SPIN LABEL DETECTION OF AGGREGATION BY DEOXYGENATED SICKLE HEMOGLOBIN UNDER NON-GELLING CONDITIONS

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1. Introduction

There has been much interest over the past few years in the aggregation or gelation mechanism of deoxygenated sickle hemoglobin (HbS). Upon deoxygenation, HbS in concentrated solutions aggregates to form extended microtubules which, in the red cell, cause sickling. Studies of the aggregation process have shown that the kinetic data exhibit a delay period, followed by large, rapid changes in such properties as heat absorption [1,2], viscosity [3,4] and turbidity [5–7]. The characteristics of the aggregation process have suggested that gelation is a multistep process, with a rate-controlling nucleation step occurring during the delay period [1,8,9].

The nucleation concept appears to be well accepted, but direct evidence for the existence of such pregelation aggregates is quite limited. Rayleigh light scattering and osmometry measurements have suggested that deoxy HbS exhibits self-association at concentrations substantially below the minimum gelation concentration [10]. Laser light scattering measurements have also observed linear [11] or exponential [9] increases in scattered intensity during the delay period before gelation. However, while these studies have given indirect evidence for the existence of pregelation aggregation states, there is thus far little information on the characteristics of these intermediate states. A further knowledge of the physical characteristics of these intermediate states is important in understanding the mechanism of pregelation aggregation and of gelation, and may suggest useful routes to inhibit sickling.

Here, we report comparative spin label EPR measurements of deoxy HbS and HbA rotational diffusion under non-gelling conditions. The results indicate that deoxy HbS in the presence of IHP exhibits an aggregation which is detectable at as low as ~3 g/dl and 1°C. The extent of this aggregation increases slowly with both temperature and Hb concentration. We suggest that the aggregation states observed here may act as nucleation intermediates under conditions where gelation can occur.

Abbreviations: HbS, sickle hemoglobin; HbA, normal adult hemoglobin; IHP, inositol hexaphosphate; EPR, electron paramagnetic resonance; ST-EPR, saturation transfer EPR; Mal-6, 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl; HFS, hyperfine separation

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2. Materials and methods

Sickle blood samples were obtained from homozygous S donors, and Hb composition checked by electrophoresis. Samples containing more than trace amounts (~5%) of HbA or fetal Hb were discarded. Membrane-free carbonmonoxy HbA and HbS were prepared and labeled with Mal-6 following the procedures in [12,13]. Mal-6 labeled Hb solutions were prepared in 0.15 M sodium phosphate buffer at pH 6.7 with IHP added to give a constant IHP:Hb molar ratio of 4:1. Samples were deoxygenated under humidified nitrogen and loaded into specifically constructed flat glass capillaries with short optical paths. After EPR measurements were completed, the visible region optical spectra were recorded, and the fractional deoxy Hb, oxy Hb and met Hb contents determined using a least square analysis analogous to the methods in [14]. Average met Hb and deoxy Hb contents were ~6% and 85%, respectively.

Conventional first harmonic EPR spectra were measured on a Varian E-4 spectrometer following standard methods [15]. Spectra were recorded using a 40 G sweep width. The apparent hyperfine separation (HFS) was obtained by first measuring the separation of the low field line from the center line, then measuring the separation of the center line from the high field peak and adding the two measurements as shown schematically in the inset to fig.1. Relative uncertainties are estimated to be ±0.1–0.2 G. Second harmonic out-of-phase ST-EPR spectra were recorded on the same spectrometer, but utilized a PAR model 126A lock-in amplifier for phase-sensitive detection. Modulation and phase-sensitive detection were at 100 and 200 kHz, respectively. Other procedures and spectrometer settings were equivalent to those of [16]. Temperature was measured with a digital thermometer and a copper–constantin thermocouple; accuracy of individual measurements is estimated to be ±0.5°C.

3. Results and discussion

Previous work has shown that the Mal-6 spin label is tightly bound to the Hb molecule, and that the resulting spin label EPR spectra can be used to monitor Hb rotational correlation times [15,17]. In particular, the apparent hyperfine separation (HFS) increases as the correlation time increases. For Brownian rotational diffusion, the correlation time is related to the effective molecular radius (for spherical geometry) by the Stokes relation:

$$\tau_R = 4\pi a^3 \eta / 3kT$$

where a, η, k and T are the molecular radius, the viscosity, the Boltzmann constant and the absolute temperature, respectively. Thus, if HbS exhibits any significant aggregation behavior under non-gelling conditions, the effective molecular radius would be larger than that of HbA, and we would expect the HFS to be increased over that of HbA.

