THE HEMOGLOBIN-HAPTOGLOBIN REACTION AS A PROBE OF HEMOGLOBIN CONFORMATION

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SUMMARY - The dissociation of hemoglobin to dimers is a prerequisite for the binding of hemoglobin to haptoglobin. This reaction, then can be used as a conformational probe of the state of the $\alpha_1\beta_2$ area of contact. With this approach, we find that mixed liganded hybrids ($\alpha_2\beta_2^*$ and $\alpha_2^*\beta_2$, where * identifies the ferric pair of hemes) can exist in a conformation that resembles deoxyhemoglobin when deoxygenated. Also stripped deoxy Hb A can exist in a form able to dimerize more readily than deoxy Hb A bound to phosphates. Finally, the data shows that deoxy Hb Hiroshima and deoxy Hb Chesapeake exist in a conformation different from that of Hb A, expressed in a shift in the equilibrium between dissociating and non-dissociating forms. This equilibrium is strongly affected by phosphates, with deoxy Hiroshima reverting to an almost normal behavior while deoxy Chesapeake still reacting rapidly with haptoglobin.

The dissociation of hemoglobin (Hb) to dimers has been shown to be a prerequisite for the reaction between liganded hemoglobin and haptoglobin (Hp) (1); in the presence of phosphates, no binding to deoxyhemoglobin has been demonstrated (2). This difference in haptoglobin binding must reflect structural changes (tertiary and/or quaternary) in the hemoglobin tetramer, having immediate effects in the α_1-β_2 contact area (3). These changes may well be linked to the major quaternary structural changes defining the oxy and deoxy structures as disclosed by x-ray analysis (4). Conversely, the haptoglobin binding reaction may be employed as a probe of hemoglobin conformation, and we describe the results of applying it to a number of hemoglobin derivatives and mutant human hemoglobins.

METHODS

The purification of Hp 1-1 from human serum was carried out as described previously (1). Judged by starch gel electrophoresis , the preparation was 85-90% pure; contaminants were

albumin and α_2 and α_2 glycoproteins. Concentration of haptoglobin was determined by spectrophotometric titration (4). Stripping of Hb of organic and inorganic phosphates was accomplished by slow passage through Sephadex G-25 equilibrated with a solution 0.05 M in bis-Tris buffer, pH 7.3 (5). Total phosphate determinations on these samples confirmed the adequacy of the "stripping" procedure (6).

Mixed ligand hybrids were prepared with α^{SH} chains isolated by the method of Geraci, Parkhurst and Gibson (7) and tetrameric β_4 hemoglobin separated by starch block electrophoresis, pH 7.0, 0.04 M phosphate buffer from a hemolysate containing 40% of Hb H. To prepared mixed liganded hybrids, either the α chains or β_4 tetramers were reacted with potassium ferricyanide at a ratio of 1.3 moles $K_3Fe(CN)_6$ per mole of heme in 5 mM KCN, to convert the hemes to the cyanmet form. Excess ferricyanide was removed by Sephadex G-25 chromatography and the appropriate ferric and ferrous chain were assembled by quantitative recombination. Absence of significant excess of isolated chains was monitored by starch gel electrophoresis.

Hb Hiroshima was prepared by column chromatography using Bio-Rad 70 equilibrated with 0.005 M ammonium phosphate buffer, pH 7. The Hb Hiroshima fraction was readily eluted with the same buffer while Hb A remained attached to the column. The Hb A was eluted with 0.5 M ammonium sulfate adjusted to pH 8.0.

Hb Chesapeake was separated by starch block electrophoresis, 0.04 M veronal, pH 8.6.

Hb Des His, a reconstituted hemoglobin assembled with β chain previously digested with carboxypeptidase A and lacking of His 146 β, was a gift of Dr. J.V. Kilmartin, M.R.C. Laboratory of Molecular Biology, Cambridge, England.

Hemoglobin binding to haptoglobin was measured by the method of Nagel and Gibson (8), based on the quenching of the aromatic amino acid fluorescence of haptoglobin by the hemes of hemoglobin.

