Proc. Nat. Acad. Sci. USA
Vol. 70, No. 5, pp. 1506–1508, May 1973

Concerted Formation of the Gel of Hemoglobin S

(hemoglobin A/sickle-cell anemia/ultracentrifugation/protein association)

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Communicated by Joseph S. Fruton, March 19, 1973

ABSTRACT Apparent weight-average molecular weights of hemoglobin A and hemoglobin S were measured at high concentrations by equilibrium ultracentrifugation. Carbonmonoxy-hemoglobin S appears to exist as a solution of unassociated molecules, as do carbonmonoxy- and deoxy-hemoglobin A. Deoxy-hemoglobin S, however, exists in a gel-like state at concentrations above 14 g/dl, but no aggregates smaller than the gel were observed in solutions that were in equilibrium with the gel. Carbamoylation of hemoglobin S produced a solution of unaggregated molecules, as did cooling of uncarbamoylated hemoglobin S to 5°. It is concluded that the gel of hemoglobin S is formed in a stoichiometrically concerted manner, and that the size of the smallest stable aggregate is greater than 20 hemoglobin molecules.

The distortion of the shape of deoxygenated erythrocytes which is characteristic of sickle-cell anemia appears to be the result of the formation of an ordered gel-like state by unliganded HbS within the cells (1–4). Electron microscopy (5–8) and x-ray diffraction (9) provide strong evidence that the gel is composed of bundles of parallel filaments, each bundle containing about six filaments in its cross-section. Formation of these structures must be the result of association between hemoglobin molecules. The viscometric measurements of Allison (10) have shown that gelation occurs rather sharply as concentration is increased, and that the phenomenon is a reversible one. Aside from these findings, little is known of the nature of the association reactions which lead to the formation of the HbS gel. This communication gives a preliminary report of measurements that bear on the question of the stoichiometry of the gelation reaction.

MATERIALS AND METHODS

HbA was prepared by the chromatographic method of Williams and Tsay (11), and HbS was prepared from the blood of an individual known to be homozygous for the sickle-cell gene by a modification of the same method. Carbamoylated HbS (about 2.0 carbamoyl groups per Hb tetramer) was prepared by reaction of oxy-HbS with 0.05 M $KN^{14}CO$ at pH 7.3 and 23° (12). Solutions of each hemoglobin were concentrated, prior to the equilibrium ultracentrifugation experiments, by vacuum ultrafiltration. The concentrated solutions were then dialyzed for at least 48 hr against a pH 7.0 buffer composed of 0.1 M NaCl–0.1 M sodium phosphate–1 mM EDTA.

In order to observe these concentrated solutions in the ultracentrifuge, a cell with an optical path of 0.75 mm was constructed. A standard Yphantis-type centerpiece (13) was cemented to a sapphire cell-window and was then cut to the desired thickness on a lathe and lapped to smoothness on a series of ground-glass plates (14). The thin centerpiece was fitted to standard cell components by means of appropriate aluminum spacers. Access for filling was obtained by removing the upper cell-window. The cell was filled with protein solution in a glove-box, following the techniques described by Kellett and Schachman (15). Sodium dithionite (0.02 mg/ml) was added to the solutions of unliganded Hb after deoxygenation. Liganded Hb was prepared by equilibration with buffer-saturated CO.

Centrifugation was conducted in a Beckman model E instrument equipped with an absorption scanner, which was used, at 541 nm, to obtain the absolute concentration near the top of the solution column. The principal observations were made with the Rayleigh interference optical system, and were greatly facilitated by the use of a helium–neon laser as a light source (16). Apparent weight-average molecular weights were computed by means of the program of Roark and Yphantis (17). The true density of the protein solution at each measured point, rather than the density of the solvent, was used in the final calculation of apparent molecular weight. Attainment of sedimentation equilibrium was verified by measurement of successive photographs taken at intervals of 4 hr. No measurable change occurred after about 20 hr, and final measurements were made from photographs taken about 28 hr after the start of an experiment.

