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STUDIES ON THE SUBUNIT DISSOCIATION OF DEOXYHEMOGLOBIN USING HEMOGLOBIN-HAPTOGLOBIN INTERACTIONS

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SUMMARY: Hemoglobin (Hb) binds to haptoglobin (Hp) as αβ dimers. The binding affinity of Hb for Hp depends on the dissociation of Hb into dimers. Hp_{1-1} has been used in order to estimate the relative dissociation into dimers of two abnormal Hbs : Hb Creteil and Hb Hope, in oxy and deoxy conditions as compared to that of Hb A and cyanmet Hb A. We have found that in oxy conditions Hb Creteil, a variant with high oxygen affinity, and Hb Hope, a variant with low oxygen affinity, dissociate into dimers, at a similar rate to Hb A. The dissociation of Hb Creteil in deoxy condition is the same as that of cyanmet Hb A indicating that in case of Hb Creteil, "R-T" equilibrium is shifted towards "R" state.

It was previously reported that the haptoglobin (Hp) molecule binds rapidly hemoglobin (Hb) dimers (αβ) but not tetramers (1). This was supported by the fact that the rate constants of the Hp-Hb binding increased with the dilution of Hb and consequently with the proportion of dimers in solution (2). The Hp-Hb reaction is essentially irreversible. Deoxy Hb, which poorly dissociates into dimers fails to combine with Hp, unless the reaction proceeds for many hours (3). The difference of haptoglobin binding between oxy and deoxy Hb reflects structural changes (tertiary and quaternary) in the hemoglobin tetramer, associated with the tetramer-dimer dissociation. These changes may well be linked to the major quaternary structural changes defining the oxy (R) and deoxy (T) structure as disclosed by X-ray analysis (4) and consequently the haptoglobin binding reaction may be employed as a probe for hemoglobin conformation (5).

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In the present work, we have used mixtures of $^{3}[H]$-labelled Hb A and Hb X in order to study their relative affinities for Hp using the competition of the two Hbs for the Hp binding sites. The haptoglobin binding, in oxy and deoxy condition, of two mutant human hemoglobins, Hb Creteil and Hb Hope was studied and compared to that of Hb A and cyanide methemoglobin A.

MATERIALS AND METHODS.

Human Hp_{1-1} was purified from pleural fluids of patients with malignant diseases. Purity of Hp preparations was tested by polyacrylamide gel electrophoresis and by immunoelectrophoresis. Hp concentration was measured using an absorption coefficient of $A_{280}^{1\%} = 12.0$ (6). Binding capacity of purified Hp was measured by fluorescence quenching titration (7) using a Fica spectrofluorimeter. Activity of the preparation used throughout this paper was of 0.85 and the quantity of Hp was given as active Hp. $^{3}[H]$-labelled Hb A was obtained by incubation of normal reticulocytes with L-$^{3}[H]$-leucine (8). Hb Creteil (β89 F5 Ser → Asn) was isolated by DEAE cellulose column chromatography (9). Hb Hope (β136 H14 Gly → Asp) was isolated by column chromatography on amberlite Biorex 70 in a 0.04 M phosphate buffer, pH 7.12 without cyanide (10). Cyanide methemoglobin A (cyanmet Hb A) was obtained as follows : potassium ferricyanide was added to oxy Hb A in a molar ratio 2:1 to obtain met Hb A. Potassium cyanide was then added in a sufficient amount to transform completely met Hb A into cyanmet Hb A. The excess of reagents was removed by passing the mixture through a Sephadex G-25 column. Deoxygenation experiments were done as follows : a tonometer with two compartments was used. One part contained a mixture of $^{3}[H]$-labelled Hb A and Hb X in equal amounts. The final quantity of Hb was 30 nmoles. The second compartment contained 15 nmoles of Hp_{1-1}. Deoxygenation was performed under vacuum and the two solutions were mixed. After 15 min. of incubation at room temperature the tonometer was opened and electrophoresis on cellulose acetate was performed. The amount of Hb A in the different electrophoretic fractions was estimated by measuring the radioactivity of the fractions since Hb A was $^{3}[H]$-labelled. Hbs were eluted from acetate cellulose by Tris-HCl 0.05 M, pH = 7.0 buffer. The $^{3}[H]$radioactivity was counted in a Beckman LS-150 liquid scintillation system after peroxydation of the samples, with adequate quenching corrections.

