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EPR Study of the Hemoglobin Rotational Correlation Time and Microviscosity During the Polymerization of Hemoglobin S

M. Lores[1], C. Cabal[1], O. Nascimento[2], and A. M. Gennaro[3]

[1] Biophysics and Medical Physics Center, University of Oriente, Santiago de Cuba, Cuba
[2] Physics Institute of Sao Carlos, University of Sao Paulo, Sao Paulo, Brazil
[3] Biochemistry and Biological Sciences Faculty, Universidad Nacional del Litoral, Santa Fe, Argentina

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Abstract. The microviscosity and the protein rotational correlation time are analyzed in samples of hemoglobin A and hemoglobin S with the intracellular concentration at 36 °C and during spontaneous deoxygenation. With this purpose, we use glutathione and carbonmonoxy hemoglobin labeled with 4-maleimido-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO) as probes and 4-maleimido TEMPO bound to the hemoglobin (A and S) as a spin label. The saturation transfer electron paramagnetic resonance experiment showed a sigmoidal behavior, and an increase (about twice) of the hemoglobin rotational correlation time and microviscosity during the polymerization process of hemoglobin S. The delay time determined by this method coincides with that obtained in proton magnetic resonance experiments. These results help to explain the temporal behavior of the proton relaxation times obtained in samples of hemoglobin A and S under the same experimental conditions.

1 Introduction

The polymerization of hemoglobin S (HbS) is the basic molecular process of sickle cell disease [1, 2]. This process causes the proton relaxation times T_1 and T_2 to decrease in samples of HbS with the intracellular concentration under spontaneous deoxygenation conditions [3, 4]. By a qualitative analysis we [4] recently stated that the increase of the correlation time τ_C of the water bound to the Hb is the principal feature determining the behavior of T_1 and T_2. Because the proton ^1H magnetic relaxation in protein solutions is determined by the fraction of the water strongly bound to the macromolecule [5], the τ_C values will be related to the Hb rotational correlation time τ_R. Nevertheless, no quantitative analysis of these physical parameters under spontaneous deoxygenation conditions and all along the HbS polymerization process has been made until now.

Following the Debye model [6], τ_R can be determined as a function of the macroscopic viscosity of the Hb solution η,

$$\tau_R = (4\pi R^3/3kT)\eta, \quad (1)$$

where R is the Hb effective radius, k is the Boltzmann constant and T is the temperature in Kelvin.

The η value can be determined by using Mooney's generalized equation up to concentrations of approximately 7 mM [6, 7]:

$$\eta = \eta_0 \exp[[\eta]C/(1 - C_f[\eta]C)]. \quad (2)$$

According to Eq. 2, η depends only on the intrinsic viscosity of Hb $[\eta]$, its concentration C, and the viscosity of the solvent η_0. The C_f factor [7] describes an effect of the interaction among the protein molecules and the interaction of the protein molecules with the solvent.

Electron paramagnetic resonance (EPR) allows one to perform studies of the molecular kinetic parameters of the protein solutions. Particularly, the use of nitroxide radicals has enabled the study of the τ_R [8, 9] and the microviscosity η_μ [7] in Hb solutions. The present article reports studies of η_μ and τ_R during the polymerization of HbS under spontaneous deoxygenation conditions performed by the spin-labeling method and probes whose size (or molecular mass) is smaller than or comparable to that of the protein. In addition, the relations between parameters η_μ, τ_R, T_1 and T_2 during the agglutination process are analyzed.

2 Materials and Methods

28 Hb A (HbA) and 21 HbS samples were obtained by classical procedures [4] from fresh venous whole blood donated by voluntary individuals and patients.

2.1 Spin Labeling Method. Measurement of τ_R.

The HbA and HbS samples to be used in the spin-labeling experiment were mixed with 4-maleimido-2,2,6,6-tetramethyl-piperidine-1-oxyl (4MT) (2:1) and incubated at 8 °C during 24 h. The labeled Hb (LHb) was washed with buffer saline phosphate and filtered through a Centricon membrane (exclusion size, 10000 Da) in order to eliminate the unbound radical (at least 5 times). Then, the LHb concentration was adjusted to the intracellular concentration (about 30 g/dl) utilizing the 10000 Da membrane and optical spectrophotometry as a verification method. 40 µl of LHb were deposited in a capillary tube that was later sealed at both extremes and introduced in an EPR quartz tube for the experiment.

A Varian E-109 Century (X-band) spectrometer was employed to determine the saturation transfer (ST-EPR) spectra of the Hb-bound radical in the HbA and HbS samples: central field, 3267 G; sweep time, 4 min, and a Varian E-231

multipurpose system for temperature control. The second harmonic out-of-phase absorption (V_2') ST-EPR spectrum [9] was obtained with a sweep field of 160 G and a time constant of 0.128 s; the modulation amplitude and frequency were of 5 G and 50 kHz, respectively.

