SPIN-LABEL STUDIES AT F9(93)β OF DEOXYHEMOGLOBIN S AGGREGATION

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SUMMARY

Iodoacetamide spin-labels bound to the F9(93)β cysteine residues of hemoglobin S are sensitive to changes in environment induced by a decrease in temperature or an increase in viscosity, yet show no change in ESR spectrum upon aggregation (gelation) of the deoxyhemoglobin. Similar results are found with two different spin-labels having different chain lengths between the nitroxide radical and the cysteine residue. The fact that the environment which the F9(93)β cysteine residues experience in the gel is indistinguishable from that experienced in solution indicates that they are not located in close proximity to any of the sites of interaction between individual deoxyhemoglobin S tetramers and that while the viscosity of a deoxyhemoglobin S solution changes enormously upon gelation, the microviscosity within the gel can not be greatly changed.

Recent studies have provided preliminary information on the molecular arrangement of deoxyhemoglobin S molecules in the aggregated (gelled) state (1,2,3). While it has been shown that the hemoglobin S molecules aggregate into long fibers, the amino acid residues on the hemoglobin tetramers which interact with one another to produce such an aggregate remain undetermined. One approach to the identification of regions of the hemoglobin S tetramer which interact during aggregation is to attach probes to specific amino acid residues on the individual tetramers and follow the observable changes which take place during deoxygenation and the resultant gelation.

Spin-labels have been used with a great deal of success to probe the conformational changes which take place within the hemoglobin molecule during the transition from the deoxygenated state to the oxygenated state (4,5). As McConnell and his co-workers have pointed out, the rotational diffusion rate of the hemoglobin tetramer is too low to affect the electron spin resonance (ESR) spectra of attached labels (6). This suggests that the transition from

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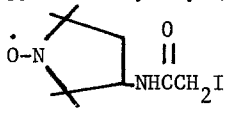
the tetramer of 64,000 molecular weight in solution to the very large macromolecular gel which occurs upon deoxygenation of hemoglobin S will not necessarily result in a change in ESR spectrum of an attached spin-label. However, spin-labels are sensitive to changes in their local environment (7) and therefore, changes in the freedom of rotation of the spin-label around the axis between it and the protein as a result of changes in the conformation of deoxyhemoglobin S molecules upon aggregation could be detected. The studies reported herein deal with the effect of aggregation of deoxyhemoglobin S on the ESR spectrum of spin-labels attached to the F9(93)β cysteine residues.

MATERIALS AND METHODS

Samples of blood from individuals with sickle cell disease were obtained from the Hematology Service of the Department of Internal Medicine, The University of Texas Southwestern Medical School. The hemoglobin was isolated from cells via the procedure of Drabkin (8) and disc gel electrophoresis was carried out on the isolated hemoglobin samples to verify their homogeneity.

The spin-labels 3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl(I) and 3-[[2-[2-(2-Iodoacetamido)ethoxy]ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl(II)



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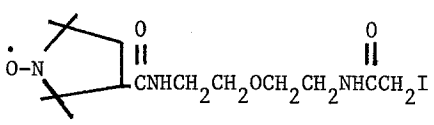

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were obtained from Syva, Inc. Incubation of isolated hemoglobin S (0.5 mM) with either iodoacetamide spin-label was carried out at 4°C in 10 mM phosphate buffer, pH 7.2, for times ranging between 16 and 42 hours. Experiments were carried out with both two-fold and ten-fold excesses of spin-label over reactive sulfhydryl groups. After incubation, free spin-label was removed from the hemoglobin solution by dialysis against 10 mM phosphate buffer, pH 7.0, and then passage through G-25 Sephadex which had been equilibrated with the same buffer. The amount of free F9(93)β cysteine residues remaining was determined on all spin-labeled hemoglobin samples by the p-mercuribenzoate titration method of Boyer (9).

The samples of spin-labeled hemoglobin were concentrated by ultrafiltration. Deoxygenation of both dilute and concentrated samples was achieved by gently shaking the sample at room temperature under an atmosphere of 95% nitrogen-5% carbon dioxide. Solutions were loaded into quartz capillary ESR tubes in a glove bag under an atmosphere of the above gas mixture. Solutions of deoxyhemoglobin S above the minimum gelation concentration were loaded into capillary tubes at 4°C and allowed to gel at room temperature before ESR measurements were carried out. All gelled samples of spin-labeled deoxyhemoglobin S could not be forced out of the capillary tubes at room temperature but would readily flow out of the tubes at 4°C. The heme concentration of all samples was determined by measuring the absorbance at 419 nm after reduction with sodium dithionite and bubbling with carbon monoxide (ε = 191 $mM^{-1}cm^{-1}$)(10). ESR measurements were carried out using a Varian E-4 spectrometer equipped with an E-257 variable temperature accessory. Samples were contained in high-purity quartz capillary tubing having an inside diameter of 1 mm (11). Titration of the reactive sulfhydryl groups on spin-labeled hemoglobin S with p-mercuribenzoate showed that depending on the incubation conditions used, 25% to 100% of the F9(93)β sulfhydryl groups were reacted with spin-labels. The shape of the ESR spectra of all oxy- and deoxyhemoglobin S samples was independent of the amount of spin-label bound.

