Viscometric and Spectrophotometric Measurements of Hemoglobin S Polymerization Kinetics

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The rates of polymerization and depolymerization of identical concentrated deoxygenated hemoglobin S (HbS) solutions following a rapid temperature change were examined by several methods. Two of these methods measured viscosity changes in either gently agitated (AGT) or nonagitated (NAGT) samples. The third method utilized a change in turbidity at 735 nm (SDT). By all three methods, a delay period, during which no observable change was detected, followed the temperature change. Gelation, as determined in nonagitated samples by a viscosity-based technique, occurred before or coincided with gelation as determined spectrophotometrically. The slope of the concentration dependence of the delay time is significantly decreased by agitation. Similar monitoring of the depolymerization reaction indicated the persistence of increased viscosity after observation of a marked decrease in turbidity.

The fundamental process that produces sickle cell anemia is aggregation of hemoglobin S (HbS) molecules. Deoxygenated HbS is relatively insoluble compared to deoxygenated normal adult hemoglobin (HbA) or oxygenated HbS and aggregates into long noncovalently linked polymers. Insights into the pathophysiology of sickle cell disease have resulted from investigation of the kinetics of the aggregation process in cell-free systems. The physiologic significance of kinetic experiments of HbS aggregation relates to the time required for an erythrocyte to traverse a capillary bed, where it becomes partially deoxygenated. Sickle erythrocytes, which develop significant quantities of intracellular aggregates, may be impeded in their ability to deform and thus traverse the capillary bed. The work of Hofrichter et al. indicates that factors that appear to have minor effects on HbS gelation equilibrium or which are difficult to control in equilibrium studies may exert major effects on the kinetics of aggregation. Kinetic studies have not only increased the understanding of the basic mechanisms of aggregation but also have permitted the study of the effects of antisickling agents on the rate and extent of gelation. Kinetic data may be used to estimate the increase in delay time required to produce significant clinical benefit, and thus, may enable in vitro evaluation of the efficacy of antisickling agents under specified conditions.

The present studies compare several of the commonly employed kinetic techniques, namely, the monitoring of polymerization by changes in viscosity and the monitoring of turbidity by absorbance in the near infrared region (735 nm) in concentrated HbS solutions. The length of the delay period and the dependence of the delay period on deoxy-HbS concentration was determined using each technique in identically prepared samples. Spectrophotometric change occurred either after or coincident with an increase in viscosity in nonagitated samples.

MATERIALS AND METHODS

Preparation of Hemoglobin Solutions

The use of human subjects was approved by the Human Research Committee, and informed consent was obtained in accordance with the provisions of the Declaration of Helsinki. Blood was obtained from individuals homozygous for sickle cell anemia. Cells were washed 3 times with 0.9% NaCl; lysis was accomplished by the addition of 1 volume distilled water and 0.4 volume toluene, followed by centrifugation 2 times at 39,000 g for 20 min at 4°C to remove cell membrane fragments. Purification of oxy-HbS was performed on DE-52 anion exchange resin (Whatman, Inc., Clifton, NJ) by the method of Huisman and Dozy, with minor modifications. All sample handling was carried out at 4°C to minimize methemoglobin formation. Purity of the HbS thus obtained was verified by cellulose acetate electrophoresis; purification in this manner also affected the removal of organic phosphates. When required, HbS was stored at −80°C.

