Aggregation and Crystallization of Hemoglobins A, S, and C

PROBABLE FORMATION OF DIFFERENT NUCLEI FOR GELATION AND CRYSTALLIZATION

Kazuhiko Adachi and Toshio Asakura

From The Children's Hospital of Philadelphia, Department of Pediatrics and Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104

The oxy and carbonmonoxy forms of Hb A and Hb S formed aggregates or gels when dissolved in phosphate buffers at concentrations above their solubility and warmed rapidly to 30°C from 0°C. Kinetic studies showed that although deoxy-Hb A and deoxy-Hb S aggregated with a clear exhibition of a delay time, the oxy and carbonmonoxy forms of Hb A and Hb S did not show a delay time. These results suggest that the deoxy forms of Hb A and Hb S aggregate according to the nucleation-controlled mechanism, while oxy- and carbonmonoxyhemoglobins aggregate by the simple linear aggregation mechanism. It was also found that the gels or aggregates of deoxy-Hb A and carbonmonoxy-Hb S could be converted to crystals by further incubation. The rate of crystallization depended upon the concentration of hemoglobin in the supernatant, with faster crystallization at higher concentrations. Similar experiments with deoxy-Hb C (86 Glu → Lys) showed that this hemoglobin also crystallized after aggregation, with both reactions accompanied by a delay time. The activation energy for the crystallization reaction of deoxy-Hb C (100 – 150 kcal/mol) was much higher than that for the aggregation reaction (20 kcal/mol). These results suggest that deoxy-Hb A, deoxy-Hb S, and deoxy-Hb C form two types of nuclei that are specific to the formation of gels (or aggregates) or crystals. The concentration of hemoglobin measured after completion of crystallization was much lower than that measured after gelation or aggregation and was independent of the initial hemoglobin concentration. This concentration is assumed to be the real solubility of hemoglobin.

Gelation of the deoxy form of sickle hemoglobin (deoxy-Hb S) is characterized by a delay time prior to aggregation (1–8). This unique kinetic reaction has led to the hypothesis that deoxy-Hb S polymerizes according to the nucleation-controlled polymerization mechanism (3, 7). Recently, we found that deoxy-Hb A, as well as deoxy-Hb S and deoxy-Hb C, formed aggregates with a clear exhibition of a delay time (9–11) when solutions of these hemoglobin in concentrated phosphate buffers with concentrations of 120 – 200% of their solubility were heated rapidly from 0–30°C. Preliminary comparative studies on the aggregation and deaggregation reactions of deoxy-Hb S and deoxy-Hb A showed no essential difference in the kinetics and the macroscopic findings of the gels, except that much higher concentrations of Hb A were required for aggregation and that aggregates of deoxy-Hb A could be converted to crystals at a faster rate (10, 11). These results suggest that hemoglobins other than Hb S and Hb A would also aggregate similarly if incubated under appropriate conditions. We investigated the polymerization properties of deoxy-Hb C because this hemoglobin has an amino acid substitution at the same site as does Hb S and is known to crystallize easily (12, 13). We also investigated the aggregation and crystallization of the oxy, carbonmonoxy, and deoxy forms of Hb A and Hb S. Differences in the mechanism of the aggregation (gelation) and crystallization of various hemoglobins with different ligand states will be discussed.

EXPERIMENTAL PROCEDURES

Hb A and Hb S were purified from Hb AS blood by column chromatography on DEAE-Sephadex as described elsewhere (10, 11). Hb C, purified from Hb C, Hb AC, and Hb SC blood by the standard chromatographic technique on CM-Sephadex and was identified by the standard cellulose acetate and starch gel electrophoresis methods and by the mechanical shaking test (10, 14). The concentration of hemoglobin was determined spectrophotometrically by the use of the millimolar extinction coefficient of moll. The concentration of hemoglobin was determined by measuring the concentration of dissolved hemoglobin after centrifugation. The stock solution of concentrated phosphate buffer for the experiments used for 3 M (KH2PO4, 92.50 g, and K2HPO4, 404.1 g/liter). The pH of the solution was determined with a pH electrode after a 10-fold dilution of the solution.