RESULTS AND DISCUSSION.

Fig. 1 represents the electrophoretic patterns on cellulose acetate of Hb A, cyanmet Hb A, Hb Creteil and Hb Hope and their

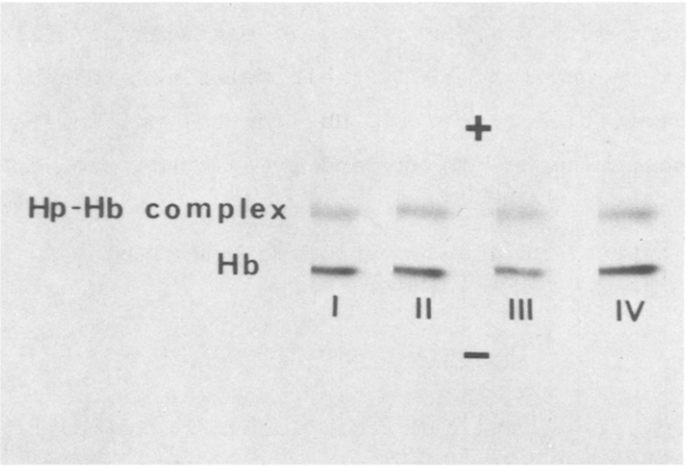

Fig. 1 Electrophoresis on cellulose acetate of Hb/Hb-Hp complex mixtures. Tris-EDTA borate buffer pH 8.9
I - Hb A
II - Cyanmet Hb A
III - Hb Creteil
IV - Hb Hope

corresponding Hp-Hb complexes. It clearly shows that all the bound Hbs have similar electrophoretic mobilities under these conditions and that the Hp-Hb complexes are well separated from the non bound Hbs. Fig. 2 shows the results obtained when equimolar mixtures of Hb A and Hb X are incubated with Hp in oxy conditions. Since Hp binds to the $\alpha\beta$ dimers of hemoglobin, a hemoglobin which has an increased tendancy to dissociate into dimers would have a higher affinity for Hp than Hb A. If the tested Hb has the same dissociation into dimers as Hb A, then $^{3}[H]$ radioactivity would be equally distributed between Hp-Hb complex and non-bound Hb under the conditions described in METHODS. The results summarized in Fig. 2 showed that Hb A, cyanmet Hb A, Hb Creteil and Hb Hope have bound to Hp, under oxy condition, at the same rate. We can conclude that the dissociation into dimers of these Hbs, is identical.

Incubation of the mixture cyanmet Hb A / $^{3}[H]$-Hb A under deoxy condition resulted in the following distribution of radioactivity : 89 % of radioactivity in free Hb fraction, 11 % in Hp-Hb complex fraction (Fig. 3 I). This means that cyanmet Hb A

