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HEMOGLOBIN SUBUNIT-SUBUNIT AFFINITY-DETERMINANT OF HEMOGLOBIN FORMATION

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ABSTRACT

Hemoglobin A_2 is often elevated in β-thalassemia and decreased in α-thalassemia. This might be due to hemoglobin subunit-subunit affinity variation. It has been inferred from the study of abnormal hemoglobins that the α subunits have higher affinity for β subunits than for δ subunits. However, only in one study has the affinity of α, β, and δ subunits for each other been measured. In this work we have attempted to measure the hemoglobin subunit-subunit affinity with somewhat different approach, i.e., hybridization of hemoglobin A and A_2. It is shown that hybridization and recombination of equal amounts of hemoglobins A and A_2 lead always to the formation of more hemoglobin A than A_2. Incubation of pure α, β, and δ subunits forms more hemoglobin A than A_2 as the availability of α subunits declines. It is concluded that hemoglobin α subunits have approximately four-fold higher affinity for β subunits than for the δ subunits under these experimental conditions. This subunit-subunit affinity difference, which has been attributed to the variation in molecular electrostatic charges, explains the variation of hemoglobin A_2 levels in thalassemia syndromes.

INTRODUCTION

Hemoglobin (Hb) A_2 level is often elevated in patients with β-thalassemia. Although its level is expressed as a percentage of the total Hb (HbA + A_2) which is variable in these patients, thence a high degree of apparent variability of HbA_2 level, the absolute amount of HbA_2 is, however, increased (Weatherall, 1972; Bunn *et al.*, 1977). In α-thalassemia, on the contrary, the level of HbA_2 is often decreased (McCormack, 1980). Higher electrostatic attraction between α and β subunits than between α and δ subunits has been suggested as a cause of this phenomenon (Bunn and McDonald, 1983; Mrabet *et al.*, 1986; Bunn, 1987). However, in only one study affinity between α and β and α and δ subunits was measured (Martinez and Menendez, 1982). The present work was undertaken to measure directly the Hb subunit-subunit affinity *in vitro*.

MATERIALS AND METHODS

Paramercuribenzoic acid (p-CMB) was obtained from Sigma Chemical Company, St. Louis, MO. Diethylaminoethyl cellulose (DE-52) was obtained from Whatman, Kent, England.

Hemoglobin A was purified from fresh human red cells by CM-Sephadex - ion-exchange chromatography (Winterhalter and Huehns, 1964). α and β subunits were prepared by incubation of HbA with p-CMB according to the method described by Bucci and Fronticelli (1965). HbA_2 was prepared by DEAE-Sephadex - ion-exchange chromatography (Huisman and Dozy, 1962). δ subunits were prepared according to the following procedure. HbA_2 was gel filtered against diluted tris-HCl buffer. Two percent p-CMB solution in 2M glycine-KOH buffer, pH 8 and $MgCl_2$ were added so that the final concentrations of p-CMB and $MgCl_2$ were 0.013 M and 0.3 M respectively. The pH was adjusted to 5.6 with 1.5 M solution of glacial acetic acid. The solution was incubated under carbon monoxide (CO) at 4 °C for about seven days. The Hb solution was gel filtered first against 5 mM NaCl solution and then against tris-phosphate-KOH buffer and applied to a column of DE-52 equilibrated with the same buffer. The column was washed with the same buffer containing (0.3 per cent v/v) of 2-mercaptoethanol under CO. After the α subunits and the non-reacted HbA_2 were eluted, the native δ subunits were eluted with 0.3 M sodium phosphate, pH 7.4. The purity of all Hb subunits was tested by cellulose acetate electrophoresis (Marengo-Rowe, 1965).

HYBRIDIZATION OF HEMOGLOBIN A AND A_2

Pure hemoglobin A and A_2 samples were dialyzed overnight against distilled water at 4 °C. The concentrations of both hemoglobin solutions were adjusted to about three percent. 0.2 ml of hemoglobin A solution was mixed with 0.2 ml of hemoglobin A_2. One-half was kept as control and the other half was dialyzed against sodium acetate buffer at pH 4.7 and 4 °C. Recombination was carried out by dialysis of the sample for about 20 hours against tris-EDTA-boric acid buffer, pH 9 at 4 °C. The percentage of various hemoglobins was determined by cellulose acetate electrophoresis and quantitation of the different bands.

