Aggregation of Deoxyhemoglobin S at Low Concentrations*  

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The self-association of deoxyhemoglobin S was measured in dilute solutions (0 to 5 g/dl) by Rayleigh light scattering at 630 nm and osmometry in 0.05 M potassium phosphate buffer (pH 7.35). Weight and number average molecular weights (\(M_w\) and \(M_n\), respectively) and the second or higher virial coefficients, \(B'\) were determined.  

No experimentally significant differences were observed between oxy- and deoxy-Hb S up to the concentration of 2 g/dl; their apparent average molecular weights were within experimental error. Above that concentration, both \(M_w\) and \(M_n\) of deoxy-Hb S were significantly different from that of oxy-Hb S. The negative second virial coefficient of deoxy-Hb S, observed by both techniques, is consistent with the self-association of this protein. The lack of effect of 0.4 M propylurea on the state of aggregation and the significant influence of 0.1 M NaCl suggests that polar interactions are involved in formation of these aggregates.  

The sickling of erythrocytes associated with sickle cell anemia is caused by the polymerization of deoxyhemoglobin S (1). The polymers consist of microtubules of about 180 Å diameter whose supermolecular structure is presently in the process of being defined (2, 3).  

The understanding of the mechanism of polymerization of Hb S has also been the subject of active research in recent years (4, 5) but a final picture is still elusive. Equilibrium ultracentrifugation (6, 7), light scattering (8, 9), and magnetic relaxation of water proton (10) studies have resulted in conflicting results on the presence or absence of pregelation aggregation of deoxy-Hb S.  

The purpose of this paper is to analyze by osmometry and light scattering methods, low concentration Hb S solutions, to establish whether or not the phase-change phenomena generally referred to as "gelation" is preceded by the formation of smaller size hemoglobin aggregates.  

**EXPERIMENTAL PROCEDURES**  

**Methods**—Heparin-treated venous blood was obtained from patients homozygous for Hb S and from normal individuals. Hemolysates were prepared by the method of Drabkin (11) with slight modifications. Hemoglobin concentration was determined on a Cary 14 recording spectrophotometer or a Gilford model 240 spectrophotometer after conversion to the cyanmet form with Drabkin's reagent, using the molar \((64,450)\) extinction coefficient of \(4.6 \times 10^4\) at 540 nm (11).  

**Osmometry**—Osmotic pressure measurements were made with a Wescan High-Speed Membrane Osmometer utilizing Schleicher and Schuell B-19 membranes as described previously (12). Measurements of osmotic pressures of deoxyhemoglobin S and A solutions were made in an atmosphere of \(N_2\) maintained in a Labconco glove box. Before the osmotic pressure determination, both solvent and solution compartments of the osmometric cells were flushed with deoxygenated buffer. Deoxyhemoglobin solution (1 ml) was used to flush the cell compartment before the measurement to remove any adsorbed oxygen. Completion of deoxygenation inside the glove box was checked by observing the gelation of a 24 g/dl solution of hemoglobin S in an open beaker located inside the box or spectrophotometrically in a tonometer (13). With this last method no more than 8% of oxy-Hb was observed. Hemoglobin solutions were not exposed to the presence of sodium dithionate.  

Osmometric pressure data were interpreted on the basis of the equation  

\[
\frac{\pi}{RT} = \frac{1}{M_w} + B'C
\]  

Where \(\pi\) is the measured osmotic pressure, \(M_w\) is the number average molecular weight, \(C\) is the concentration of the protein (in grams per liter), \(R\) is the gas constant, \(T\) is the absolute temperature, and \(B'\) is the second virial coefficient.  

**Light Scattering**—Determination of the turbidity were made in a light scattering photometer of Brice's design (12) made by Wood Manufacturing Co. The instrument was modified by the manufacturer to measure light scattering of 630 nm. Previously dialyzed solutions of hemoglobin were filtered through a double set of Millipore (0.2 μ) filters (12). Stoppered square cells of 10 × 10 × 35 mm were used. The acceptability of solutions was judged according to criteria discussed previously (12). All determinations were corrected for absorption and depolarization.  