Fig.1 compares the apparent HFS of Mal-6 labeled HbA and HbS at 1°C and 30°C over 2–16 g/dl. The data in this figure indicate that the HFS is essentially the same as that of HbA at the lowest concentrations, but is significantly larger at >5 g/dl. This increased HFS for HbS is observed at both temperatures, but the increase of the HbS HFS over that of HbA at

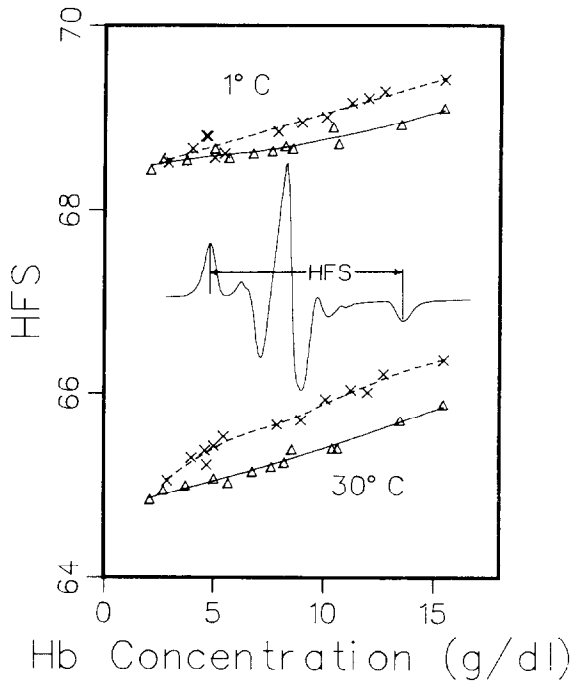

Fig.1. Hb concentration dependence of the observed HFS of Mal-6 labeled deoxy HbA (–△–) and deoxy HbS (–X–) at 1°C and 30°C. Values were measured as shown in the inset spectrum. The lines shown through the data are spline fits, and have no theoretical significance. None of the samples measured here exhibited gelation; sample tubes were broken open after EPR measurements, and all samples found to be completely fluid. The approximate 4 G difference in HFS observed over 1–30°C is due in part to the intrinsic HFS temperature dependence [22], and in part to the increased viscosity at lower temperature. The HbA/HbS comparisons were performed at constant temperature under identical solution conditions. Thus the observed HFS diffusion between HbS and HbA must reflect differences in their rotational diffusion characteristics.

30°C and 15 g/dl (~0.6 G) is about twice the difference observed at 1°C (~0.3 G).

One possible interpretation of this difference is that there is a structural difference between the spin label binding sites of HbA and HbS. However, the Mal-6 spin label is covalently attached to the Cys β93 residue, a position which is well separated from the HbS β6 mutation site, and X-ray studies have observed no significant differences between the tertiary structures of HbA and HbS [18]. Furthermore, other spin label studies of HbA and HbS have shown the β93 label site to be unaffected by the HbS mutation [19]. Thus it is unlikely that the observed HFS differences