The Hb rotational correlation time was determined experimentally with a calibration curve of τ_R as a function of the ratio between the amplitudes of the central lines of the ST-EPR spectrum C'/C [9]. In order to construct this curve, C'/C was obtained by recording V_2' in samples of LHb dissolved in glycerol-water mixtures of known viscosity [8] and the τ_R value was calculated with Eq. (1). The interdependence between these parameters was fitted [8] with a fourth-order polynomial regression ($r = 0.99$, $N = 12$, $P < 0.0001$) to obtain

$$\tau_R = 9.6 \cdot 10^{-7} + 2.5 \cdot 10^{-6} C'/C + 2.9 \cdot 10^{-6} (C'/C)^2 + 1.6 \cdot 10^{-6} (C'/C)^3 + 3.2 \cdot 10^{-7} (C'/C)^4. \quad (3)$$

2.2 4MT plus Glutathione Spin Probe Method. Measurement of η_μ

A stock solution of 4MT in ethanol (20 mM) was prepared. 25 µl of this solution were deposited in the bottom of a tube (volume, 2 ml) and the solvent was evaporated under N_2. 20 µl of a solution of glutathione (Glu) in buffer saline phosphate (25 mM) was added, and after 30 min the buffer was evaporated under N_2 to obtain the 4MT plus Glu probe. The samples for the EPR experiment were prepared by mixing 50 µl of Hb with the probe in the 2ml tube and were deposited in a capillary tube that was later sealed at both extremes and introduced into an EPR quartz tube.

The EPR spectrum (X-band) of the 4MT plus Glu probe dissolved in HbA and HbS samples was obtained on a Bruker ER 200 spectrometer: central field, 3390 G; sweep field, 100 G; modulation amplitude, 0.32 G; attenuation, 18 dB; time constant, 100 ms. The correlation time τ_{c4MT} of the 4MT plus Glu was calculated with the standard formula for isotropic high-mobility regime measurements at X band [7].

For the calculation of η_μ around the 4MT plus Glu probe, we employed a calibration curve of τ_{c4MT} as a function of viscosity that was established by recording the spectra of the probe in sucrose solutions of known concentrations [7]. A straight line was obtained in the measured range of sucrose viscosities (1–9 mPa·s), and the linear regression $r = 0.994$, $N = 13$, $P = 9 \cdot 10^{-12}$ yielded a slope of $(8.10 \pm 0.13) \cdot 10^{-8}$ s $(\text{Pa} \cdot \text{s})^{-1}$ ($T = 25$ °C). The η_μ values were corrected by applying Eq. (1), taking into account the temperature change.

2.3 4MT plus Carbonmonoxy Hb Spin Probe Method. Measurement of η_μ

The carbonmonoxy Hb (HbCO) was prepared by placing the Hb solution in a carbon monoxide CO atmosphere for 30 min. To obtain the HbCO plus 4MT

probe, the HbCO (HbACO or HbSCO) samples were labeled with 4MT by the same procedure explained above for HbA and HbS samples. 20 μl of HbACO plus 4MT or HbSCO plus 4MT were mixed with 80 μl of HbA or HbS, respectively, and 40 μl of the mixture were deposited in a capillary tube that was later sealed at both extremes and introduced into an EPR quartz tube for the EPR experiment. Optical spectrophotometry was used as a control method to verify the transition from oxygenated Hb ($\lambda_2 = 577$ nm and $\lambda_3 = 541$ nm) to HbCO ($\lambda_2 = 569$ nm and $\lambda_3 = 540$ nm) and to determine the Hb concentration ($\lambda_1 = 405$ nm, $\xi = 393\ \mathrm{mM^{-1}cm^{-1}}$).

The η_μ value around the HbCO plus 4MT was calculated by taking the correlation time of this probe dissolved in water ($1.7 \cdot 10^{-8}$ s) and the viscosity of water (0.7052 mPa · s) at 36 °C

$$\eta_\mu = (\tau_C^*/1.7 \cdot 10^{-8})0.7052. \tag{4}$$

The rotational correlation time τ_C^* of the HbCO plus 4MT probe in the Hb solution was determined with Eq. (3), where τ_R is substituted by τ_C^*. In this case, the C'/C ratio was obtained starting from the ST-EPR spectrum of the 4MT contained in the HbCO plus 4MT probe.

At the beginning, all the samples studied were in the solution state and completely oxygenated. All the EPR measurements were performed during 8 h at approximately intracellular Hb concentrations under spontaneous deoxygenation conditions and at 36 °C. The errors in the determination of τ_R, η_μ around the 4MT plus Glu and η_μ around the HbCO plus 4MT were of 2.2%, 2.7% and 4.9%, respectively.