Our light scattering results were analyzed using the Debye equation:  

\[
\frac{H'C}{\tau} = \frac{1}{M_w} + 2B'C
\]  

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where $r$ is the excess turbidity of the protein solution over the dialysate, $M_w$ is the weight average molecular weight, $H'$ is the light scattering constant:

$$H' = \frac{32\pi n^2}{3N\lambda^2} \frac{(6 + 6\rho)}{(6 - 7\rho)}$$

where $n$ is the refractive index of the solvent, $(dn/dc)_o$ is the refractive index increment used, $\rho$ is the depolarization ratio, and $(6 + 6\rho)/(6 - 7\rho)$ is the Cabanes factor appropriate for the instrument of Brice's design. The Rayleigh ratio $R_s$, representing the ratio of scattered light at 90 to 0° angle, is the actually measured experimental quantity.

Differential Refractometry—The refractive index increments at constant chemical potential $(dn/dc)_o$ were determined using differential refractometer attachment in a Wood light scattering photometer at 630 nm as described by Elbaum and Herskovits (12).

Table I summarized the refractive index data for oxy-Hb A and oxy-Hb S used for light scattering determinations. The same table contains ranges of absorption and depolarization corrections used for determination of weight average molecular weight. Reversibility of aggregation of chromatographed (Sephadex G-75) Hb S solutions upon reoxygenation was studied by osmometry and results are summarized in Table II. Qualitatively similar results measuring light scattering were obtained on unchromatographed Hb S solution.

**RESULTS**

Osmotic pressure determinations of oxy-Hb A, deoxy-Hb A, oxy-Hb S, and deoxy-Hb S are presented in Fig. 1. While the slopes of both liganded forms of Hb A and of Hb S on the $\pi/CRT$ versus $C$ plot were expectedly positive, deoxy-Hb S was the only one with a negative slope. Similar observations were made measuring light scattering of hemoglobin solutions (Fig. 2). The negative slope on the $H'C/r$ versus $C$ plot was apparent also only for deoxy-Hb S solutions.

Table III summarizes the osmometric and light scattering data obtained from Fig. 1 and Fig. 2. The second virial coefficient ($B'$) obtained from the slope of $H'C/r$ versus $C$ ($2B'$) and $\pi/CRT$ versus $C$ ($B$) were averaged and are also presented in the table.

The effect of concentration of NaCl on the $H'C/r$ of deoxy-Hb A and deoxy-Hb S solutions (2 g/dl) is summarized in Fig. 3. While there is a relatively small effect of NaCl on deoxy-Hb A, addition of 0.1 M NaCl to the 0.05 M potassium phosphate buffer (pH 7.35) changes the weight average molecular weight of deoxy-Hb S from 83,300 to 67,600. The presence of propylurea, a hydrophobic agent, did not change significantly the value of $H'C/r$, while previous work (14) has demonstrated that it inhibits the polymerization of Hb S when judged by minimum gelling concentration. In order to study if the stabilization of the "T" state of Hb S has an effect on its molecular
weight, a 2-fold molar excess of inositol hexaphosphate was added to the protein solution. The results are shown in Fig. 4. The state of aggregation of deoxy-Hb S was undistinguishable in the presence and absence of inositol hexaphosphate.

The degree of polydispersity (ratio of number and weight average molecular weights) is summarized in Fig. 5. The polydispersity of deoxy-Hb S solutions increases at higher protein concentration.

**DISCUSSION**

The most significant observation made on the basis of osmometric and light scattering determinations presented here is that the slope of the relation between the reciprocal molecular weight and the deoxy-Hb S concentration, is negative. Under experimental conditions studied, this is consistent with the self-association of this protein. Note should be made that neither oxy-Hb A and oxy-Hb S nor deoxy-Hb A have shown this behavior. In addition, the data presented here suggest that the self-aggregation of Hb S at low concentrations of protein is not predominately hydrophobic in nature, but rather polar or electrostatic (or both) in its mode of bonding.