Deoxy Hb binding to haptoglobin was also followed at 430 mμ by the stopped flow method

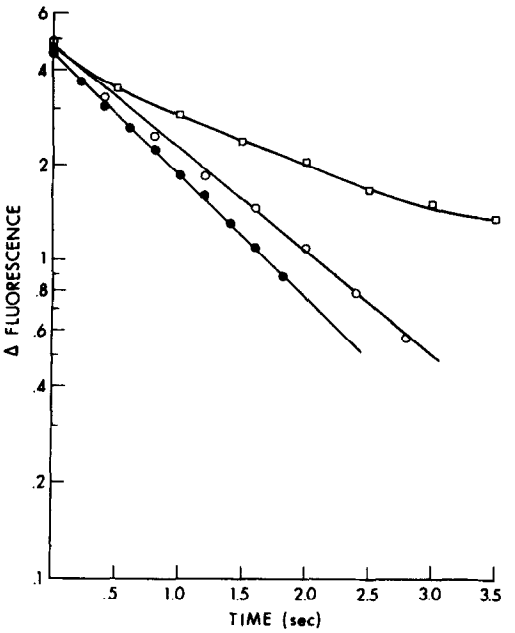

Fig. 1.

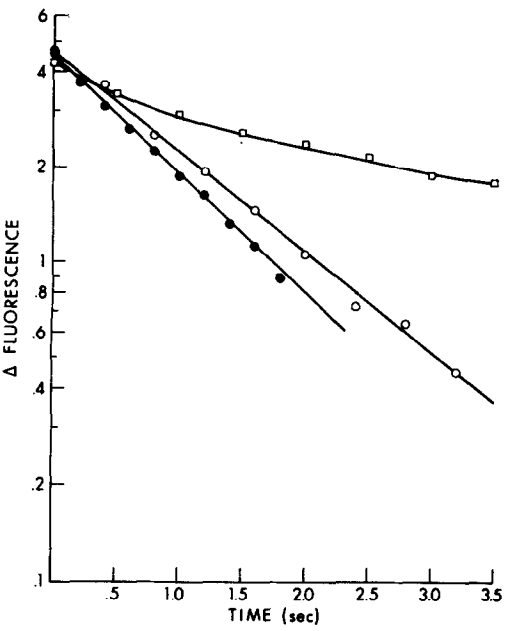

Fig. 2.

Figure 1: The binding of $\alpha_2\beta_2^*$ mixed ligand hybrid to haptoglobin followed by fluorescence quenching. Concentrations of reactants before mixing: Hb 27 μM (in tetramer); Hp, 3.5 μM. Open circle: oxy $\alpha_2\beta_2^*$ vs. Hp. Open square: deoxy $\alpha_2\beta_2^*$ vs. Hp. Solid circle: oxy Hb A vs. Hp. All solutions were at pH 7.0, 0.1 M potassium phosphate buffer. Temperature 22°C.

Figure 2: The binding of $\alpha_2^*\beta_2$ mixed ligand hybrid to haptoglobin followed by fluorescence quenching. Open circle: oxy $\alpha_2^*\beta_2$ vs. Hp. Open square: deoxy $\alpha_2^*\beta_2$ vs. Hp. Solid circle: oxy Hb A vs. Hp. Same conditions as Figure 1.

using the apparatus of Gibson and Milnes (9) with some modifications. This procedure is based on the ΔOD at 430 mμ observed between deoxy Hb and deoxy Hb-Hp complex (10).

RESULTS

1. The binding of haptoglobin to mixed liganded hybrids.

Fig. 1 depicts the binding of $\alpha_2\beta_2^*$ to haptoglobin as followed by fluorescence quenching. The oxygenated mixed liganded hybrid has a rate of reaction, at pH 7.0, 0.1 M PO_4 very much like native Hb A. The initial first order rate constant is 0.86 sec^{-1} for oxy

Hb A and 0.81 sec^{-1} for oxy $\alpha_2\beta_2^*$. When the hybrid is deoxygenated, a significant reduction of the rate of reaction occurs and the reaction is no longer pseudo first order.

Fig. 2 depicts the binding of $\alpha_2^*\beta_2$ to haptoglobin under the same conditions as the alternate hybrid. The result is essentially identical. The oxy hybrid behaves very similarly to native hemoglobin in its rate of reaction with haptoglobin (rate constant = 0.77 sec^{-1}). When deoxygenated, a slowing of the reaction is observed agina, to an even further extent.