RECOMBINATION OF HEMOGLOBIN SUBUNITS

Various amounts of hemoglobin subunits (α,β,δ) in concentration of 0.2 mM were mixed in such a way that the final proportions of different subunits of the total mixture was as is shown in the following: a) $\alpha = \beta = \delta = 33.3$ percent; b) $\alpha = 50$ percent, $\beta = 25$ percent, $\delta = 25$ percent; c) $\alpha = 20$ percent, $\beta = 40$ percent, $\delta = 40$ percent; d) $\alpha = 10$ percent, $\beta = 45$ percent, $\delta = 45$ percent. The mixture a,b,c, and d was dialyzed against tris-EDTA-boric acid buffer, pH 9, in order for the subunits to recombine (Huisman, 1977). At different time intervals, small samples were taken and were subjected to electrophoresis on cellulose acetate strip in order to separate HbA from A_2. In order to quantitate the separated hemoglobins, the bands were cut and inserted in buffer to elute the hemoglobin. The eluted hemoglobins were quantitated by determination of absorption of 420 nm.

RESULTS

When hemoglobin A and A_2 were hybridized and the final percentage of hemoglobin A and A_2 were measured, it was found that the proportion of hemoglobin A is higher in all 19 experiments performed in spite of wide variability of the results (Fig. 1).

Since the total of hemoglobin A and A_2 recovered was taken as 100 percent in every experiment, the percentages in each experiment are mirror images. It should be mentioned that there was no visible precipitate in any of the hemoglobin solutions after the hybridization.

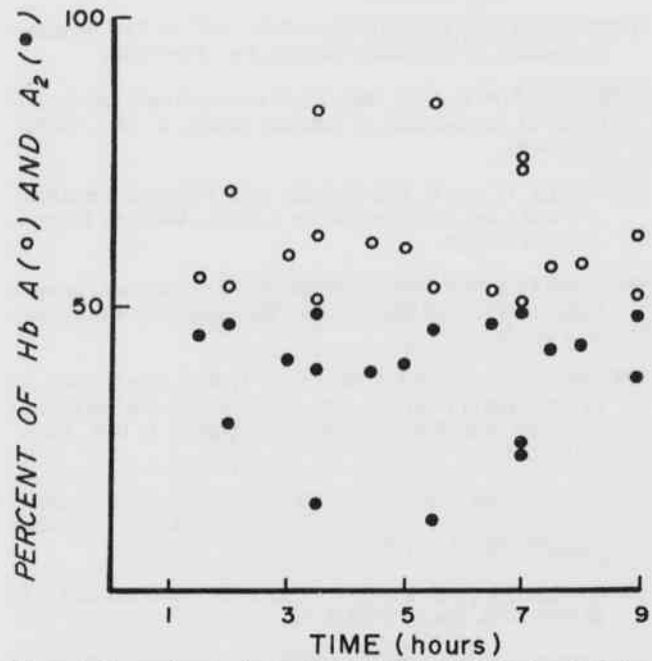

Figure 1. Separation and quantitation of hemoglobin A and A_2 by cellulose acetate electrophoresis after hybridization and recombination at various time intervals. The total quantity of hemoglobin A and A_2 is taken as 100 percent in every experiment.

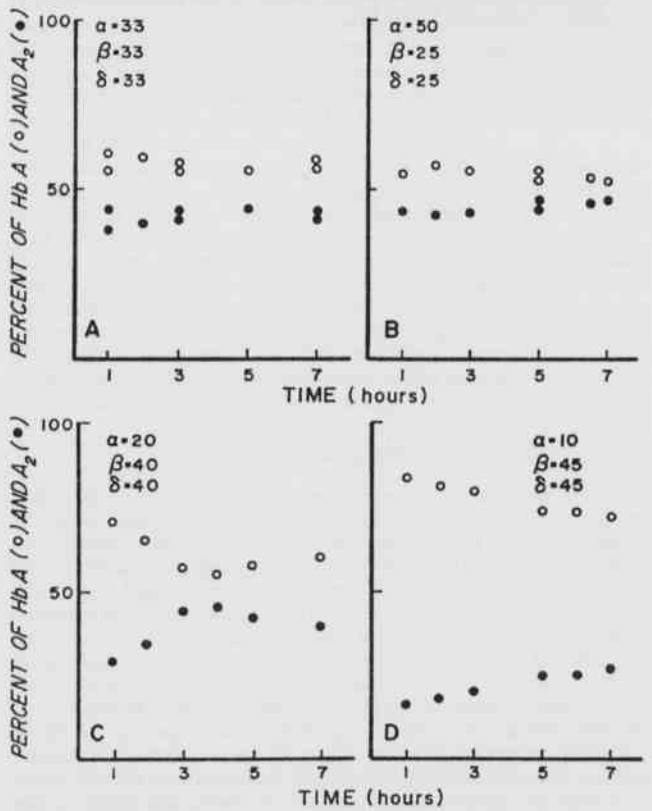

Figure 2. (A,B,C,D) Recombination of various hemoglobin subunits at different ratios of α,β, and δ at various time intervals. Again, the total recovered hemoglobin A and A_2 were taken as 100 percent.