The absorption spectrum of each solution was measured, after appropriate dilution, at the end of each run to permit estimation (18) of the fraction of methemoglobin present in the solution. This fraction never exceeded 5%.

RESULTS

Hemoglobin A

In Fig. 1 are shown the values obtained for HbA of apparent weight-average molecular weight ($M_{w,app}$), plotted against the local concentration (c) within the centrifuge cell. It is evident that nonideal effects are important at the extremely high concentrations being observed. Although these nonideal effects will be dealt with in detail in a future publication, it is useful to note here that they can be adequately described by a truncated virial series expansion (19):

$$\frac{1}{M_{w,app}} = \frac{1}{M} + 2Bc + 3Cc^2 + 4Dc^3$$

where M is the molecular weight of the hemoglobin tetramer, and B, C, and D are nonideal colligative virial coefficients. Values of the coefficients obtained for liganded hemoglobin A were $B = 5.9 \times 10^{-5}$ cm^3·mol/g^2, $C = 1.7 \times 10^{-5}$ cm^6·mol/g^3, and $D = 6.6 \times 10^{-4}$ cm^9·mol/g^4.

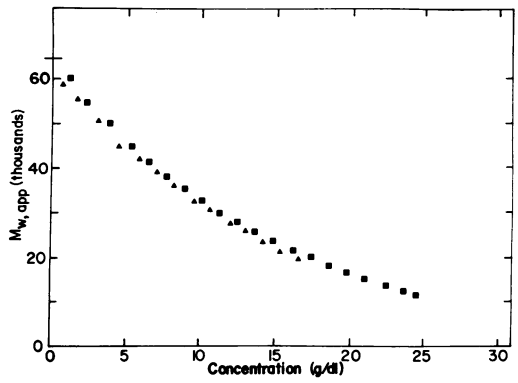

FIG 1. HbA, liganded (■), and unliganded (▲). Apparent weight-average molecular weight plotted against local concentration in the centrifuge cell. (Concentration is expressed as grams of protein per 100 ml of solution.) Initial concentrations were about 10 g/dl; rotor speed, 26,000 rpm; temperature, 20.0°; time, after start of run, 24 hr. The molecular weight of the hemoglobin tetramer is indicated by a horizontal mark on the ordinate.

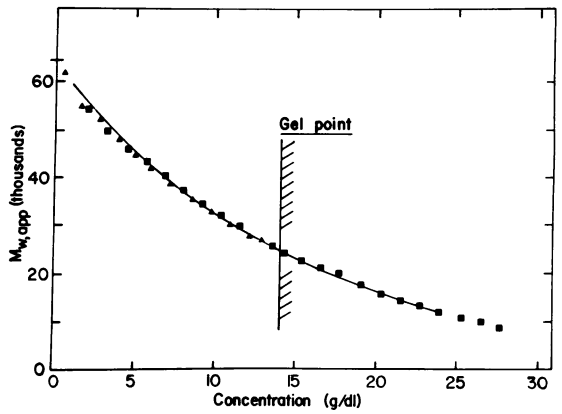

Fig. 3. HbS, liganded (■), and unliganded (▲). Apparent weight-average molecular weight is plotted against local concentration in the centrifuge cell. The concentration at which the gel-solution interface is observed is indicated. The *curve* represents the data for liganded HbA drawn from the points shown in Fig. 1. Other conditions are as in Fig. 1.

Fig. 1 also shows that the behavior of unliganded HbA is closely similar, if not identical, to that of the liganded protein. The small systematic difference between the two curves is of the same order of magnitude as the standard error of measurement of a single point, and is probably not significant.

