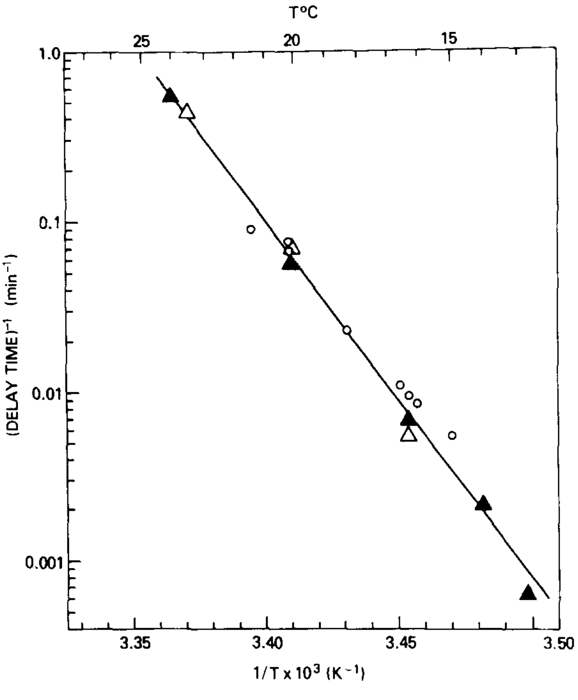

Figure 4: Comparison of reciprocal delay times measured by NMR (open circles), birefringence (closed triangles), and turbidity (open triangles) as a function of the reciprocal temperature. The reciprocal delay times from NMR represent single experiments, while each point for the turbidity and birefringence results represent the average reciprocal delay time from at least three experiments at each temperature.

on the same straight line. This result shows that the delay time measured by the different techniques is the same for identical samples at the same temperature.

DISCUSSION

The progress of the gelation reaction has been observed by monitoring five different physical properties - birefringence (7), heat absorption (7,8), turbidity (9), viscosity (10,11), and now water proton magnetic resonance linewidths. Understanding the change in each of these properties is complicated by the fact that at equilibrium the polymers are not randomly oriented, but are aligned in an ordered phase (7,8,19). Only in the case of birefringence is the interpretation reasonably clearcut. The birefringence measures only aligned polymers, and Hofrichter has presented a theory which shows that the extent of formation of aligned polymers is simply proportional to the square root of light transmitted between crossed polars (20). In contrast, the bulk of the heat absorption probably comes from the polymerization process, with little or no heat associated with alignment (8). There are probably two major contributions to the turbidity. Turbidity can originate from light scattering by individual polymers, and from domains of aligned polymers as a result of discontinuities in the refractive index at the domain-solution interface or at interfaces between domains. Because the scattering is only a very weak function of wavelength, the latter mechanism may dominate in our experiments. There are also at least two possible sources of broadening of the water proton magnetic resonance line. The first mechanism, discussed by Thompson et al. (14), and more recently by Zipp et al. (21), utilizes a model in which water is rapidly exchanged among three distinguishable environments. According to this model, the transverse relaxation time (T_2) is determined by a small fraction of the water that is tightly bound to the protein, and has a correlation time comparable to the rotational diffusion time for the protein. Since the rotational diffusion time of polymerized hemoglobin is considerably longer than that of the monomer, it is likely that the observed increase in linewidth results at least in part from the increased correlation time of the tightly bound water fraction (14,21,22). A second source of broadening upon gelation could be the magnetic field inhomogeneities caused by the formation of polymer domains having magnetic susceptibilities different from the bulk solution, as well as by the anisotropy in the magnetic susceptibility of the aligned polymers. This mechanism has been discussed for other systems (23-26). It predicts that the linewidth will increase nearly linearly with increasing field strength, in accordance with our observations ($\Delta\nu_{\frac{1}{2}}$ = 3.7 Hz at 60 MHz and 10.0 Hz at 220 MHz for ungelled samples; $\Delta\nu_{\frac{1}{2}}$ = 6.5 Hz at 60 MHz and 17.5 Hz at 220 MHz for gelled samples at 19°C) and those of Cameron and Block (15) (see also ref. 14). Finally, the viscosity results are complicated by the large shear dependence (11), and no attempt has yet been made at a detailed theoretical interpretation.

The major result of the experiments presented here is that the onset of the gelation reaction is found to occur simultaneously by NMR, birefringence, and turbidity methods. We have previously found, moreover, that the delay time for the appearance of heat absorption and birefringence is also the same for identical samples (7). The minimal conclusion from these results is that the change in all of these physical properties is rate-limited by the same process. We have already proposed that this rate-limiting process is the formation of thermodynamically unstable nuclei for individual polymers, and that each nucleus consists of about 30 hemoglobin molecules (7). Since the nuclei are predicted to be at very low concentration, the observation of a delay using a variety of techniques that are sensitive to the aggregation of hemoglobin provides strong support for a nucleation mechanism. Nucleation can quantitatively account for the concentration and temperature dependence of the delay time (7). A nucleation mechanism could also account for the finding that increasing the shear rate decreases the delay time in viscosity experiments (11), and the preliminary observations that in scanning experiments the viscosity change precedes the changes in other physical properties (11,12).

The shearing force could break up polymers as they form, generating multiple nuclei in an autocatalytic fashion and thereby shortening the delay time.

In order to gain more insight into the structural mechanism of the gelation reaction it would seem important to directly observe the formation of nuclei during the delay period. This will require detailed investigations of the small changes that have been observed thus far, such as the slight sharpening of the NMR water line prior to the large broadening. It would also be important to extend the kinetic measurements to much shorter time scales, in order to separate the polymerization steps from the alignment process. We have observed overshoots in the turbidity (27) in progress curves with delay times less than about 100 sec. This result suggests that sequential steps in the gelation reaction might become well separated by decreasing the delay time to 0.1 - 1 sec., and we are currently extending our kinetic measurements into this time range.

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