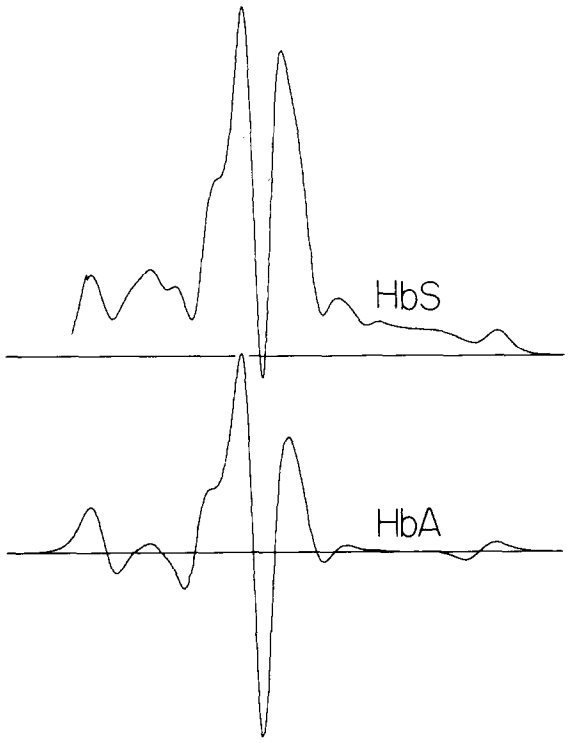

Fig.2. ST-EPR spectra of Mal-6 labeled deoxy HbA and deoxy HbS in the gel state, both at 30°C and ~25 g/dl. The HbA spectrum suggests apparent correlation times $\sim 10^{-7}$ s [16]. The HbS spectrum suggests apparent correlation times $\sim 10^{-5}$–10^{-4} s, 2–3 orders of magnitude longer than that of HbA. However, gelled HbS appears to be a two-phase system [23], with both monomer and polymer components contributing to the spectrum. Thus a simple single correlation time interpretation of the HbS spectra would be inaccurate.

reflect differences in the local spin label binding site structures.

A more probable interpretation of this behavior is that HbS exhibits an association at concentrations and temperatures well below the minimum gelation point. To further check this interpretation, we have also measured both the HFS and the second harmonic ST-EPR spectra of deoxy HbS and HbA at 30°C and ~25 g/dl. HbS is gelled under these conditions. Under these conditions the HFS of HbS is >1 G larger than that of HbA, approximately twice the difference exhibited by the non-gelled samples at 30°C and 15 g/dl. Fig.2 compares a ST-EPR spectrum of gelled HbS with that of HbA under the same conditions. From the reference spectra of [16] it can be seen that the HbA spectrum is essentially at the fast motional sensitivity limit of the ST-EPR method, while the HbS spectrum indicates substantially slower motion, in agreement with the motional restrictions to be expected from the gelation process. (For the gelled system, the ST-EPR display is more sensitive than the HFS as an indicator of aggregation due to the fact that ST-EPR is a non-linear method in which the signal will be dominated by those HbS molecules undergoing very restricted motion, while the HFS is a linear average from all HbS molecules in the system.)

The interpretation that the observed HFS differences reflect differences in the average HbA/HbS rotational behaviors is thus also supported by the observation that the HbA and HbS HFS appear to be essentially equivalent at low concentration (where one would expect intermolecular interactions to be diminished) (fig.1), and by the observation that the difference in HFS is increased significantly upon gelation. The larger HFS for HbS thus implies that HbS rotational diffusion is slower than that of HbA (i.e., the average HbS correlation time is longer than that of HbA) under equivalent conditions. Since the HbS mutation does not appear to induce any significant alterations in the Hb tertiary structure [18], and since HbA and HbS appear to be hydrodynamically equivalent at low concentrations (fig.1), the slower HbS rotational diffusion observed at higher concentrations must be an aggregation-induced increase in the effective molecular radius.

The EPR measurements described here appear to provide a particularly sensitive method for probing pregelation events in the sickling process. The high sensitivity of this method probably arises from the fact that the correlation time is proportional to the third power of the molecular radius. A doubling of the molecular radius, for example, will increase the correlation time by a factor of 8. Thus the changes in the EPR spectral parameters described here could be produced by relatively small changes in HbS aggregation.

Thermodynamic calculations using data derived from solution studies of the gelation process have suggested that the critical nucleation intermediate is probably an aggregate of ~10 HbS molecules [8]. Equilibrium ultracentrifugation studies have also indicated that any stable HbS aggregation states existing below the minimum gelation concentration must be smaller in size than ~20 HbS molecules [20]. The results presented here are consistent with the suggested existence of finite-size aggregates under equi-

librium conditions at concentrations and temperatures below the minimum gelation point. It appears likely that, as HbS concentration and/or temperature increase toward the gelation point, these small aggregates will increase in size until a stable aggregate structure is formed (the critical intermediate [8]) and fiber formation begins. More quantitative information about this pre-gelation aggregation under various experimental conditions should, in principle, be obtainable from EPR measurements such as these when combined with the development of a correlation time/HFS calibration curve [15] for deoxy Hb molecules, and the construction of aggregation models based on various possible structures [21].

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