

Polymerization of Hemoglobin in Sick Trait Erythrocytes and Lysates*

(Received for publication, December 12, 1980, and in revised form, February 20, 1981)

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We report $^{13}\text{C}/^1\text{H}$ magnetic double resonance measurements of polymer in sickle trait erythrocytes and direct measurements of solubility on lysates of these cells as a function of oxygen saturation. Polymerized hemoglobin is detected by these two methods only at oxygen saturation values below 60% and increases to 0.4 of the total hemoglobin at complete deoxygenation. These results confirm the validity of the NMR approach in determining values of polymer fraction. We have also found that the thermodynamic theory, that successfully describes gelation in sickle erythrocytes, does not predict the experimental results for sickle trait erythrocytes and must be refined.

We have previously demonstrated the usefulness of $^{13}\text{C}/^1\text{H}$ magnetic double resonance in studying gelation of hemoglobin S in solution as well as within the SS erythrocyte¹ (1-3). Measurements of the polymer fraction in deoxyhemoglobin S solutions in the concentration range 22 g/dl to 32 g/dl correlate well with the amounts predicted from the solubility measured using ultracentrifuge sedimentation (2), a technique already established for studying gelation of hemoglobin S solutions (4-6). We also found that the amount of polymer within SS erythrocytes (3) increased monotonically with decreasing oxygen saturation and that polymer could even be detected at very high oxygen saturations (>95%). The measurements agree with the behavior predicted by the thermodynamic analysis of gelation by Minton and Ross (7, 8) which takes into account the highly nonideal behavior of hemoglobin at the concentrations found within the erythrocyte. The ^{13}C NMR studies are also consistent with filterability studies of SS erythrocytes (9) and the qualitative measurements of intracellular gelation using water-proton relaxation (10).

Intracellular gelation as a function of oxygen saturation appears to behave differently compared with cell sickling as a function of oxygen saturation (3). In order to establish further the relationships among NMR measurements of intracellular gelation, cell sickling, and the pathophysiology of sickle cell disease, we have studied the behavior of erythro-

cytes from heterozygous individuals (AS cells). We have measured the amount of polymer formed within AS erythrocytes as a function of oxygen saturation using $^{13}\text{C}/^1\text{H}$ double resonance methods (11). The solubility of AS cell lysate was also measured as a function of oxygen saturation using ultracentrifuge sedimentation. The amounts of polymer predicted for these samples using the thermodynamic theory of Minton (7) were calculated and compared with the experimental measurements, as were data on the sickling behavior of AS erythrocytes.

MATERIALS AND METHODS

AS erythrocytes were prepared from blood obtained from individuals with sickle trait. Hemoglobin electrophoresis of the samples used gave levels of 61% hemoglobin A, 39% hemoglobin S, and hemoglobin F <1% for B.H.; and 58%, 42%, and <1% respectively for M.S. The cells were washed three times with Earle's balanced salt solution without bicarbonate (12), with the addition of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (13) and a pH of 7.2. The cells were then suspended by the addition of buffer equal to twice the volume of packed cells. Each sample was prepared by placing 2 ml of the cell suspension into a spinning cup tonometer (IL-273, Instrumentation Laboratory, Lexington, MA) at 37 °C and equilibrating with various gas mixtures (0-20% oxygen and the balance nitrogen; Medgas Lif-O-gen, Inc., Cambridge, MD). The total oxygen saturation was determined by withdrawing 10 μl of sample and inserting it into the Micro Blood Analyzer (Advanced Products SRL, Milan, Italy) (14). Samples were transferred anaerobically using a gas-tight Hamilton syringe into 8-mm NMR tubes filled with the same gas mixture and fitted with a gas-tight cap. The cells were packed and the excess buffer was removed anaerobically giving a final sample volume of 0.65 ml.

Natural abundance ^{13}C spectra (15.09 MHz) were recorded using a Nicolet TT-14 spectrometer modified for experiments in solids (11) as described in Ref. 3. All spectra were recorded at a sample temperature of 37 °C. The dipolar proton resonant field was 13 G ($\gamma_1\text{H}_2/2\pi = 55$ kHz) for the proton-enhanced spectra and the Hartman-Hahn condition ($\gamma_1\text{H}_1 = \gamma_2\text{H}_2$) was matched using adamantane as a standard. Relative polymer fractions were determined from difference spectra of the proton-enhanced spectra. The absolute magnitude was determined by comparing the scalar-decoupled spectrum (the standard 90° - t sequence) of the oxygenated sample with the spectrum of the fully deoxygenated sample for each individual.