RESULTS AND DISCUSSION

The results of studies using spin-label I in an effort to detect deoxyhemoglobin S aggregation are shown in Fig. 1. Curves A and B are the ESR spectra obtained with spin-labeled oxy- and deoxyhemoglobin S respectively. The samples used for these curves contained 53 mg/ml hemoglobin, far below the minimum gelation concentration. The spectra are very similar to those obtained by others with spin-labeled hemoglobin A (5). Curves C and D of Fig. 1 were obtained with samples of spin-labeled oxy- and deoxyhemoglobin S at a concentration of 283 mg/ml. At room temperature this concentrated deoxyhemoglobin S sample is a gel, yet the ESR spectrum (curve D) is indistinguishable from that

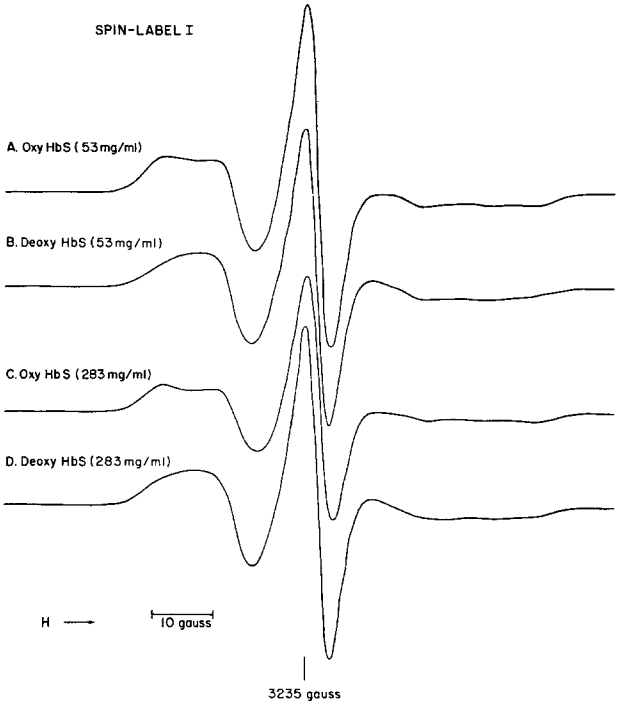

Figure 1: ESR spectra of hemoglobin S spin-labeled with 3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (spin-label I) at F9(93)β cysteine residues. A, oxyhemoglobin S (53 mg/ml); B, deoxyhemoglobin S (53 mg/ml); C, oxyhemoglobin S (283 mg/ml); D, deoxyhemoglobin S (283 mg/ml). The spectra were obtained at room temperature (23°C).

shown in curve B for a deoxyhemoglobin S solution. The oxyhemoglobin S sample at both concentrations is a solution.

The results in Fig. 1 show that the freedom of rotation of spin-label I bound to the F9(93)β cysteine residues of deoxyhemoglobin S is not changed by aggregation. While spin-label I is not found to be sensitive to aggregation when attached to deoxyhemoglobin S, it was found that this label was sensitive to changes in viscosity induced by the addition of glycerol or to changes in temperature. As shown in curve A of Fig. 2, spin-label I when bound to deoxyhemoglobin S gives a more immobilized type of ESR spectrum when the viscosity of the solution is increased by the addition of glycerol. A more immobilized type of ESR spectrum also results from lowering the temperature. It is well

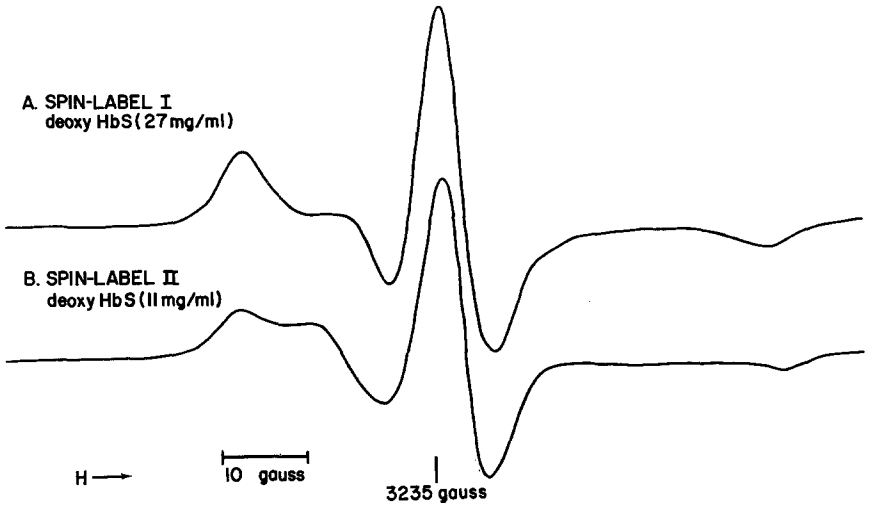

Figure 2: ESR spectra of deoxyhemoglobin S spin-labeled at F9(93)β cysteine residues in 50% (v/v) glycerol. A, 3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (spin-label I). B, 3-[[2-[2-(2-Iodoacetamido) ethoxy]ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (spin-label II). The spectra were obtained at room temperature (23°C).

known that by decreasing the temperature to 4°C, a deoxyhemoglobin S gel reverts to a solution (12). The temperature dependence of the transition to a more immobilized type of ESR spectrum was measured on dilute and concentrated oxy- and deoxyhemoglobin S samples and found to be similar for all samples even though at about 15°C the concentrated deoxyhemoglobin S sample goes through the gel-sol transition.