HbS samples were dialyzed against 0.25 M potassium phosphate, pH 7.7, and concentrated by pressure ultrafiltration (Amicon ultrafiltration unit, PM10 membrane, Amicon Corp., Lexington, MA) to a concentration giving a conveniently measurable delay time. This was usually 16–20 mM heme. Hb concentrations were measured as the cyanmet Hb derivative and were expressed as mmole heme/liter, based on ε = 11.5 mmole⁻¹ cm⁻¹ per heme at 540 nm. Samples were deoxygenated under nitrogen; complete deoxygenation was assured by the addition of sodium dithionite to a final concentration of 50 mM. The final pH was verified at the end of each experiment using a Corning Digital 160 Blood Gas System. If required, dilution of aliquots was achieved by the addition of small volumes of deoxygenated 0.25 M potassium phosphate, pH 7.0, or bovine serum albumin (BSA) solution. BSA (Sigma Chemical Co., St. Louis, MO) solutions were prepared at a concentration equal to that of the HbS solution after dithionate addition; the required amount of BSA was added.
was dissolved in 0.25 M potassium phosphate, pH 7.0, and deoxy-
genated. Concentration of the BSA solution was determined by spectrophotometric measurement at 280 nm (ε = 0.613 mg⁻¹ cm⁻¹ ml⁻¹). Thorough mixing at all stages of sample handling eliminated concentration heterogeneity. Methemoglobin concentration was determined by the method of van Assendelft and was less than 5% in all experiments.

2,3-Diphosphoglycerate (2,3-DPG) was obtained from Sigma as the pentacyclohexylammonium salt; it was converted to the H⁺ form on AG50W-X8 (BioRad), lyophilized, and dissolved in 0.25 M potassium phosphate, pH 7.7. An appropriate volume was added to HbS prior to dithionite addition; final 2,3-DPG concentration was measured by the method of Keitt. Inositol hexaphosphate was obtained from Sigma.

Aggregation Kinetics

Agitated gelation time (AGT). Measurement of the AGT was performed by gently agitating a 250-μl sample of deoxygenated HbS in a sealed 12 × 75 mm nitrogen-filled test tube after the tube had been transferred from an ice bath to a water bath equilibrated at 30°C, and the time required for the solution to cease to flow was observed. The half-time for thermal equilibration following a temperature jump from 2°C to 30°C was 16 sec. When values within a given experiment were compared, the coefficient of variation was 11%; the variability in this measurement in a series of experiments was 17%.

Nonagitated gelation time (NAGT). Verification of the time of physical gelation in a nonagitated system was performed in two ways. In the first, a series of sealed 12 × 75 mm nitrogen-filled tubes, each containing 200 μl of deoxygenated HbS, was transferred from an ice bath to a water bath at 30°C. The first tube of the series was inspected by gently tipping at intervals of 6–18 sec to determine the time required for formation of a physical gel. When gelation was observed, the next tube in the series was similarly handled. The time of physical gelation in this nonagitated system was established as that time at which a tube in the series contained firmly gelled HbS on the occasion of the first inspection of that tube. This inspection would have occurred 6–18 sec after gelation in the preceding tube, which had had more than one tip. The intraassay variability in this measurement was 2%. In a second method, estimation of the extent of physical gelation in the flow cell described below was made. This was done by observation of movement of a nitrogen bubble in the partially filled cell. Freedom of movement of the nitrogen bubble in the cell equilibrated at 2°C was verified. Samples were rated as "gel" if, after a single inversion of the flow cell, no movement of the nitrogen bubble could be detected, and as "firm gel" if no displacement of the nitrogen bubble was observed after vigorous agitation.

Spectrophotometric delay time (SDT). Continuous monitoring of turbidity and determination of the spectrophotometric delay time (SDT) was made in a Beckman Acta V spectrophotometer. A QS169 water-jacketed flow cell (Hellma Cells Inc., Jamaica, NY) with path length 2 mm and internal volume of 250 μl was used in all experiments. The cell was connected to two circulating water baths held at temperatures appropriate for inducing gelation by a sudden rise in temperature. The half-time for thermal equilibration following a temperature jump from 2°C to 30°C was 5.3 sec, within 5.3 sec, with full thermal equilibration occurring within 30 sec. A gentle stream of nitrogen was directed at both exterior windows of the cell to remove condensation. The cell was equilibrated at 2°C and purged with nitrogen. Samples of deoxygenated HbS were injected anaerobically into the flow cell. The sample volume was 250 μl for SDT determinations and 180 μl for estimation of the extent of physical gelation in the flow cell. Completeness of deoxygenation was verified by scanning the sample between 700 and 825 nm. SDT was determined at 735 nm with a slit width of 0.5 mm. Aggregation kinetics were monitored by the change in apparent absorbance due to turbidity following a change in temperature. SDT was defined as the time between the temperature change and the point described by the intersection of lines extended from the baseline and from the tangent to the inflection point in the rapid phase. The coefficient of variation when values within a given experiment were compared was 6%. The interassay variability was 15%; this variability in the SDT is comparable to that reported by Hofrichter et al.