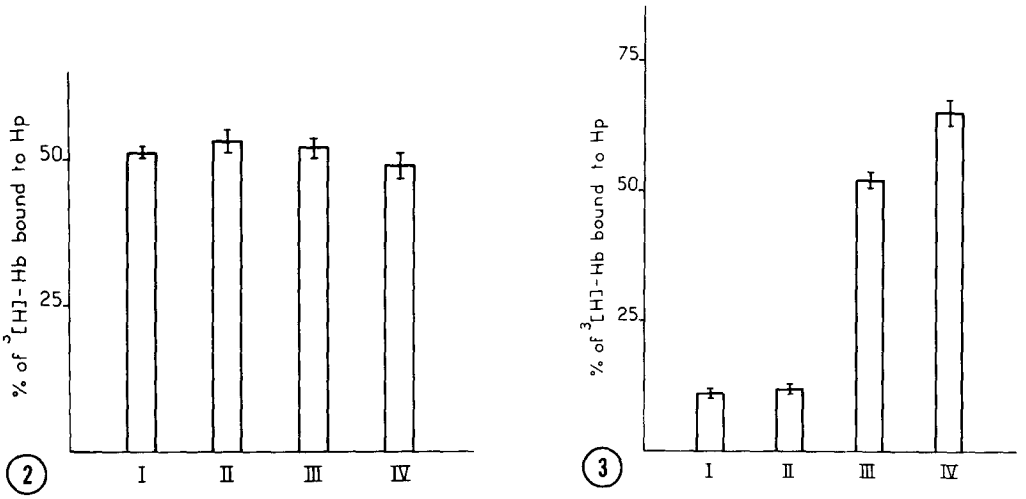

Fig. 2 Relative haptoglobin binding of various human Hbs in oxy conditions. To a mixture containing equal amounts of the test Hb and Hb X, Hp_{1-1} was added in a molar ratio Hp /Hb 1/2.

I) ^{3}[H]-Hb A/cyanmet Hb A
II) ^{3}[H]-Hb A/Hb Creteil
III) ^{3}[H]-cyanmet Hb A/Hb Creteil and
IV) ^{3}[H]-Hb A/Hb Hope.

Fig. 3 Relative haptoglobin binding of various human Hbs in deoxy conditions. See Fig. 2.

preferentially binds to Hp. Cyanmet Hb A is considered as being in "R" distorted state (11). The constant of dissociation ($K_{4,2}^{app}$) of this hemoglobin, in absence of phosphate, was found to be of 2.4×10^{-4} (12), indicating a dissociation similar to that of oxy Hb A. The result we have obtained agree with these facts since it gives another indication that the dissociation of cyanmet Hb A is similar to that of oxy Hb A. In case of incubation in deoxy condition of a mixture in equal amounts of labelled Hb A and Hb Creteil, the amount of radioactivity found in the Hp-Hb fraction was 12 % (Fig. 3 II). This result was similar to that obtained for Hb A/cyanmet Hb A mixture. Two hypotheses could explain the results obtained for Hb Creteil : a) during reoxygenation of the mixture Hb A/Hb Creteil, O_2 binds preferentially to Hb Creteil, which results to a dissociation of Hb Creteil

greater than that of Hb A and consequently Hb Creteil binds preferentially to Hp, b) under deoxy conditions the equilibrium between dissociating and non dissociating forms of Hb Creteil is shifted towards the dissociating form. In order to conclude, a competition test was performed between $^{3}[H]$-labelled cyanmet Hb A and Hb Creteil in deoxy conditions. The result (Fig. 3 III) demonstrated that the two Hbs bind at the same rate to Hp. This means that, since the cyanmet Hb A dissociates similarly to oxy Hb A, this is also the case of deoxy Hb Creteil. These results fit very well with the functional properties (9) of Hb Creteil which lead us to conclude that the "R-T" equilibrium of Hb Creteil is shifted towards the R state.

Hb Hope, a low O_2 affinity variant (13) was also investigated. The incubation of the mixture of $^{3}[H]$ labelled Hb A/Hb Hope under deoxy condition resulted in an unequal distribution of radioactivity : 35 % for free Hb and 65 % for bound Hb (Fig. 3 IV). Two hypotheses could explain the results obtained for Hb Hope.
a) under deoxy conditions some amount of Hb A was bound to Hp ;
b) during reoxygenation of the mixture, Hb Hope binds slower than Hb A to Hp. It was tested that under deoxy conditions Hb A does not practically bind to Hp after 15 min. of incubation (3). Consequently the binding of hemoglobin A to Hp takes place during reoxygenation of the mixture. The decreased oxygen affinity of Hb Hope is responsible for the relatively low binding of this Hb as compared to Hb A. Similar experiments have previously been used in order to estimate the relative dissociation into dimers of some Hbs (14) in oxy conditions. It has been observed an increased binding to Hp in case of Hb Kansas and a decreased one in case of Hb Chesapeake and Yakima. In our case we have not observed different binding for the abnormal Hbs we have studied. This means that Hb Creteil or Hb Hope are not more dissociated in their oxy form than Hb A. In contrast great differences have been observed in deoxy conditions and they reflect structural changes in the hemoglobin tetramer. This experiment can be used as an additional proof of modifications of the molecular conformation.

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