RESULTS

Aggregation of Oxy and Carbonmonoxy Forms of Hb S and Hb A—As shown previously (10, 11), solutions of deoxy-Hb S and deoxy-Hb A in concentrated phosphate buffer aggregated with a clear exhibition of a delay time. If the hemoglobin concentration was 6 g/dl or more, gels were formed, while below this concentration, suspensions of amorphpously aggregated hemoglobin were formed. In order to investigate if the oxy and carbonmonoxy forms of Hb S have properties similar to those of deoxy-Hb S and deoxy-Hb A, oxy- and carbonmonoxy-Hb S were dissolved in concentrated phosphate buffer at various concentrations at 0°C and warmed rapidly to 30°C. As shown in Fig. 1, both oxy- and carbonmonoxyhemoglobins aggregate, but without a distinct delay time prior to aggregation. Quite interestingly, when the solution was further incubated overnight or longer, the aggregates converted to crystals. As a result, the turbidity increased sharply (Fig. 1). The minimum hemoglobin concentration required for the aggregation of carbonmonoxy-Hb S (1.8 g/dl) in 1.8 M phosphate buffer was slightly higher than that of oxy-Hb S (1.5 g/dl). The rates of crystallization depended on the
Aggregation and Crystallization of Hb A, Hb S, and Hb C

![Graph A](image1.png)

**Fig. 1. Kinetics of aggregation of liganded Hb S.** A, effect of hemoglobin concentration on the rate of aggregation of oxy-Hb S in 1.8 M phosphate buffer, pH 7.4. Hemoglobin solutions (A, 2.27 g/dl; B, 1.80 g/dl; C, 1.65 g/dl; D, 1.59 g/dl; E, 0.93 g/dl) were heated from 0–30°C at time zero. The turbidity was measured spectrophotometrically by recording the absorbance at 700 nm. B, effect of hemoglobin concentration on the rate of the aggregation of CO Hb S (A, 2.13 g/dl; B, 1.95 g/dl; C, 1.85 g/dl). Experimental conditions are the same as those shown in Panel A.

![Graph B](image2.png)

**Fig. 2. Effect of hemoglobin concentration on the rate of aggregation of oxy- and carbonmonoxy-Hb A.** Solid lines indicate results for oxy-Hb A and dotted lines for carbonmonoxy-Hb A (A, 4.1 g/dl; B, 3.7 g/dl; C, 3.4 g/dl; A’, 6.35 g/dl; B’, 5.9 g/dl). Experimental conditions were the same as those shown in Fig. 1A.

dotted line in Fig. 3). Since supernatant hemoglobin does not absorb light at 700 nm, the optical density decreased to near zero (Fig. 3). After about 2 h, the crystallization reaction began and was completed after further incubation for 40 min. No crystallization occurred if amorphous aggregates alone were incubated. Amorphous aggregates were converted gradually to three-dimensional crystals only in the presence of the soluble fraction. If the initial concentration of deoxy-Hb C was decreased to 0.5 g/dl, there was no aggregation reaction, but crystallization occurred after a delay time of 41 h. This suggests that a specific hemoglobin concentration is required for aggregation and that, below this level, Hb C forms crystals without first forming amorphous aggregates. The relationship between changes in optical density at 700 nm (turbidity) and initial hemoglobin concentration to aggregation and crystallization is shown in Fig. 4. The turbidity caused by both the aggregation and crystallization reactions increased linearly with increases in the initial hemoglobin concentration (Fig. 4). The length of the delay time for both the aggregation and crystallization reactions also depended on the initial hemoglobin concentration.

**Size of Nuclei for Aggregation and Crystallization of Deoxy-Hb C**—Hofrichter et al. (3) and Kowaleczkowski and Steinhardt (15) reported that the size of a nucleus produced during the delay time could be related to the slope (n value) of a curve obtained by the logarithmic plot of the delay time versus hemoglobin concentration. As shown in Fig. 5, the n
values for the aggregation and crystallization reactions of deoxy-Hb C in 2.1 M phosphate buffer, pH 7.4, at 30°C are clearly different. The value for aggregation is 1.2, while that for crystallization is 3.0. These data suggest that nuclei formed prior to aggregation are different from those for crystallization. These values are also different from those obtained for deoxy-Hb A and deoxy-Hb S (10). The size of nuclei formed prior to the aggregation of deoxyhemoglobin C is shown in Fig. 6. These experiments were carried out on the rates of both the aggregation and crystallization reaction. The size of nuclei formed prior to the aggregation of deoxyhemoglobins S and C is strongly affected by the type of amino acid at the sixth position of the β chain.