2. The binding of haptoglobin to Hb A stripped of phosphates.

In Fig. 3, we examine the reaction of deoxy Hb A stripped of phosphate and the effects of addition of phosphates to these solutions. A surprising result was the observation of binding of deoxy Hb A stripped of phosphates to haptoglobin at a slow rate. The addition of phosphates stops this reaction, rendering hemoglobin incapable of reacting with haptoglobin as previously reported (2). This finding was subsequently confirmed by following the reaction by fluorescence quenching in the presence of small amounts of dithionite.

The addition of 2,3-Diphosphoglycerate (2,3-DPG) also inhibits greatly the reaction between stripped deoxy Hb A and haptoglobin, in the same manner as inorganic phosphates.

3. The binding of human hemoglobin variants in different ligand states in the presence and absence of phosphates.

We have previously reported that oxy Hb Hiroshima, in 0.1 M phosphate, pH 7.0, binds haptoglobin with a slightly faster rate than Hb A in the same conditions (4). Fig. 5 depicts the binding of oxy Hb Chesapeake, in 0.1 M PO_4 to haptoglobin. In the conditions of the experiment, oxy Hb A binds with a rate constant of 0.81 sec^{-1}. Oxy Hb Chesapeake, however, binds more slowly with a rate constant of 0.19 sec^{-1}. Of special significance is that Hb Chesapeake in the presence of phosphate binds in the deoxy form, unlike Hb A, but a very slow rate, and without conforming with pseudo first order kinetics as can be observed from the graph.

When these mutants are previously stripped of organic and inorganic phosphates,

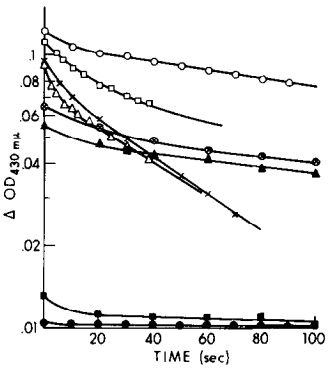

Fig. 3.

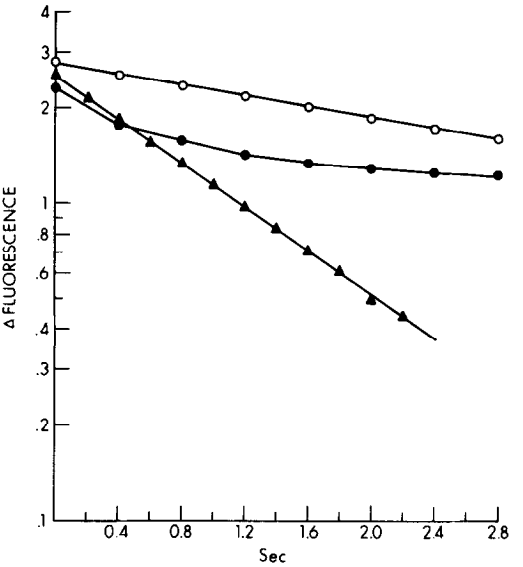

Fig. 4.

Figure 3: Binding of stripped deoxy hemoglobins to haptoglobin. Open circle: stripped deoxy Hb A vs. Hp. Open square: stripped deoxy Hb Hiroshima vs. Hp. Open triangle: stripped deoxy Hb Chesapeake vs. Hp. X: stripped deoxy Hb Des His vs. Hp. All previous solutions were at pH 7.0, 0.05 M bis-Tris. Solid symbols and ⊗: same as corresponding symbols but with the addition of 0.1 M phosphate, pH 7.0 to the Hb solution. Hemoglobin concentrations were 2.2 μM (tetramer) and haptoglobin concentration was 4.05 μM. Temperature 22°C.

Figure 4: The binding of Hb A and Hb Chesapeake in different ligand states to haptoglobin. Open triangle: oxy Hb A vs. Hp. Open circle: oxy Hb Chesapeake vs. Hp. Solid circle: deoxy Hb Chesapeake vs. Hp. All solutions at pH 7.0, 0.1 M phosphate. Hemoglobin concentration: 6 μM (tetramer) Haptoglobin 3 μM. Temperature 22°C.

interesting aspects of these reactions with haptoglobin in the deoxy state are uncovered. In Fig. 4, we can follow the reaction of deoxy Hb Hiroshima and Hb Chesapeake previously stripped from phosphates and after the readdition of phosphate. Stripped deoxy Hiroshima binds haptoglobin with a slightly faster rate, maintaining the same difference observed when non-stripped Hb A is compared to Hb Hiroshima. The addition of phosphate to stripped Hb Hiroshima greatly slows the binding of this mutant to Hb, making its behavior very similar

to that of Hb A. The case, of stripped Hb Chesapeake, is different: Deoxy stripped Hb Chesapeake is capable of binding Hp at a faster rate than Hb A and Hb Hiroshima. But the addition of phosphates only reduces the rate slightly.