RESULTS

A typical curve for the change in absorbance at 735 nm with time is seen in Fig. 1. This example is representative of the curves obtained in all experiments, although variation was seen in the amplitude of the signal change. Gelation, as determined in this experiment by AGT and NAGT, occurred 70 and 21.6 sec, respectively, before gelation as determined by the SDT.

Decreasing pH and increasing 2,3-DPG or inositol hexaphosphate (IHP) concentration potentiated the aggregation of deoxygenated HbS as evidenced by the reduced concentration required to produce a constant spectrophotometric delay time (Table 1). The spectrophotometric method utilized in these studies detected the effects of pH and organic phosphates on gelation as described by others using similar or different methods. Thus, this technique was capable of demonstrating effects known to be important in gelation of HbS.

Experiments were done to compare the AGT, NAGT, and SDT in identical samples under a variety
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of conditions. We studied a range of HbS concentrations from 14.33 to 15.75 mM heme at pH 6.9–7.0 and performed the temperature jump from 2°C to a variety of higher temperatures, ranging from 22.5°C to 30°C. These results are presented in Table 2. Experiments were generally performed on different days and under varying conditions. Factors such as decreasing deoxy-HbS concentration or decreasing the final temperature generally increased the length of the observed delay period. Attempts were made to reduce day-to-day variation by controlling factors such as Hb composition, presence of organic phosphates, pH changes after dithionite addition, temperature, etc. Despite these efforts, day-to-day variability existed, and inconsistencies in the data are noted in comparing experiments C and D, and E and F. Inspection of the data indicates that, in 8 of 10 experiments, the NAGT was less than the SDT by 3–94 sec. In 2 experiments, NAGT was greater than SDT by 3.6 and 7 sec. Statistical analysis of these 10 experiments was performed by analysis of the differences by the paired t test. This test used as the response for an experiment the average of the replications done for that experiment. This method of analysis was chosen recognizing that experiments were performed under different conditions, that day-to-day assay variability existed, and that, in a number of experiments, repetitive measurements of the same parameter on a given day were not available. Statistical analysis revealed that the AGT is significantly less than both the NAGT (p < 0.01) and SDT (p < 0.01) and that the NAGT is significantly less than the SDT (p < 0.05). Inspection of the sample in the flow cell, as described in Materials and Methods, was performed in three experiments to ascertain the state of physical gelation. Results of this method were in good agreement in all three experiments with the determined NAGT. In experiment A, this inspection confirmed the existence of a firm gel in this nonagitated system at ≤273 sec (n = 2) (NAGT = 291 sec). In experiment B, this time was ≤246 sec (n = 2) (NAGT = 244.5 sec), and in experiment D, ≤153 sec (n = 6) (NAGT = 158 sec).

The depolymerization reaction following the temperature change from 30°C to 2°C was also monitored spectrophotometrically. The deoxy-HbS remained a physical gel, even after the return of the absorbance tracing to baseline. In experiment D (Table 2), the spectrophotometric depolymerization time, determined analogously to SDT, was 82.4 ± 5.8 sec (n = 5). Visual inspection of the sample in the flow cell confirmed the existence of a firm gel at 156–162 sec (n = 3) and a partial gel at 165–172 sec (n = 3). The sample did not flow freely until 170–178 sec (n = 3). The gel