**Effect of Temperature on the Aggregation and Crystallization Reactions of Deoxy-Hb C**—The effect of temperature on the rates of both the aggregation and crystallization reactions is shown in Fig. 6. These experiments were carried out in 2.1 M phosphate buffer, pH 7.4, with a constant hemoglobin concentration (1.35 ± 0.014 g/dl). The temperature was raised from 0°C to the temperatures shown in Fig. 6. The delay times and the amplitudes for both aggregation and crystallization reactions were temperature-dependent. The lower the temperature, the longer the delay time and the smaller the amplitude. The same tendency is seen in the polymerization of deoxy-Hb A and deoxy-Hb S (10). Arrhenius plots of the dependence on temperature of the delay times for both the aggregation and crystallization reactions are shown in Fig. 6. The energy of activation for the delay time of the aggregation reaction is calculated as 20 kcal/mol. The curve for the delay time for crystallization bends slightly at higher temperatures.

The energies of activation for the crystallization reaction range from about 100 kcal/mol at higher temperatures (near 30°C) to as high as 150 kcal/mol at lower temperatures (near 20°C), which are much higher than those for aggregation. These results support the idea that the mechanisms for the aggregation and crystallization of deoxyhemoglobin are temperature-dependent and different. The substitution of lysine for glutamic acid at the β position of deoxy-Hb C inhibits aggregation (gelation) but accelerates crystallization.

**Deaggregation of Deoxy-Hb C Aggregates and Reliquefaction of Crystals**—Both amorphously aggregated deoxy-Hb C and crystalline deoxy-Hb C could be liquefied by cooling and by diluting the phosphate buffer. As shown in Fig. 7, amorphous aggregates of deoxy-Hb C deaggregated upon dilution of phosphate buffer with water at a rate much faster than did crystals.

**Solubility of Hb S and Hb A with Different Ligands**—The concentration of hemoglobin in the supernatant after the aggregation reaction has reached a plateau is called the “solubility” of the hemoglobin in that solution. As reported previously (11), the solubility of deoxy-Hb S in 1.8 M phosphate buffer was independent of the initial deoxy-Hb S concentration. The solubility of deoxy-Hb S determined by measuring the concentration of the supernatant deoxy-Hb S after gelation in low phosphate buffer was also reported to be inde-
Aggregation and Crystallization of Hb A, Hb S, and Hb C

Solubilities of Deoxy Forms of Hb A and Hb C—The apparent solubilities of deoxy-Hb A, oxy-Hb S, and carbonmonoxy-Hb S at the plateau of the aggregation curves were dependent on the initial hemoglobin concentration. However, solubilities measured after the completion of the crystallization reaction were independent of the initial hemoglobin concentration. Similar results were obtained for the measurement of the apparent solubility of deoxy-Hb C in 2.1 M phosphate buffer, pH 7.4, at 30°C (Fig. 9). The solubility measured after completion of the crystallization reaction was 0.16 ± 0.02 g/dl. Under the same condition, the apparent solubilities of deoxy-Hb A were 0.35 ~ 0.41 g/dl after aggregation and the solubility measured after crystallization was 0.016 g/dl. Thus, hemoglobin concentrations in the supernatant of deoxy-Hb C after the aggregation and crystallization reactions were much higher than those of deoxy-Hb A. The value of the solubility measured after crystallization of deoxy-Hb C coincides well with that obtained by extrapolating zero excess turbidity on a plot of O.D. at 280 against total hemoglobin concentration (Fig. 4).

Fig. 7. Deaggregation of aggregates and reliquefaction of crystals of deoxy-Hb C in 2.1 M phosphate buffer. The hemoglobin concentration of deoxy-Hb C for the solubilization curve for crystals was 0.6 g/dl and that for aggregates was 1.0 g/dl. The aggregates and crystals of deoxy-Hb C were melted by the dilution of phosphate molarity with water at 30°C. The broken line is the solubilization curve for aggregates and the solid line is that for crystals.

Fig. 8 (left). Relationship between the solubility of HB S and HB A with different ligands, and the initial hemoglobin concentration in 1.8 M phosphate buffer, pH 7.4. The solubility was determined by measuring the concentration of soluble hemoglobins in the supernatant after completion of aggregation (gelation) and crystallization.

Fig. 9 (right). Relationship between the solubility of deoxy forms of HB S, HB A, and HB C and the initial hemoglobin concentration in 2.1 M phosphate buffer, pH 7.4. The solubility was determined by measuring the concentration of soluble hemoglobins in the supernatant after completion of the aggregation and crystallization of deoxy-Hb C.