3 Results

The temporal behavior of the τ_R obtained in HbA and HbS samples in the ST-EPR experiment are shown in Fig. 1. A constant behavior is observed in HbA ($\tau_R = (5.1 \pm 0.13) \cdot 10^{-8}$ s), in contrast to HbS, which shows a sigmoidal behavior with three stages: stages I and III, in which the rotational correlation time of Hb remains practically invariable, and stage II, in which τ_R increases twice on the average.

Figure 2 shows the typical temporal behavior of η_μ around the 4MT plus Glu probe for samples of HbA and HbS. The η_μ values remain approximately constant all through the course of the experiment in HbA (2.14 ± 0.06 mPa · s), as well as in HbS (1.99 ± 0.05 mPa · s).

Figure 3 shows the typical temporal behavior of the η_μ around the HbACO plus 4MT and HbSCO plus 4MT probes dissolved in HbA and HbS samples, respectively. A constant behavior is observed in HbA ($\eta_\mu = 2.06 \pm 0.1$ mPa · s), in contrast to HbS, in which the results show a sigmoidal behavior with three stages: stages I ($\eta_\mu = 2.06 \pm 0.1$ mPa · s) and III ($\eta_\mu = 3.79 \pm 0.19$ mPa · s), in which the microviscosity around the probe remains practically invariable, and stage II, in which η_μ increases up to 1.8 times its initial value.

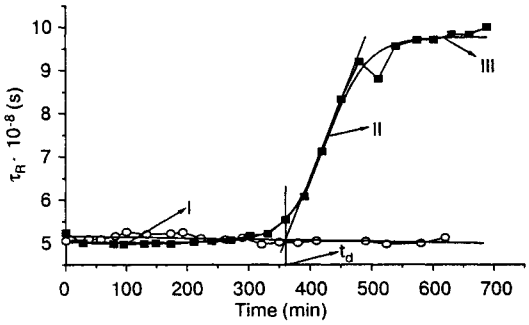

Fig. 1. Typical behavior of τ_R in the samples studied. The solid lines represent the sigmoidal and linear fittings that characterize the results obtained in HbS and HbA, respectively (open circles, HbA, 30.1 g/dl; closed squares, HbS, 29.9 g/dl). τ_R increases 1.9 times in the HbS sample for $t > t_d$, while in that of HbA, it remains constant.

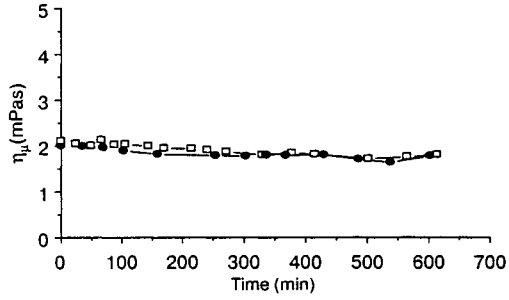

Fig. 2. Typical temporal behavior of η_μ around the 4MT plus Glu probe in samples of HbA and HbS (closed circles, HbS, 30 g/dl; open squares, HbA, 29 g/dl).

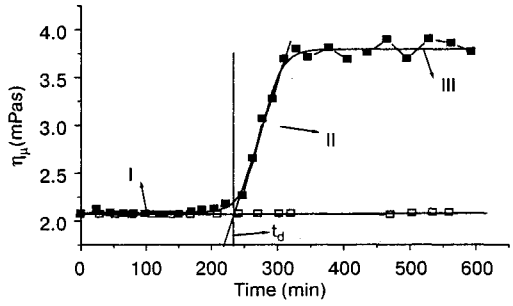

Fig. 3. Typical temporal behaviour of η_μ around the HbCO plus 4MT probes in samples of HbA and HbS (open squares, HbA, 30.2 g/dl; closed squares, HbS, 29.8 g/dl).

4 Discussion

The τ_R values in HbA and in HbS during stage I ($\tau_R = (5 \pm 0.11) \cdot 10^{-8}$ s) coincide with those reported in the literature (about 10^{-8} s) [10]. The constant behavior of the Hb rotational correlation time in HbA samples and its increase in HbS samples confirm the decrease of HbS mobility as a result of the polymerization. For HbS during stage II, τ_R increases twice on the average, while in stages I and III, the processes that take place do not alter the average dynamic parameters of the system, τ_R included. This increase of τ_R is smaller than that obtained by other authors under complete deoxygenation conditions [8], which can be explained considering that during 800 min of experiment (under spontaneous deoxygenation conditions) we only obtain a partially deoxygenated Hb solution. This fact results in obtaining a partially polymerized HbS sample in which τ_R is the average of the contributions from polymerized and nonpolymerized HbS molecules.