<table>
<thead>
<tr>
<th>Experiment</th>
<th>AGT (sec)</th>
<th>NAGT (sec)</th>
<th>SDT (sec)</th>
<th>Deoxy-HbS Concentration (mM Heme)</th>
<th>Final Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>135.2 ± 11.0 (3)</td>
<td>291.0 ± 4.2 (2)</td>
<td>385 (1)</td>
<td>14.33 ± 0.23</td>
<td>30°C</td>
</tr>
<tr>
<td>B</td>
<td>114.0 ± 1.7 (2)</td>
<td>244.5 ± 6.4 (2)</td>
<td>260 (1)</td>
<td>14.74 ± 0.13</td>
<td>30°C</td>
</tr>
<tr>
<td>C</td>
<td>96.2 ± 0.4 (3)</td>
<td>153.6 ± 0.8 (2)</td>
<td>150 (1)</td>
<td>14.90 ± 0.19</td>
<td>30°C</td>
</tr>
<tr>
<td>D</td>
<td>109.6 ± 12.0 (8)</td>
<td>158.0 ± 5.6 (2)</td>
<td>179.6 ± 11.8 (5)</td>
<td>15.25 ± 0.16</td>
<td>30°C</td>
</tr>
<tr>
<td>E</td>
<td>58.5 ± 14.0 (2)</td>
<td>96 (1)</td>
<td>89 (1)</td>
<td>15.36 ± 0.04</td>
<td>30°C</td>
</tr>
<tr>
<td>F</td>
<td>78.6 (1)</td>
<td>120 (1)</td>
<td>123 (1)</td>
<td>15.75 ± 0.11</td>
<td>30°C</td>
</tr>
<tr>
<td>G</td>
<td>132.6 (1)</td>
<td>156 (1)</td>
<td>205 (1)</td>
<td>15.75 ± 0.11</td>
<td>27°C</td>
</tr>
<tr>
<td>H</td>
<td>159.6 (1)</td>
<td>246 (1)</td>
<td>260 (1)</td>
<td>15.75 ± 0.11</td>
<td>24.5°C</td>
</tr>
<tr>
<td>I</td>
<td>165.0 (1)</td>
<td>264 (1)</td>
<td>272 (1)</td>
<td>15.75 ± 0.11</td>
<td>23.8°C</td>
</tr>
<tr>
<td>J</td>
<td>175.8 (1)</td>
<td>402 (1)</td>
<td>440 (1)</td>
<td>15.75 ± 0.11</td>
<td>22.5°C</td>
</tr>
</tbody>
</table>

Identical Hb samples were used in measuring AGT, NAGT, and SDT in a given experiment. All experiments were conducted in 0.25 M potassium phosphate buffer, pH 7.0. The pH after dithionite addition was 7.0 in all experiments, except D, where the final pH was measured to be 6.9. Initial temperature in each experiment was 2°C. Numbers in parentheses indicate the number of replicates. Values are given as mean ± 1 SD.
state thus persisted as long as 90 sec after the absorbance tracing had returned to baseline values.

Further experiments evaluated the concentration dependence of the polymerization reaction as determined by the various kinetic techniques. The reaction order determined by the viscosity-based techniques (AGT and NAGT) was compared to that determined by the spectrophotometric method (SDT). If the rate of the reaction is defined as the reciprocal of AGT or SDT, the slope of a plot of the log of the reaction rate versus the log of the HbS concentration specifies the formal order of the reaction, assuming solution ideality. Excluded volume effects, however, lead to non-ideality in HbS solutions at the high protein concentrations used in these experiments. Protein activity coefficients increase with the increasing protein concentration; the true reaction order may be determined from a plot of log 1/reaction time versus log HbS activity. The reaction order can be determined from the HbS concentration if the activity coefficient of HbS is constant as the concentration varies.

Results of a typical experiment investigating the dependence of both AGT (Fig. 2, A and B) and SDT (Fig. 2, C and D) on HbS concentration are seen in Fig. 2. In Fig. 2 (A and C), the HbS concentration was varied, but the total protein concentration kept constant by the addition of appropriate amounts of BSA. In Fig. 2 (B and D), the HbS concentration was varied by the addition of small amounts of phosphate buffer. In this experiment, using measurements of the AGT, the slopes were 13.1 in the absence of BSA and 2.7 in the presence of BSA. Using measurements of SDT, the slopes were 27.2 and 5.8 in the absence and presence of BSA, respectively. In a similar experiment, the concentration dependence of the NAGT was 16.7 in the absence of BSA. The combined results of a series of experiments investigating the concentration dependence of the AGT showed the slopes to be 11.8 ± 2.6 (n = 7) in the absence of BSA and 3.1 ± 1.4 (n = 5) in samples diluted by the addition of equimolar BSA. A similar series of experiments investigating the concentration dependence of the SDT demonstrated the slopes to be 31.3 ± 8.1 (n = 7) and 5.3 ± 0.6 (n = 5) in the absence and presence of BSA, respectively.