AS lysate was prepared from another sample of sickle trait blood (hemoglobin A and S levels were 60% and 40%, respectively, determined by electrophoresis; hemoglobin F <1%) by washing the cells three times with 0.15 M potassium phosphate buffer, pH 7.4. The cells were lysed by the addition of distilled water equal to 20 times the volume of packed cells. The membranes were removed by centrifugation and the lysate was dialyzed three times with 0.1 M potassium phosphate buffer at pH 7.5. The lysate was concentrated to 34 g/dl by ultrafiltration and vacuum dialysis and then dialyzed three times with 0.15 M potassium phosphate buffer at pH 7.4. Samples at various oxygen saturations were prepared in 1-ml aliquots in the tonometer as described above. Using a gas-tight Hamilton syringe, 300 μl of each sample was transferred into a quartz tube (Wilmad PQ-701, 5-mm outside diameter and 45 mm in length) under a layer of mineral oil. The samples were allowed to gel at 37 °C. The samples were then spun in the ultracentrifuge (Beckman SW50.1 rotor with an 0.8-ml adapter, 40,000 rpm, 1 h, 37 °C). The supernatant concentration and oxygen saturation were determined by withdrawing 10 μl of supernatant within 1 to 2 mm from the top of the pellet for each measurement. The hemoglobin concentration was determined by dilution of 201-fold into buffer, conversion to CN-methemoglobin, and reading the absorbance at 540 nm (15).

RESULTS

Samples of AS erythrocytes were prepared at oxygen saturation values ranging from complete oxygenation to complete

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† Recipient of a Burroughs Wellcome Fellowship.

¶ The abbreviations used are: SS erythrocytes, sickle erythrocytes; AS erythrocytes, sickle trait erythrocytes.

deoxygenation. The amount of polymerized hemoglobin at each oxygen saturation was measured using ^{13}C proton-enhanced nuclear magnetic resonance spectroscopy. We found that no increases in signal amplitude above base-line could be detected above 60% oxygen saturation, indicating the absence of polymer formation at these oxygen saturation values. The results for cells from two patients are shown in Fig. 1. Below about 60% oxygen saturation, the amounts of polymer formed increase monotonically with decreasing oxygen saturation to about 0.4 of the total hemoglobin present at complete deoxygenation.

In order to compare these results of intracellular gelation in AS erythrocytes with results expected for solutions of hemoglobin mixtures of similar composition (at 34 g/dl and 37 °C), we prepared AS hemolysate for solubility measurements. Al-

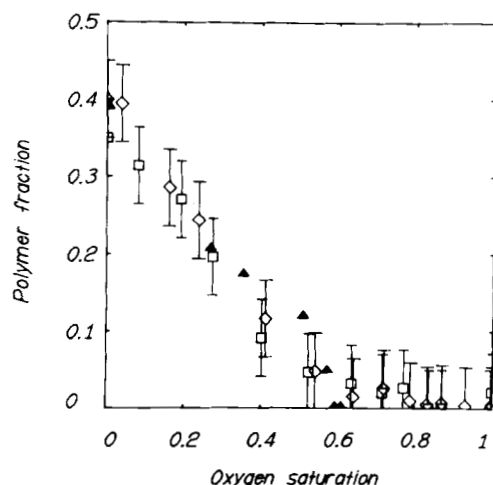


FIG. 1. Polymer fraction as a function of oxygen saturation in AS erythrocytes measured using the ^{13}C proton-enhanced spectra. (The absolute magnitudes were determined using the scalar decoupled spectra for the fully oxygenated and deoxygenated samples.) \square and \diamond represent two different individuals (M.S. and B.H., respectively). \blacktriangle represents the polymer fractions calculated from the sedimentation solubilities of AS lysate using a total hemoglobin concentration of 34 g/dl, close to the mean corpuscular hemoglobin concentration for the samples used in this study (34 g/dl for M.S. and 35 g/dl for B.H.).

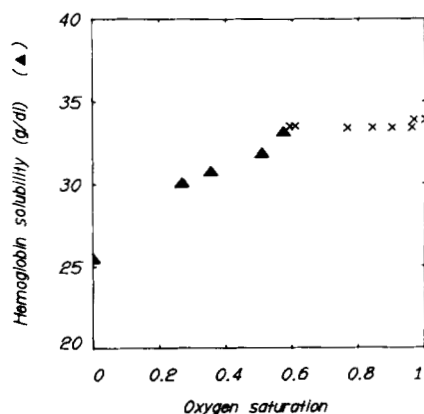


FIG. 2. Hemoglobin solubility at 37 °C as a function of oxygen saturation determined by ultracentrifuge sedimentation of AS lysate. \blacktriangle represents the solubility determined on gelled solutions of AS lysate (60% A; 40% S). \times represents the total hemoglobin concentration of the AS lysate samples that did not gel after incubation at 37 °C for 24 h. (The solubility must be close to or greater than the total concentration of these ungelled samples.)