DISCUSSION

Mechanism of Gelation of Deoxy-, Oxy-, and Carbonmonoxyhemoglobins in Concentrated Phosphate Buffer—The results shown in this paper demonstrate that the kinetics of the aggregation of the deoxy forms of Hb A and Hb S is different from the kinetics of the oxy and carbonmonoxy forms of Hb A and Hb S. The aggregation reactions of the deoxy forms of Hb A and Hb S were accompanied by a delay time, while those of the oxy and carbonmonoxy forms of Hb A and Hb S were not. In 1962, Oosawa and Kasai (19) analyzed the linear and helical aggregation of macromolecules such as actin, microtubules, bacteriophage tails, etc. With the linear aggregation mechanism, there is an end-to-end association of aggregates (linear polymers). In the equilibrium state, the solution contains dispersed monomers and linear polymers of various lengths. A delay time does not accompany this reaction. With the helical aggregation mechanism, the aggregates begin to appear when the protein reaches a critical concentration which is determined by solvent conditions. For further growth of helical polymers, monomers attach to the helical nucleus. Above the critical concentration, long helical aggregates co-exist in equilibrium with a constant concentration of dispersed monomers and a small amount of simple linear aggregates. There is a delay time prior to polymer formation. Recently, Hofrichter et al. (3) reported that the gelation of deoxy-Hb S occurred by nucleation-controlled polymerization, a mechanism similar to the helical aggregation mechanism proposed by Oosawa and Kasai (19). In the gelation of deoxy-Hb S, nucleation is the rate-limiting step and each fiber, which may be considered a microcrystal, grows from a separate nucleus and monomers. More recently, we found (9-11) that relatively diluted solutions of deoxy-Hb S and deoxy-Hb A aggregate with a clear demonstration of a delay time in concentrated phosphate buffer. Kinetic studies of deoxy-Hb S in concentrated phosphate buffer suggested that the size of nuclei produced during the delay period is smaller in concentrated phosphate buffer and further decreases with increases in phosphate concentration (10).

As shown in this paper, deoxy-Hb C, deoxy-Hb A, and deoxy-Hb S aggregate by a similar mechanism, while the oxy and carbonmonoxy forms of Hb A and Hb S aggregate without a delay time. The latter hemoglobins appear to aggregate by the simple linear aggregation mechanism (15, 20), which can
be described by a single equilibrium stoichiometry: \( nA \rightleftharpoons A_n \)
where \( A \) is the monomer and \( A_n \) is a large polymeric aggregate.

The gelation or aggregation of deoxy-Hb A and deoxy-Hb C with a delay time suggests that the hydrophobic amino acid substitution (Val) at the \( \beta \) position is not essential for the formation of nuclei. This mutation simply accelerates the gelation or aggregation of the deoxy form of those hemoglobins having it.

Briehl (21) reported that deoxy-Hb S (0.73 g/dl) formed a gel in 1.7 M phosphate buffer. White and Heagan (22) prepared the gels of the oxy and deoxy forms of Hbs A and S in 2.33 M phosphate buffer. We also observed the gelation of various ligand forms of Hb A and Hb S in concentrated phosphate buffer. These results suggest that any type of hemoglobin can form gels which are characterized by the immobilization of gelled proteins upon tilting the test tube. Our results clearly showed that the gelation of oxy- and carbonmonoxymoglobin does not require a delay time. However, we found that aggregates of deoxy-Hb S and deoxy-Hb A formed in concentrated phosphate buffer showed optical birefringence, while aggregates of oxy- and carbonmonoxymoglobin did not unless these hemoglobins were crystallized. This suggests that hemoglobins with \( T \) structure can form three-dimensional polymers (gel) and that the structure of such aggregates is different from that of the aggregates of oxy- and carbonmonoxymoglobin.

**Relationship between Aggregation (Gelation) and Crystallization**—Gels or amorphously aggregated deoxy-Hb A, deoxy-Hb C, oxy-Hb S, and carbonmonox-Hb S produced in concentrated phosphate buffer were found to convert to crystals upon further incubation for overnight or longer. Wishner et al. (23) and Hofrichter et al. (24) reported that gels of deoxy Hb S converted to suspensions of three-dimensional crystals after standing for several months. Pumphrey and Steinhardt (5, 25) reported that the needle-like particles formed in stirred solutions of sickle hemoglobin are a crystalline form of the protein which is thermodynamically more stable than the deoxy-Hb S gel and that the deoxy-Hb S gel is not in a true equilibrium state, being unstable with respect to crystalline form. They proposed that the growth of Hb S crystals in a stirred solution is controlled by a secondary nucleation process (25). As shown in this paper, we found that supernatant solutions of deoxy-Hb A or deoxy-Hb C at the plateau of the aggregation curve produced crystals upon further incubation. The supernatant solution of oxy- and carbonmonox-Hb S also produced crystals even if the aggregation reaction was not accompanied by a delay time. These results and those reported by Pumphrey and Steinhardt (25) suggest that both gelation and crystallization occur according to the nucleation-controlled mechanism and that nuclei for gelation (tentatively called Nuclei G) and nuclei for crystallization (Nuclei C) are different. Nuclei C formed in the supernatant would be required for crystallization because the aggregates can not be converted directly to crystals unless the supernatant fraction is added. We propose the following two schemes for the aggregation and crystallization of hemoglobins.