**DISCUSSION**

Upon rapid warming of a concentrated deoxygenated HbS solution, the solution becomes increasingly viscous and exhibits well-documented changes in a number of characteristics, such as viscosity,^12,13,18,19 turbidity,^3,5,14,20 heat absorption,^1,4^ birefringence,^3,4,20 nuclear magnetic resonance water line width,^20^ and transverse water proton relaxation times.^^^^21 Gelation kinetics have also been observed following laser photolysis of carbon monoxide from HbS; these experiments have extended observations to short time ranges and very small sample sizes. Following a temperature jump or loss of carbon monoxide, a delay period ensues, during which no apparent change occurs in the physical feature being observed. This delay is followed by a phase of rapid signal change. Attempts to correlate observations from the various methods described above are confused by the use of different experimental conditions (pH, ionic strength, organic phosphate concentrations, degree of purification of HbS, etc.) used by each investigator, and by the differences inherent to the various methods of measurement (time required for thermal equilibration, degree of agitation, etc.). Eaton et al. compared the results of birefringence, turbidity, and water proton magnetic resonance experiments using identically prepared samples and found that all three techniques see the onset of gelation simultaneously. Similarly, Ross et al. observed identical delay times in calorimetric and birefringence experiments. Until the present study, however, no report has correlated viscosity changes in a system similar to that used to measure other changes in the physical properties of deoxy-HbS after a rapid temperature increase. The studies described in this report.

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**Fig. 2.** Dependence of the AGT (A, B) and SDT (C, D) on HbS concentration. The effect of protein environment on reaction order measured by each technique is seen. Curves of deoxy-HbS diluted by addition of 25.1 g/dl BSA solution (final pH 6.9) are shown by open circles. Curves of deoxy-HbS diluted by addition of 0.25 M potassium phosphate (final pH 7.0) are shown by closed circles.
determine the onset of a marked increase in viscosity relative to an abrupt change in turbidity. Our studies, using a series of identical samples, showed that a marked increase in viscosity, as measured in a gently agitated system (AGT), preceded an alteration in turbidity (SDT) \((p < 0.01)\). Paired analysis of the differences between the NAGT and the SDT indicated that the NAGT is significantly less than the SDT \((p < 0.05)\). The observation that aggregation within certain limits reduces the lag time and accelerates gelation has previously been reported.\(^{12,18,21}\)

We have also monitored the depolymerization reaction in order to determine the sequence of events when the deoxy-HbS is again cooled. Several investigators have determined that the depolymerization reaction exhibits different kinetics from the polymerization reaction.\(^{3,14}\) Our experiments confirm this observation. When monitored spectrophotometrically, the polymerization time (SDT) in one experiment was 179.6 \(\pm\) 11.8 sec; similar monitoring of the depolymerization reaction gives a value of 82.4 \(\pm\) 5.8 sec. Further, it appears that the expected decrease in viscosity does not occur at this time, but may require as long as an additional 90 sec. It thus appears that the spectrophotometric change during depolymerization precedes the viscosity change. This sequence is the reverse of that observed in monitoring the polymerization process.

