ACCELERATION OF THE RATE OF DEOXYHEMOGLOBIN S POLYMERIZATION BY THE ERYTHROCYTE MEMBRANE

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1. Introduction

One rationale for the difference between the frequently severe clinical course observed in individuals with sickle cell anemia and the benign clinical course observed in individuals with sickle cell trait may be found in the profound difference in the rate of deoxyhemoglobin S polymerization between the two states [1]. The kinetic properties of this polymerization process have been shown to be altered by both pH and the degree of oxygenation of hemoglobin S (HbS) [2]. However, the influence of the erythrocyte membrane on the kinetics of polymerization has not been investigated. It might be clinically significant if polymerization could be delayed during the time period required for an erythrocyte to traverse the circulation back to the lungs where reoxygenation of the hemoglobin would occur [3]. For this reason the effect of the erythrocyte membrane on the kinetics of deoxy HbS has been studied by measurement of transverse water proton relaxation times (T_2) [4–6]. The rate of polymerization in erythrocytes having a normal hemoglobin concentration is too fast to be measured by available techniques. However, in studies with S/S erythrocytes containing a reduced hemoglobin concentration, the rate of polymerization is much more rapid than in solutions of hemoglobin S at the same hemoglobin concentration. Addition of erythrocyte membrane fragments to hemoglobin S solutions also results in a decreased delay time and a more rapid rate of polymerization. These results show that the erythrocyte membrane accelerates the rate of deoxyhemoglobin S polymerization perhaps by acting as a template for the polymer formation.

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2. Materials and methods

All samples of erythrocytes and solutions of HbS used in these studies were from individuals homozygous for HbS as determined by polyacrylamide gel electrophoresis. Hemoglobin solutions were equilibrated with 0.25 M potassium phosphate buffer at pH 7.2 by dialysis and concentrated via ultrafiltration prior to use. Deoxygenation of HbS solutions was accomplished in nuclear magnetic resonance (NMR) tubes at 4°C by addition of sodium dithionite to 45 mM final conc. and the tubes were then sealed under a 95% N_2–5% CO_2 atmosphere. The hemoglobin concentrations were determined at the end of each experiment.

Reduction in the mean corpuscular hemoglobin concentration (MCHC) of intact erythrocytes was achieved by a modification of the method in [7,8]. Variation in the MCHC was achieved by varying the lysate hemoglobin concentration. The resultant cells were found to have normal mean corpuscular volumes (MCV) and significantly reduced MCHC values. The modified cell samples were placed on a Ficoll gradient and gradually centrifuged at 50 × *g* for 5 min followed by a slow increase to 250 × *g* for 7 min. The gradient used consisted of equal volumes of 14.0%, 15.0%, 15.2%, 15.4% and 17.0% Ficoll [9]. Normal erythrocytes and modified cells having MCHC values $\gtrsim$23% are pelleted while cells with very small MCHC values of 11% or below remain at the top of the gradient. Each modified cell sample within this range of MCHC values formed a sharp band in the gradient and the

higher the MCHC value the farther into the gradient the cells moved.

Modified cells were suspended in phosphate-buffered saline (pH 7.4) and deoxygenation was achieved by gentle degassing with 95% N_2–5% CO_2. The concentration of oxyhemoglobin was measured by a CO-Oximeter (model 182 from Instr. Labs) and was found to be <5% in all cases. Red cell indices (MCHC and MCV) of the erythrocyte samples were measured at the end of each experiment using a model S Coulter counter from Coulter Electronics. The pH of each erythrocyte sample was measured at room temperature at the end of an experiment. A freeze–thawing method was employed to measure the intracellular pH [10]. Red cell membranes from normal adult erythrocytes were prepared as in [11], dialyzed against 0.25 M potassium phosphate buffer (pH 7.2) and then sonicated for 30 s at a dial setting of 1 on a Sonifier Cell Disruptor Model W185D from Branson Sonic Power Co. The concentration of membrane protein was measured by the method in [12]. Measurement of T_2 as a function of time was carried out by the temperature-jump method in [1,2].

3. Results and discussion

Erythrocyte sickling at zero pO_2 has been reported to be altered by changes in MCHC, pH and temperature [13]. It is necessary, therefore, to use samples which are matched as closely as possible with respect to these parameters in order to compare the kinetic profile of the polymerization process inside erythrocytes with that in isolated hemoglobin solutions. In fig.1, the kinetic profile at 37°C obtained with sickle cells having a reduced MCHC value (21.5%) is compared with that of a solution of HbS (216 mg/ml) at pH 6.9. The T_2 values at 4°C of both samples are 200 ms. From the initial observation time, 10 s after jumping from 4°C to 37°C, the T_2 values of the cell sample decrease and the change is complete by 100 s. On the other hand, the solution containing an equivalent concentration of HbS has a 1000 s delay time suggesting a much slower rate of polymerization of deoxy HbS in solution as compared to the same HbS concentration inside an erythrocyte.

Figure 2 shows the kinetic profile of 4 erythrocyte samples of different MCHC values. In the case of the 2 lowest MCHC values (18.1% and 19.1%), as the temperature is jumped to 37°C a rapid increase in T_2 is initially observed, indicating the presence of a delay time, followed by a drop in T_2 to a constant value which is dependent on the hemoglobin concentration. The changes are nearly complete within the time required for temperature equilibration of the sample (60 s). It can be seen that at a normal MCHC (33%) polymerization is so rapid that it is complete within a very few seconds.