Hemoglobin S

When unliganded HbS was observed at centrifugal equilibrium, a region near the base of the centrifuge cell was nearly opaque, as shown in Fig. 2. The line of demarcation between this region and the region of solution, where the fringes are easily visible, is quite sharp. The opaque region disappeared when the solution was cooled to 5°, and reappeared upon warming to 20°; undoubtedly, it represents the gel of HbS. (Highly viscous material can be removed from the cell at the end of experiments with unliganded HbS, and this material becomes fluid on exposure to air.)

In Fig. 3 are shown results of experiments with liganded and unliganded HbS. The unliganded protein is similar in its behavior to the liganded protein throughout the entire region where gel is not present. Furthermore, HbS behaves indistinguishably from HbA throughout the observed region of concentration. In experiments conducted with different *initial* protein concentrations in the centrifuge cell, identical values of $M_{w,app}$ were obtained at the same *local* concentrations in the cell, and the concentrations at which gelation was observed were not dependent on the initial concentration. It can thus be concluded (13, 17) that the gel is in rapidly reversible equilibrium with the solution.

Results of preliminary experiments under three conditions that disrupt the gel of HbS are shown in Fig. 4. HbS at 5° and carbamoylated HbS at 20°, both unliganded, behave similarly to liganded HbS at 20°, as does a mixture of 75% HbS with 25% carbamoylated HbS. The systematic difference between the behavior observed at 5° and at 20° is probably significant, and may well represent a variation with temperature of the partial specific volume or virial coefficients of the protein.

DISCUSSION

The most striking feature of these results is that unliganded HbS, up to its gelling concentration, behaves essentially indistinguishably from liganded HbS and from liganded or unliganded HbA. The simplest interpretation of these findings is to assume that all four proteins simply form nonassociating and nonideal solutions, but that unliganded HbS, in addi-

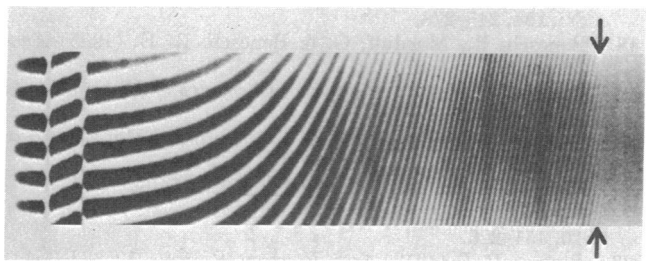

FIG. 2. Interference fringes obtained at centrifugal equilibrium from unliganded HbS. *Arrows* mark the gel-solution interface. Rotor speed was 26,000 rpm. Centrifugal acceleration is from *left* to *right*, the height of the solution column in this direction is 2.6 mm, and the concentration at the meniscus of the solution is 0.28 g/dl.

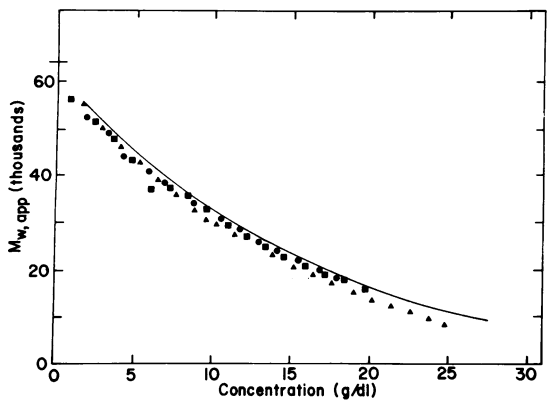

FIG. 4. Carbamoylated HbS (■), a mixture of 25% carbamoylated HbS with 75% HbS (●), and HbS at 5° (▲), all unliganded. The *curve* represents the data for liganded HbS drawn from the points shown in Fig. 3. Other conditions are as in Fig. 1.

tion, forms a gel in a highly concerted fashion at a concentration near 14 g/dl. The alternative explanation, that at concentrations below the gelling concentration of HbS all four proteins associate in an identical fashion, is improbable.