Finally, we can observe in Fig. 4, that Hb Des His, (an artificial compound generated by the removal of His β 146 with carboxypeptidase A) has a characteristic reaction with haptoglobin. When stripped Hb Des His in the deoxy state is reacted with haptoglobin, one observes a rather fast rate of reaction as compared with Hb A. The addition of phosphates slows the reaction considerably but does not suppress it. Thus, although there are some similarities between Hb Des His and Hb Hiroshima, they behave differently in their reaction towards haptoglobin.

DISCUSSION

The data presented here refer to three different aspects of the conformation of hemoglobins as probed by the hemoglobin-haptoglobin reaction. We will discuss these aspects separately.

a) The Conformation of Stripped Hb A.

Biochemical data had suggested in the past that the central cavity of hemoglobin was the likely binding site for DPG (12, 13). This hypothesis has been recently confirmed by x-ray crystallography (14) and in addition to the alpha amino groups of the beta chains, residues His β2, His β143 and Lys β82 have been demonstrated to be involved in the binding site. The stoichiometry of 1:1 binding between 2,3-DPG and the hemoglobin tetramer and the preferential affinity for deoxyhemoglobin seems now well established (15, 13).

The evidence presented here demonstrates that stripped deoxy-Hb A can exist in a form able to dimerize more readily than deoxy Hb A bound to phosphate. Hence the $\alpha_1\beta_2$ area of contact is probably affected by the binding of 2,3-DPG and inorganic phosphate to hemoglobin.

b) The Conformation of Mixed Liganded Hybrids.

The evidence presented here suggests that these hybrids can exist in a conformation that resembles that of deoxyhemoglobin when one pair of subunits (alpha or beta) are free from

ligand. Moreover, the complex kinetics of the haptoglobin binding to the deoxy hybrids may possibly reflect the presence of two deoxy forms not in rapid equilibrium.

These findings are in excellent agreement with the studies of Caldwell and Nagel (16) on the binding of 2,3-DPG to mixed liganded hybrids and in general agreement, with the data obtained with a spin-labelled triphosphate compound by Ogata and McConnell (17). They are also consistent with the data of Brunori et al. (18) that describes a full alkaline Bohr effect for both types mixed liganded hybrids ($\alpha_2\beta_2^*$ and $\alpha_2^*\beta_2$). This last result implies that both chains participate in conformational changes in accordance with the result of Kilmartin et al. (19) that assigns about half of the Bohr effect to each chain. This finding has bearing on the problem of the mechanism of heme-heme interaction, and suggests that two unliganded sites might be sufficient to favor the deoxy conformation or more appropriately a quaternary structure that resembles the unliganded state.

c) Conformation of Hb Hiroshima, Hb Chesapeake and Hb Des His.

The data for stripped Hb Hiroshima and Hb Chesapeake show that their deoxy forms exist in a conformation different from that of hemoglobin A, expressed in a shift in the equilibrium between dissociating and non-dissociating forms. This equilibrium is not rapidly attained, however, since the kinetics of the reaction with haptoglobin are markedly heterogeneous in both cases. The equilibrium is strongly affected by inorganic phosphates and by 2,3-DPG. Nevertheless Hb Hiroshima responds more readily than Hb Chesapeake, which still reacts quite rapidly with haptoglobin in 0.15 M phosphate.

In the absence of phosphates, Hb Des His is similar to hemoglobin Hiroshima, as would indeed be expected on structural grounds. Unlike Hiroshima, it shows relatively little response to the addition of phosphates.

As a general conclusion it can be stated that the hemoglobin haptoglobin reaction can be used to demonstrate differences in conformation between various deoxyhemoglobins. The nature and significance of these differences requires correlation with other functional properties of the hemoglobins.

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