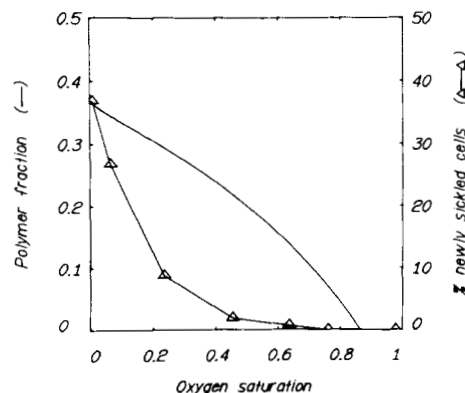


FIG. 3. Theoretical calculation of polymer fraction and cell sickling as a function of oxygen saturation for a 60% A:40% S hemoglobin mixture of 34 g/dl. 16.05 g/dl is used for the solubility of pure deoxyhemoglobin at 37 °C (16). For e_x , the relative tendency for hemoglobin x to be incorporated into the polymer, we use $e_{\text{SS}} = 1$ for deoxyhemoglobin S, $e_{\text{AS}} = 0.26$ for the hybrid deoxyhemoglobin AS (17), and $e_{\text{AA}} = 0$ for deoxyhemoglobin A. To take into account the various liganded intermediates, we use the framework of the allosteric model with $e_1 = 1$ for unliganded T state, $e_2 = 0.2$ for liganded T state, and $e_3 = 0$ for R state. (The T and R states are calculated by using $L = 100,000$, $K_R = 160$, and $K_T/K_R = 0.01$ parameters from Ref. 16.) Δ represents the percent of newly formed sickled shapes from AS erythrocytes as a function of oxygen saturation from Ref. 19.

iquots of the hemoglobin solution were equilibrated with various oxygen gas mixtures, transferred anaerobically to quartz tubes under a layer of mineral oil, incubated at 37 °C, and allowed to gel. We found that samples with oxygen saturation greater than 60% did not gel. The samples were then centrifuged at 37 °C and $179,000 \times g$ for 1 h. After centrifugation, the oxygen saturation and concentration of the hemoglobin of the supernatant was determined by sampling the supernatant within 1 to 2 mm above the pellet. (This minimizes the effect on solubility measurements of monomer depletion of the supernatant due to ultracentrifugation (2).) Below 50% oxygen saturation, the solubility decreased with decreasing oxygen saturation to 25.4 g/dl at complete deoxygenation (Fig. 2). Using these hemoglobin solubility values, we calculated the polymer fraction f_p expected for an initial hemoglobin concentration of $C_T = 34$ g/dl using $f_p = C_p / (C_T - C_o) / (C_T (C_p - C_o))$ (3) where $C_p = 70$ g/dl is the polymer concentration (16, 17) and C_o is the hemoglobin solubility in grams/dl. The results of these measurements are also shown in Fig. 1. The calculated polymer fraction using the sedimentation solubility for mixtures of hemoglobins S and A are in very good agreement with the ^{13}C NMR measurements for AS cells.

DISCUSSION

Intracellular gelation in AS erythrocytes should be nearly identical with gelation of comparable solutions of hemoglobin A and S mixtures. In cell-free gelled mixtures of hemoglobin, the solubility of hemoglobin can be measured using ultracentrifugation and the amount of polymer can be calculated from the solubility. Using the solubilities measured for a 60%:40% mixture of hemoglobins A:S at 34 g/dl and at varying oxygen saturations (Fig. 2), we calculated the amounts of polymer present and compared these predicted values with the intracellular measurements by nuclear magnetic resonance spectroscopy (Fig. 1). The agreement is excellent.

For AS erythrocytes and mixtures, no polymer formation is detected until the oxygen saturation falls below 60%. As the

oxygen saturation is further decreased, the polymer fraction continues to increase to 0.4 of the total hemoglobin at full deoxygenation. This behavior is in contrast to the detection of polymer at high oxygen in SS erythrocytes and the increase of polymer fraction to about 0.6 or 0.7 of the total hemoglobin at full deoxygenation (3). These experimental results on oxygen dependence of intracellular gelation can be compared with published data on oxygen dependence of cell sickling for AS erythrocytes (18, 19). In Fig. 3, data from published AS cell sickling studies (19) are presented. Comparison of Fig. 1 with Fig. 3 indicates that the behavior of intracellular gelation and of cell sickling as a function of oxygen saturation are different. As with our earlier results for SS erythrocytes (3), these data further suggest that intracellular gelation does not necessitate cell sickling.