Nonideality in these solutions probably arises primarily from excluded volume. An approximation (20) of the magnitude of the second virial coefficient to be expected from a spherical molecule the size of hemoglobin gives $B \cong 4\bar{v}/M = 4.7 \times 10^{-5}$ cm^3·mol/g^2, where $\bar{v}$ is the partial specific volume of the molecule. The observed value, $B = 5.9 \times 10^{-5}$ cm^3·mol/g^2, is not much larger than the estimated value, indicating that the Donnan effect is probably of secondary importance in these solutions, as might be expected from the small electrostatic charge borne by hemoglobin at pH 7.0 (21, 22). It seems unlikely, therefore, that the magnitude of nonideality in HbS differs greatly from that in HbA, since the excluded volume of the two molecules must be nearly identical. It is thus likely that a difference of $M_{w,app}$ between HbA and HbS would constitute a qualitative reflection of differences in the state of association of these molecules.

The stoichiometrically concerted formation of the gel from single hemoglobin molecules implies that in a formal reaction scheme (23):

$$\begin{aligned} A_1 + A_1 &\rightleftharpoons A_2 \\ A_2 + A_1 &\rightleftharpoons A_3 \\ &\vdots \\ A_{n-1} + A_1 &\rightleftharpoons A_n \end{aligned}$$

which describes the formation of a large polymeric aggregate, A_n, from monomers, A_1, the equilibrium constants are of a magnitude such that intermediate polymers, A_2, A_3, ..., A_{n-1}, are not present in significant amounts. Such concerted reactions are seen, for instance, in the formation of detergent micelles (24), and can be described by a single equilibrium stoichiometry: $nA \rightleftharpoons A_n$. It is difficult to assign limits to the size and amounts of intermediate polymers that might be detectable in the experiments reported here. Under the assumptions that excluded volume is largely responsible for nonideality and that liganded HbA is unassociated, it can be conservatively estimated that less than 10% of the protein present in the solution of unliganded HbS at concentrations near 13 g/dl is present as polymers of 2–20 Hb molecules. Intermediate polymers of mass greater than about 20 Hb molecules would become difficult to detect because the steep concentration gradient which they would produce would be difficult to distinguish from the sharp gel–solution interface. It is thus possible only to conclude that no significant amount of intermediate polymer of size less than 20 Hb molecules exists in equilibrium with the gel of HbS.

The simplest scheme for forming the observed (5–8) filaments of the HbS gel is an "isodesmic" (25) linear association, in which the free energy of addition of a monomer to a growing filament is independent of the length of the filament, and side-to-side association is of secondary energetic importance. At least two proposals (8, 26) of schemes for the mode of formation of the gel of HbS have emphasized linear association, and appear to imply an isodesmic scheme. Such a scheme is wholly incompatible with the results reported here, since it requires that intermediate aggregates be present in large quantity in equilibrium with the gel. In addition, the finding that the (nongelling) mixture of 25% carbamoylated HbS with 75% noncarbamoylated HbS contains no short truncated chains (26), but rather is a solution of unassociated molecules, strengthens the conclusion that simple linear association is not the sole energetically important mode of association of HbS.

Since it is difficult to imagine a mode of *linear* association of molecules that differs greatly from the isodesmic scheme, it is likely that the *bundles* of filaments, rather than the filaments themselves, are directly in equilibrium with the HbS molecules in solution. The bundles must be formed in a concerted fashion, with the minimum stable aggregated unit consisting of at least 20, and perhaps many more, molecules. The concerted nature of the reaction seems to require that side-to-side aggregation be taken into account in any detailed proposed mode of filament formation, as it is in the scheme of Bertles *et al.* (8). A detailed description of the mode of formation of the gel of HbS must await the establishment of the size of the minimum stable associated unit.

The sickle-cell blood used in this study was the generous gift of Dr. P. N. Gillette. Miss Helen Kim rendered valuable technical assistance. The study was supported by Grant HL 12901 from the National Institutes of Health.

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