Figure 3 shows that the addition of sonicated A/A erythrocyte membrane fragments to solutions of deoxy HbS reduces the delay time of polymerization

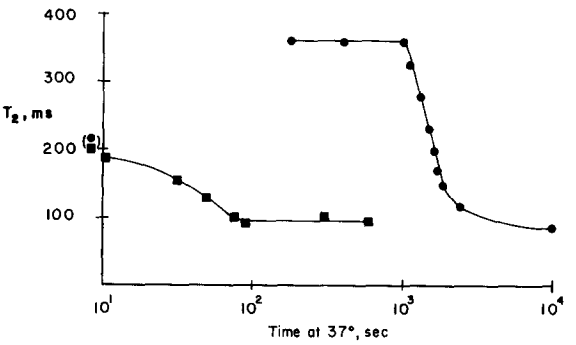

Fig.1. The transverse water proton relaxation times (T_2) as a function of time at 37°C in deoxygenated S/S erythrocytes having a reduced MCHC (21.5%) (■) and in a deoxygenated solution containing an equivalent concentration of HbS (216 mg/ml) (●). The time zero T_2 values were measured at 4°C and are shown in parenthesis. The final pH in both samples was 6.9.

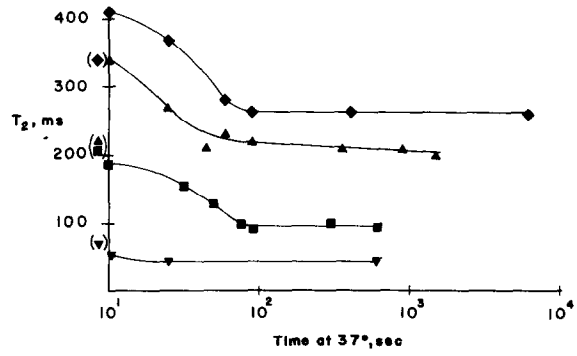

Fig.2. Transverse water proton relaxation times (T_2) as a function of time in deoxygenated S/S erythrocytes having reduced MCHC values. MCHC values are: 18.1% (◆); 19.1% (▲); 21.5% (■); 33.0% (▼). The time zero T_2 values were measured at 4°C and are shown in parenthesis. The final pH in all samples was 6.9.

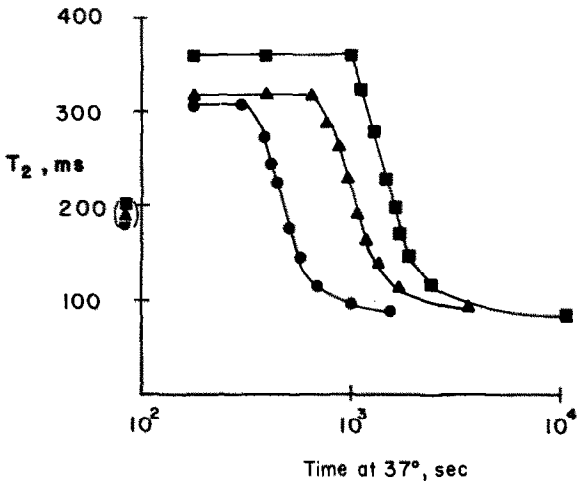

Fig.3. Transverse water proton relaxation times (T_2) as a function of time at 37°C in deoxygenated HbS solutions containing erythrocyte membranes. Erythrocyte membranes were isolated from normal adult erythrocytes, sonicated and then added to the hemoglobin solution. The HbS concentrations are 216 mg/ml (■); 217 mg/ml (▲); 213 mg/ml (●). The membrane concentrations are: 0 (■); 2.5 mg/ml (▲); 4.0 mg/ml (●). Following deoxygenation with sodium dithionite at 4°C, T_2 values were measured as a function of time at 37°C. The values in parenthesis were obtained at 4°C just prior to the temperature jump used to initiate polymerization. The final pH was 6.9. The kinetic parameters determined from this data are given in table 1.

and that as more membrane is added, the delay time becomes shorter. The delay times and rate constants determined from this data are shown in table 1. As can be seen, the delay time is shortened by the addition of membrane fragments and the pseudo-first order rate constant of polymerization is increased. This is in agreement with the report [14] that addition of erythrocyte membrane decreases the delay time of polymerization. When S/S erythrocyte membrane fragments were used similar results were observed. Furthermore, the factor by which the delay time is decreased exactly corresponds to the factor by which the rate constant is increased. This direct correlation of the two kinetic parameters has been observed as a function of pH, another effector of the rate of polymerization [12].

Table 1
Effect of addition of erythrocyte membrane fragments on the kinetics of deoxy-HbS polymerization

[Membrane] (mg/ml)[a]	pH	[Hb] (mg/ml)	t_d[b] (s)	k[c], (s^{-1})
0	6.9	216	1060	1.17×10^{-3}
2.5	6.9	217	750	1.65×10^{-3}
4.0	6.9	213	350	3.56×10^{-3}

[a] Expressed as protein concentration
[b] Delay time prior to the onset of polymerization
[c] Pseudo-first order rate constant for polymerization

A large number of high affinity hemoglobin binding sites have been reported to be specific for the inner surface of the red cell membrane [15,16]. These sites are electrostatic in nature [15,17] and may provide the explanation as to why deoxy HbS polymerization proceeds much more rapidly inside intact erythrocytes than in isolated hemoglobin solutions. Perhaps, these hemoglobin binding sites can serve as a template for the polymerization process. In this way the rate of formation of the critical concentration of nucleation species may be accelerated leading to an increased overall rate of polymerization.

In summary, the polymerization of deoxy HbS inside intact erythrocytes is found to be much more rapid than in solutions of hemoglobin at corresponding hemoglobin concentrations. This may result from interaction between HbS molecules and the erythrocyte membrane which can serve as a template for the formation of the polymer. In light of the suggestion that lengthening the delay of the polymerization process might be of therapeutic value, it seems possible that chemical intervention in the interaction between hemoglobin and the erythrocyte membrane might have the desired effect.

Acknowledgements

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