The thermodynamic theory for hemoglobin S gelation developed by Minton which neglects long range interactions and includes the nonideal behavior of concentrated protein solutions (7, 8) is very satisfactory in the analysis of mixtures of deoxyhemoglobin S with hemoglobin S liganded with CO or oxygen (20, 21). The theory is also very satisfactory for data of intracellular gelation of SS erythrocytes (3) and for data on gelation mixtures of deoxyhemoglobin S with other proteins such as hemoglobin H (β_4) and bovine serum albumin (22-24). The theory also fits data on fully deoxygenated mixtures of hemoglobin S with hemoglobins A and F (17, 22, 24). We have used the theory in its present form to calculate the predicted amounts of polymer for a hemoglobin A:S mixture of 60%:40% at 34 g/dl as a function of oxygen saturation (Fig. 3). We use a value of $e_{AS} = 0.26$ as previously reported as the tendency of AS hybrids ($\alpha_2\beta\beta^S$) to be incorporated into the polymer (17). At zero oxygen saturation, the calculated polymer fraction is 0.4, in good agreement with the ^{13}C NMR intracellular measurement and the comparable value determined from the sedimentation solubility.

As the oxygen saturation increases, the polymer fraction is predicted to decrease and to become zero around 85% oxygen saturation (Fig. 3). It is clear that theory predicts a much more gradual decrease in polymer fraction with increasing oxygen saturation than either the ^{13}C NMR or sedimentation data indicate. Changing the value of e_{AS} has a greater effect on the magnitude of polymer fraction than on the oxygen saturation value for zero polymer and the comparison between experiment and theory is not significantly improved. For hemoglobin A, e_{AA} is assumed to be zero. Positive values for e_{AA} would increase the amounts of polymer formed, further increasing the discrepancy between theory and experiment; negative values for e are not physically plausible.

There are several possible explanations for the discrepancy between theory and data. First, the actual partition of hemoglobin hybrids (i.e. SS, AS, and AA) may not follow the assumed binomial distribution (25, 26). However, with the parameters used in the calculation, the effect of changes in hybrid distribution is of the same order as changes in e_{AS} which cannot account for the discrepancy. Second, the calculation assumes that hemoglobins A and S in solution have identical intrinsic oxygen affinities. However, if the intrinsic oxygen affinity of hemoglobin S at these high concentrations were greater than hemoglobin A, then the magnitude of polymer fraction at zero oxygen saturation would remain the same, but the zero polymer-intercept would shift to lower oxygen saturations, in closer agreement with experiment.

A third possibility is that the values of e must be modified. For example, e_x , the relative tendency for hemoglobin x to be incorporated into the polymer, is assumed to be constant, independent of polymer or solution composition (with $e_{SS} \equiv 1$ for deoxyhemoglobin S). Recently, Benesch *et al.* (27) have

reported that hemoglobins A, A_2 , and F, in addition to being incorporated into the polymer ($e_x > 0$), have a solubilizing effect on deoxyhemoglobin S ($e_{SS} < 1$). Thus, neglecting long range forces is invalid. This is the case in mixtures of hemoglobin if small aggregates inhibit formation of the large fibers found in the deoxyhemoglobin S polymer. These three possibilities must be further examined by a more fully developed thermodynamic theory for hemoglobin mixtures.

It has long been appreciated that individuals with sickle trait do not experience the clinical manifestations associated with sickle cell anemia. The behavior of intracellular gelation as a function of oxygen saturation for SS erythrocytes and AS erythrocytes are very different. In the physiologically relevant region of oxygen saturation (~60%-100% oxygen saturation), the differences between SS and AS erythrocytes are most striking. In this region, polymer can be detected in the SS erythrocytes (even at oxygen saturation values >95% (3)), whereas no significant amount of polymer is detected in the AS erythrocytes (Fig. 1). In this range of oxygen saturation, the AS erythrocytes should be similar to normal erythrocytes. This correlates well with the benign course of sickle trait.

We anticipate that these ^{13}C NMR methods should be useful in studying intracellular polymer formation in the various sickle syndromes (sickle/ β -thalassemia, sickle/hereditary persistence of fetal hemoglobin, etc.) and should better define the relationship between polymer formation and clinical manifestations of disease.

Acknowledgment—We thank Dr. Geraldine P. Schechter for samples of blood from individuals with sickle cell trait.

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