The η_μ values obtained in HbA and HbS with 4MT plus Glu as a probe (Fig. 2) coincide with the macroscopic viscosity values expected for Hb samples of the intracellular concentration at 36 °C according to Mooney's law [6, 7]. Gennaro et al. [7] demonstrated that in Hb solutions, the η_μ value around 4MT plus Glu can be described by an equation similar to Eq. (2), regarding the factor that represents the interactions among Hb molecules as a null one ($C_f = 0$). On the other hand, using Mooney's equation, a similar consideration can be made for η in our concentration range (≤ 5 mM). Therefore, it is consistent for our experimental values to coincide with the theoretical values expected according to Mooney's equation. Considering that the η_μ values around the 4MT plus Glu are not sensitive to the interactions among the Hb molecules, their constant behavior in the HbS samples (Fig. 2) can also be explained in spite of the polymerization process that takes place in these samples under our experimental conditions. We state as a hypothesis that the insensitivity of the η_μ value around 4MT plus Glu to the interactions among the macromolecules is related to the small volume of this probe ($0.033 \cdot 10^{-19}$ cm^3) [11] with respect to Hb ($1.02 \cdot 10^{-19}$ cm^3) [9]. Its volume is approximately 31 times smaller than that of Hb, which could cause it to remain in the solvent without being affected by the interactions among the Hb molecules, a fundamental element during HbS polymerization.

The behavior of the η_μ value around the 4MT plus Glu probe in the HbS samples shows that the polymerization process does not modify either η_0 or, obviously, $[\eta]$ or C.

Taking into account what has been discussed above, a probe with dimensions similar to those of Hb (HbCO plus 4MT) was employed. In this case (Fig. 3), we can affirm that the values of $\eta_\mu \approx 2$ mPa·s in HbA and HbS (stage I) coincide with those obtained with 4MT plus Glu as a probe and with the η values for the concentrations and temperature used. In the HbS samples, η_μ approximately doubles its initial value, which is related to the increase of τ_R during the polymerization of HbS (Eq. (1)).

The sigmoidal behavior of the η_μ value around HbSCO plus 4MT during the polymerization of HbS and the magnitude of its variation (about twice its initial value) coincide with the results reported here for τ_R and with those reported in previous papers for T_1 and T_2 [4]. This confirms the relation among the physical magnitudes mentioned.

The three stages that characterize the behavior of τ_R and η_μ values around HbSCO plus 4MT in HbS samples correspond to the three phases of the polymerization process identified by other methods [3, 4]: nucleation (I), irreversible polymerization (II), and finally, the formation of inhomogeneous structural microdomains (III) [1]. The results shown during phase I match with the solution state of Hb. The start of phase II is defined by the delay time t_d [1], a temporal parameter identified with the beginning of the irreversible HbS polymerization. The t_d values determined on the basis of the ST-EPR experiment coincide with the results obtained in other magnetic resonance experiments under spontaneous deoxygenation conditions [4, 12], taking into account the variability of approximately 21% among individuals. During phase III, the polymer alignment and growth do not cause changes in the macromolecular mobility and the viscosity of the solution which could be detected by our experimental procedure.

The possibilities of the HbSCO plus 4MT used as probe to be sensitive to the microviscosity changes in its environment during HbS polymerization are due to the fact that its dimensions are comparable to those of the macromolecules of the solution studied. Therefore, intermolecular interactions, like those taking place during the molecular aggregation process, affect the mobility of this probe.

5 Conclusions

The ST-EPR method enables one to determine the increase of the Hb rotational correlation time and the microscopic viscosity (about twice their initial values) during the polymerization of HbS under spontaneous deoxygenation conditions, and to confirm the three characteristic phases of this process: nucleation, polymerization, and the formation of microdomains. The t_d value calculated by this method coincides with that obtained in proton relaxation experiments, taking into consideration a variability of approximately 21% among individuals. These results support the qualitative analysis of the behavior of T_1 and T_2 reported in ref. 4 under the same experimental conditions.

Both probes used (4MT plus Glu and HbCO plus 4MT) allowed us to determine the macroscopic viscosity in HbA and HbS samples with intracellular concentration at 36 °C and during the spontaneous deoxygenation process. It was demonstrated that during the polymerization the viscosity of the solvent does not vary. Furthermore, the employment of probes with dimensions similar to those of Hb showed to be a more effective tool in the study of the microviscosity and the interactions among macromolecules in Hb solutions.

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Authors' address: Manuel Lores, Biophysics and Medical Physics Centre, University of Oriente, Patricio Lumumba, s/n, CP 90 500 Santiago de Cuba, Cuba.
E-mail: manuel@cbm.uo.edu.cu