**Scheme 1. Deoxyhemoglobins.**

| Monomeric Hb (T) | Nuclei G | helical polymers | gels |
| Monomeric Hb (R) | Nuclei C | helical polymers | crystals |

**Scheme 2. Oxy- and carbonmonoxymoglobin.**

This simple mechanism appears to explain various kinetic patterns as well as the ease of gelation and crystallization of various hemoglobins. In Scheme 1, both gelation and crystallization occur by the nucleation-controlled mechanism, as these reactions are accompanied by a distinct delay time. Whether a hemoglobin solution forms gels or crystals is determined by the ease of formation of a specific type of nucleus. The 

| 1828 Aggregation and Crystallization of Hb A, Hb S, and Hb C |

**Scheme 3. Oxygenated and Carbonmonoxymoglobin.**

| Monomeric Hb (T) | Nuclei G | helical polymers | gels |
| Monomeric Hb (R) | Nuclei C | helical polymers | crystals |

As mentioned above and also previously (11), the formation of gels or amorphously aggregated hemoglobin is not the final step in the polymerization reaction of hemoglobin even when the aggregation curve reaches a plateau, because the aggregated hemoglobin eventually converts to crystals. This conversion requires the presence of free monomeric
hemoglobin, as the aggregates themselves cannot be converted to crystals. The conversion of aggregates to crystals occurs only after the addition of the supernatant fraction to the aggregates. Actually, the incubation of the supernatant fraction can produce crystals. The conversion of aggregates to crystals occurs only after the addition of the supernatant fraction to the supernatant containing deoxy-Hb aggregates. Actually, the incubation of the supernatant fraction can produce crystals. The rate of crystallization from the may be attributed to the decreased concentration of deoxy-
As mentioned above, the slow crystallization of deoxy-Hb Hb in 1.8
contrast to the extremely slow rate from that of deoxy-Hb A in 1.8
The solubility of deoxy-Hb C measured in high and low phosphate buffers are conflicting. Itano (29) reported that deoxy-Hb C had a higher solubility than deoxy-Hb A in concentrated phosphate buffer. In contrast, Charache et al. (12) reported that Hb C is less soluble than Hb A in low phosphate buffer.
We found that the solubility of hemoglobin depends highly on the method of measurement.

One method which has been used to determine the solubility of a protein is the salting out method (29). The solubility of hemoglobin can also be determined after crystallization by measuring the supernatant hemoglobin concentration. We found that solubility can be determined after aggregation or gelation which is accompanied by a delay time and that solubilities measured by these three methods, particularly with non-sickle hemoglobins, differed significantly. In addition, the solubility of hemoglobin was affected by the initial hemoglobin concentration. The solubility measured after salting out was highest, followed by that measured after gelation (or aggregation) by the temperature jump method. The solubility measured after crystallization was always the lowest. We assume that the solubility measured after salting out or aggregation is affected by the concentration of monomeric hemoglobin and soluble polymers for the formation of Nuclei C.

We can now explain why the solubility of deoxy-Hb C measured in concentrated phosphate buffer was higher than that of deoxy-Hb A, while the opposite result was obtained in low phosphate buffer (12). Measurements of the solubility of deoxy-Hb C in low phosphate buffers were carried out by measuring the remaining soluble hemoglobin after crystallization, while the solubility of deoxy-Hb A was measured before the formation of crystals (14). We assume that the solubility of deoxy-Hb C in low phosphate buffer would be higher than that of deoxy-Hb A if the solubilities of both hemoglobins were measured after crystallization. These data suggest that the solubility measured after crystallization is the actual solubility of hemoglobin.

Acknowledgments.—We are grateful to Janet Fithian for editorial assistance and Billie Corbett for secretarial help in the preparation of the manuscript.

REFERENCES

Aggregation and Crystallization of Hb A, Hb S, and Hb C