Observation of HbS polymerization by any of the means described here (AGT, NAGT, SDT) indicates that a discrete lag or delay period occurs between the time of the temperature jump and an observable change. The magnitude of the delay period is sensitive to temperature, HbS concentration, pH, degree of oxygenation, and organic phosphates; in samples not corrected for solution nonideality, this delay period is sensitive to a high power of the Hb concentration. Hofrichter et al.\(^{3,24}\) found that the delay period is inversely proportional to the 30–40th power of the HbS concentration in turbidity experiments similar (\(\lambda = 800 \text{ nm}\)) to those described in this report. The present study found a concentration dependence of 31.3 \(\pm\) 8.1 for buffer-diluted deoxy-HbS solutions when gelation was measured by a change in turbidity at 735 nm (SDT). The concentration dependence of the lag phase is 21st order\(^{19}\) or 26th order\(^{22}\) in viscosity experiments in which samples are diluted by buffer addition. In this report, the concentration dependence of the AGT in buffer-diluted samples is 11.8 \(\pm\) 2.6. The reduced concentration dependence in agitated samples may be explained by the disruption of newly formed polymers by shear forces, a process that may increase the number of nucleation sites available for polymer growth.\(^{23}\) The concentration dependence of the NAGT is 16.7 in buffer-diluted samples. Viscosity-based techniques demonstrate a reduced concentration dependence relative to spectrophotometric determinations.

These calculations of concentration dependence, however, presume that the HbS molecules behave as an ideal solution; this condition is not valid at the high HbS concentrations used. At these concentrations, or in the red cell, the hemoglobin molecules are very large relative to low molecular weight ions and closely packed. This molecular crowding requires that the activity, rather than the concentration, be used to calculate the delay time dependence. Alternatively, the concentration dependence may be accurately assessed in a series of samples in which the activity coefficient remains constant as the HbS concentration varies; this is achieved by the addition of an equimolar BSA solution. Samples diluted by addition of BSA in the present study demonstrated the concentration dependence to be 3.1 \(\pm\) 1.4 and 5.3 \(\pm\) 0.6 for AGT and SDT, respectively. With correction for solution nonideality, Behe and Englander\(^{17}\) estimate the nucleation reaction to be approximately tenth order, using a technique based on changes in viscosity. Sunshine et al.\(^{26}\) also conclude that nonideality contributes significantly to the observed power dependence (concentration dependence equals 16 with correction for nonideality, versus 34 without). In general, after correction for nonideality, viscosity-based techniques appear to demonstrate a reduced concentration dependence relative to spectrophotometric techniques.

The kinetics of the polymerization process are, as described above, characterized by a lag period followed by a phase of rapid signal change, indicating that the polymerization process occurs via a nucleation-controlled reaction. Aggregates with fewer than a critical number of subunits dissociate spontaneously, whereas aggregates of critical size can rapidly align into fibers. Attempts have been made to correlate the reaction order with the size of the critical nucleus and the number of strands in models of HbS fibers.\(^{2,3,12,14,26}\) Polymerized HbS is a microtubular structure originally described as consisting of six\(^{27}\) to eight\(^{28}\) strands. More recent structural analyses indicate that the polymerized Hb fiber is a helical structure with 14–16 tetramers in each layer.\(^{29,30}\) Determination of the reaction order of the polymerization reaction has produced wide ranges of results, depending on the particular method and conditions employed; the relationship of...
the reaction order to the structure of the polymer remains to be determined.

We have thus observed that a marked increase in viscosity precedes or closely coincides with the spectrophotometrically determined delay time, and that the reaction order, as determined by viscosity-based techniques, is smaller than that determined by the spectrophotometric method. These experiments indicate that significant events in the aggregation process may occur before any observable spectrophotometric change. It is likely that changes continue to take place in the gel long after its initial formation. Elasticity studies have demonstrated that the assembly of the network continues for a significant time after the first detection of elasticity and that the rate of increase of elasticity is dependent on the HbS concentration, with higher concentrations resulting in more rapid elasticity increases. It is possible that our choice of wavelength contributed to this reduced sensitivity; however, the detection of smaller particles requires the use of a lower wavelength, at which instrument noise resulting from higher background absorption may obscure results. The spectrophotometric technique accurately measures the effects of known inhibitors or stimulators of gelation. Additionally, simple viscosity-based techniques, such as those described by Behe and Englander and in this article, appear to be as useful and as sensitive to early events in the polymerization process as the spectrophotometric method.

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