Because spin-label I was sensitive to such changes in environment and yet insensitive to gel formation, the spin-labeling studies were repeated with spin-label II which contains a much longer chain between the attaching group and the nitroxide radical. The results of these studies are shown in Fig. 3. It can be seen that because of the longer chain length between the attaching group and the nitroxide radical ($\sim$ 10 Å), spin-label II is less immobilized when bound to hemoglobin S than is spin-label I. The oxy- and deoxyhemoglobin S spectra at 12 mg/ml are shown in curves A and B while the oxy- and deoxyhemoglobin S spectra at 252 mg/ml are shown in curves C and D. The

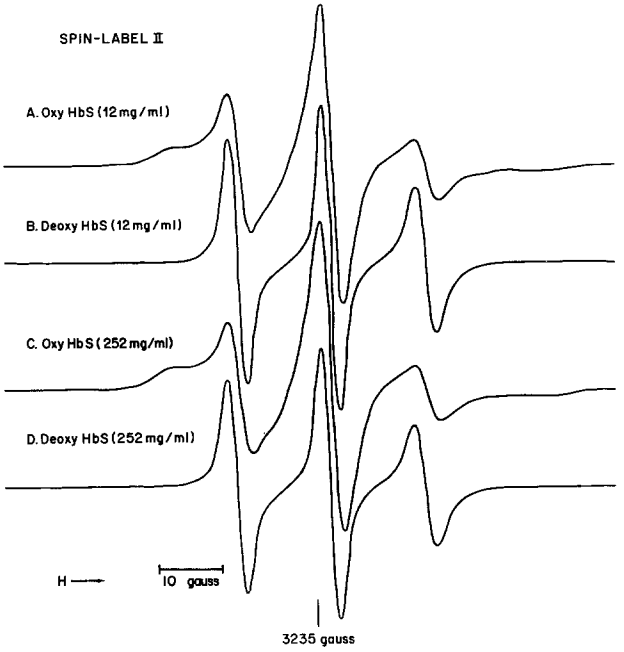

Figure 3: ESR spectra of hemoglobin S spin-labeled with 3-[[2-[2-(2-Iodoacetamido)ethoxy]ethyl]carbamoyl]-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (spin-label II) at F9(93)β cysteine residues. A, oxyhemoglobin S (12 mg/ml); B, deoxyhemoglobin S (12 mg/ml); C, oxyhemoglobin S (252 mg/ml); D, deoxyhemoglobin S (252 mg/ml). The spectra were obtained at room temperature (23°C).

deoxyhemoglobin S sample in curve D is a gel at room temperature and yet has an identical ESR spectrum to that in curve B where the deoxyhemoglobin S sample is a solution. While these results show that spin-label II is not sensitive to deoxyhemoglobin S aggregation it was found that this label when bound to hemoglobin S was sensitive to changes in viscosity induced by the addition of glycerol as seen in curve B of Fig. 2. Again, it can be seen that increasing the viscosity by the addition of glycerol results in a much more immobilized type of ESR spectrum.

Since the iodoacetamide spin-labels are bound to the F9(93)β cysteine residues on the exterior of the hemoglobin tetramer, it must be concluded that these residues are not located in close proximity to the site of any of the three interactions (ring formation, ring stacking, and parallel alignment

of the fibers) necessary to give a hemoglobin S aggregate corresponding to the model proposed by Finch, et al. (2). It has been shown that the viscosity of a deoxyhemoglobin S solution changes enormously upon gelation (13). This viscosity change is due to the sol-gel transition. However, the present spin-label studies show that the microviscosity within the gel can not be greatly changed. The environment which the spin labeled F9(93)β cysteine residues experience in the deoxyhemoglobin S gel is indistinguishable from that experienced in a deoxyhemoglobin S solution. Therefore, the interactions between individual deoxyhemoglobin S tetramers which result in aggregation are not expressed over the whole surface of the tetramers and the deoxyhemoglobin S aggregate appears to consist of some regions which are indistinguishable from individual tetramers in solution. The difference in ESR spectra between oxy- and deoxyhemoglobin as shown by curves A and B in Fig. 1 have been attributed to the conformational changes which take place during the oxy-deoxy transition (6). The fact that no change in ESR signal is observed upon aggregation of deoxyhemoglobin S (curve D, Fig. 1) indicates that the shift in the oxygenation curve as a result of cell sickling (14,15) is not the result of a spin-label detectable conformation change of the individual tetramers upon aggregation.

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