When isolated α, β, and δ hemoglobin subunits were mixed and incubated in such a way that there were as many α subunits as β and δ subunits together, the proportion of HbA formed was somewhat higher than HbA_2 in all experiments (Fig. 2B). The reference point (100 percent) is derived by the addition of HbA and A_2 concentrations in each experiment. In this way the total sum of HbA and A_2 is always equal to 100 percent. This has the advantage that regardless of the concentration of subunits, the changes in the proportions of HbA and A_2 closely correspond to their synthesis. As the relative concentration of α subunits to β and δ subunits decreased, the proportion of HbA_2 decreased. In Figure 2A there is a slight deficiency of α subunits. Where there were 33 percent of α subunits, there was a tendency for slight decrease in HbA_2 formation. As the concentration of α subunits decreased further, there was less and less HbA_2 formed (Fig. 2C,D). It should be noted that there was no appreciable loss or precipitation of subunits during the experiments. All solutions were visually clear and free from precipitates. The optical baseline also did not change significantly.

DISCUSSION

Hemoglobin A is a tetrameric molecule with two α and two β polypeptide subunits which are usually synthesized in equal amounts in the red cell precursors. In α and β thalassemias there is an excess of β or α hemoglobin subunits respectively which precipitates and, through a complex mechanism, damages the cell membrane. One could expect that the absolute amount of hemoglobin A_2 should remain the same since there is a very small amount of δ chains available. However, the amount of hemoglobin A_2 increases in most cases of β thalassemia and decreases in some cases of α thalassemia (McCormack, 1980). Although this could be due to variation in synthetic rate of δ chains, the experiments in this work suggest strongly that the α subunits have a higher affinity for β subunits than for δ subunits. This must play a significant role in the determination of HbA concentration *in vivo* similar to what has been reported by Shaeffer *et al.* in regard to HbA and S in patients with sickle cell trait (Shaeffer *et al.*, 1978). The magnitude of this affinity difference is not certain, but the results of experiments which are presented in Figure 2D where the concentration of α subunits was the lowest suggest that the α subunits have at least four-fold higher affinity for β than for δ subunits. This is much lower than 7.5-fold difference shown by Martinez and Menendez (1982). It can be said that recombination properties of the isolated subunits might be different from those of the native Hb subunits. This might introduce errors in the results; however, it has been shown in the past that when Hb subunits are fresh and completely devoid of p-CMB, they behave in similar fashion as native subunits. Utmost care was exercised in these experiments to remove p-CMB completely from the subunits. Since the method of preparation of δ subunits is different from that of α and β subunits, it might bring an artifactual difference in the subunit affinity. This was probably not the case here because the δ subunits are very stable and the presence of CO during the incubation confers to them more stability. One factor which has not been taken into consideration is the rate of monomeriztaion of subunits which might affect rate of binding together. For example, the β subunits are in tetrameric form. In order that they combine with α subunits, they have to monomerize. It is quite unlikely that *in vivo* the subunits are synthesized in the tetrameric form and then monomerize and combine with α subunits. *In vivo,* the subunit synthesis and their combination to form hemoglobin are probably synchronized. Variation of affinity between various subunits has been attributed mainly to their charge difference, i.e., electrostatic attraction between relative positive and negative charges (Rahbar and Bunn, 1987). It has been inferred from the study of abnormal Hbs that the affinity of α subunits is lower for δ than for β subunits since δ subunits have higher positive charge than the β subunits. In only one study the affinity between subunits has been measured. In the present study, we have demonstrated *in vitro* with a different approach that the higher affinity of α subunits for β than δ subunits might in fact be a major contributing factor in determination of the levels of Hbs A and A_2. Whether the variation between Hb

subunit affinity is only due to variation in their electrostatic charges or whether other factors are involved is not known and needs further investigation.

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