By considering that aggregation of deoxy-Hb S follows a single step of two or more (m) Hb molecules aggregating to form dimers, tetramers, or higher polymers, m Hb₄ = (Hb₄)ₙ, theoretical H'Ch versus C curves can be constructed assuming a fixed equilibrium constant (K₉₄ or K₉₆₄)¹ to describe the aggregation or polymerization equilibrium. Fig. 6 presents the experimental results and theoretical curves based on this simple polymerization model. None of the curves, monomer-dimer (1→2), monomer-tetramer (1→4), monomer-hexamer (1→6), or higher m-mer forms (curves not shown) give sufficiently satisfactory account of the observed data, suggesting the co-existence of monomers and several aggregating species in solution. It is significant that monomer-hexamer and higher polymeric forms give the poorer fit of data, suggesting that the aggregation might lead to low molecular weight forms of complex structure (2, 3). The lack of success in attempting to describe the polymerization of Hb S in terms of a single-species aggregate, with a characteristic single equilibrium constant,¹ prompted us to search for other models of polymerization that could account for our results.

Flory (15) has derived an equation for weight and number average molecular weights (M_w, M_n) on the basis of statistical consideration for macromolecules during condensation polymerization:

\[ M_w = M_n \left( \frac{1 + P}{1 - P} \right) \]

(4)

¹ For an associating system at equilibrium consisting of monomers and a single polymeric species of m aggregates, Equation 2 can be expressed in the form (12)

\[ \frac{H'C}{\tau} = \left[ (1 - \alpha)m \right] + 2BC \]

\[ = \left[ (m - 1) \alpha \right]^+ + 2BC \]  

(2b)

where (1 - \(\alpha\)) represents the weight fraction of associated Hb tetramers of molecular weight (Hb₄)ₙ (m times 64,460) and \(\alpha\) is the fraction of undissociated tetramers, free in solution. In order to fit the experimental data the required \(\alpha\) values at any concentration \(C\) have to be estimated, employing the "best fit" estimates of the equilibrium constant. Using the same notation as before (19), the constant appropriate for hemoglobin association is

\[ K_{94} = (K_{94})^{-1} = \left[ \frac{m^2 \alpha^2 \rho (C^{-1})^2}{\left[ (1 - \alpha)(M_n)_{1-m} \right]} \right] \]

(2c)

and from Equation 5

\[ M_n = \frac{M_w}{1 + P} \]

(5)

Where \(M_w\) is the molecular weight of the monomer and \(P\) is the fraction of the ends of the monomeric units joined to form aggregates. From Equation 4, after rearrangement one can obtain:

\[ P = \frac{M_w}{M_n} - 1 \]

(6)
The presence of a sequential phase change in gelation of hemoglobin has been suggested by Briehl (17) who studied gelation of deoxyhemoglobin by sedimentation of gels and determination of concentrations of the protein in the solution phase. Conflicting with this, is the data of Williams (6) who did not find significant amounts of higher weight molecular species in deoxy-Hb S solutions, when studied by equilibrium ultracentrifugation.

The data presented here are consistent with the presence of aggregates in low concentration solutions of deoxy Hb S. Experiments with propylurea, changes in ionic strength, and the effect of inositol hexaphosphate suggest that these aggregates have different properties from the Hb S gel or polymer (18). The bonding is apparently mostly polar instead of hydrophobic and organic phosphate effectors do not seem to influence the extent of aggregation. It is conceivable that preaggregation could be stabilized by different interacting sites than the ones found in the polymer. It is critical to investigate the range of concentrations between 10 g/dl and 25 g/dl of deoxy-Hb S to establish if these aggregates are the precursors of the polymer or an additional property of the mutated hemoglobin.

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