The Binding of Hemoglobin to Haptoglobin and Its Relation to Subunit Dissociation of Hemoglobin*

(Received for publication, July 17, 1970)

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SUMMARY

The binding of liganded hemoglobin to haptoglobin, a plasma α2-glycoprotein, is an irreversible and stoichiometric reaction that occurs physiologically at the micromolar concentration level.

The study of the reaction between deoxyhemoglobin which does not bind haptoglobin and a haptoglobin solution saturated with carbon monoxide indicates that hemoglobin tetramer is incapable of binding haptoglobin and its dissociation to dimers is a prerequisite for the reaction. The reaction between haptoglobin and the liganded dimers proceeds with a rate constant of about $5.5 	imes 10^6 \text{M}^{-1} \text{sec}^{-1}$ and the results can be fitted with a dissociation constant of $1.5 	imes 10^{-6} \text{M}$ for the hemoglobin tetramer.

The study of the reaction of isolated α- and β-hemoglobin chains towards haptoglobin half-saturated by α chains or Hb A, has permitted a detailed analysis of this reaction. The haptoglobin, on the basis of the data presented here, probably contains four binding sites, two for each hemoglobin dimer (αβ). These two pair of sites are independent and noninteracting but within each pair a strong interaction is observed between the α-specific site and the allosterically induced β site.

A detailed model of the reaction under physiological conditions is proposed on the basis of these results.

Haptoglobin is a α2-glycoprotein first described by Smithies (1) occurring in human serum as a polymorphic character and comprising three common phenotypes Hp1, 2, and 3. These types have been further subdivided into six subtypes on the basis of information provided by the analysis of the polypeptide chains constituting these molecules (2).

Haptoglobin 1-1, the type used in these studies, has a molecular weight of 100,000 and is probably a tetramer formed by two α and two β chains. The β chains probably contain the binding site for hemoglobin (3–5).

The physiological importance of haptoglobin has not been fully explored but its most striking characteristic is its capacity to bind extracorpuscular hemoglobin. The reaction probably occurs in the intravascular compartment, where the concentration of Hp is approximately 25 μM and that of hemoglobin is normally about 6 μM in heme (6).

The mechanism of the binding of hemoglobin to haptoglobin has been the subject of several recent investigations. The reaction has been regarded classically as irreversible, the globin moiety of hemoglobin binding with a stoichiometry of one hemoglobin tetramer for each haptoglobin molecule.

The kinetics of the reaction, first studied by Nagel and Gibson (7), suggested that the reaction proceeded rapidly and in all like-lihood through hemoglobin subunits. A strong indication of this was that the rate of the reaction did not increase in a linear manner with increasing hemoglobin concentration. More intriguing was the apparent potentiation of the binding of isolated hemoglobin β chains by isolated hemoglobin α chains. Hemoglobin H (84) and isolated βHMB chains have only a very low affinity for haptoglobin, but when αHMB chains are added to the mixture in sufficient amounts to fill one-half of the sites, the binding of both α and β chains proceeds normally to full saturation of the haptoglobin (7). These findings have been fully confirmed in extensive studies by Chiancone et al. (8) and Alfsen et al. (9), who in addition have called attention to the fact that, on dilution of haptoglobin, there is some increase in the rate constant for the reaction.

Another line of evidence has been developed by Kagiyama, Ogawa, and Kawamura (10) and by Hamaguchi (11) who isolated an intermediate of the form Hpαβ from partially saturated Hp solutions. Utilizing ultracentrifuge, immunological, and heme content data, they have shown that the minimum effective subunit of Hp is able to bind two chains of hemoglobin (one α and one β chain).

In spite of these facts no detailed scheme for the reaction has been put forward because of uncertainty about the dissociation of hemoglobin into subunits, and the lack of sufficient experimental data on the binding of isolated chains, which left it unclear whether monomers, dimers, and tetramers of hemoglobin must all be taken into account as participants in the reaction.

Recently, however, it has been shown (12) that there is no sig-
significant formation of monomers in the micromolar range of hemoglobin concentration and that ligand-bound and deoxyhemoglobins differ widely in their dissociation constants. It then became feasible to examine the following questions experimentally.

(a) Can the hemoglobin tetramer bind haptoglobin or can it, with the monomer, be excluded as a participant in the reaction when hemoglobin is at the micromolar level, which is the physiological level of hemoglobin concentration in plasma (6)? (b) What is the number and specificity of the Hp sites for hemoglobin subunit binding?

**Methods**

**Materials**—Purification of Hp 1-1 from human sera was carried out as described previously (13). The preparation was considered to be 85 to 90% pure; albumin and α- and α2-glycoproteins were the main contaminants of the preparation, as examined by starch gel electrophoresis and ultracentrifuge analysis. The concentration of the haptoglobin solutions was determined by spectrophotometric titration according to Roy, Shaw, and Connell (14).

Hemoglobin was prepared by the method of Drabkin, with minor modifications (15). The αHMB and βHMB chains were obtained by the method of Geraci, Parkhurst, and Gibson (16), kept in the carbon monoxide-ligated form and used within 48 hours. Their purity was controlled by cellulose acetate or starch gel electrophoresis. The concentrations were calculated on a heme basis with the following millimolar extinction coefficients (17): for CO αHMB at 576 μm, 15.15 molar cm⁻¹; for CO αHMB at 569.5 μm, 14.2 molar cm⁻¹; for CO βHMB at 569.5 μm, 14.4 molar cm⁻¹; and at 540 μm, 14.2 molar cm⁻¹. For oxyhemoglobin A the millimolar extinction coefficient at 540 μm was 15.0 molar cm⁻¹. All concentrations referred to in the text are before mixing in the stopped flow apparatus.

**Kinetic Studies**—The reaction of hemoglobin and haptoglobin can be readily measured because of the quenching of aromatic amino acid fluorescence of the haptoglobin by the heme groups of the heme groups of which have effective quenching radii of some 42 Å (7). Stopped flow kinetic measurements were made with the apparatus of Gibson and Milnes (18), with a fluorescence cell attachment of the type described by Gibson et al. (19). Excitation at 287 nm was used. Light was obtained from a direct current xenon lamp and a Bausch and Lomb 250-nm grating monochromator. Stray light was removed from the exciting beam with a 2-mm Corning glass filter No. 7-54. Protein fluorescence was observed with an EMI photomultiplier type 9225-B and a Corning glass filter No. 7-90. The data were collected directly into the memory of a PPD-SI computer, with the
FIG. 3. First-order combination rate constants calculated from Fig. 2 (O) and from previous data (7) (A) plotted as a function of Hb dimer concentration (Hb2) calculated with a $1.5 \times 10^{-6}$ M dissociation constant for the hemoglobin tetramer.

FIG. 4. Combination rates of a 3.41 $\mu$m solution of haptoglobin and $\alpha_{HMB}$ chains ranging from 100 to 6.25 $\mu$m (in heme). For comparison the combination rate of the same Hp solution with Hb A (13.7 $\mu$m concentration) is also shown, O——O. Rates followed by fluorescence quenching. All concentrations are before mixing. Temperature 22°. Solutions in 0.1 M potassium phosphate buffer, pH 7. Concentration of $\alpha_{HMB}$: O—O, 100 $\mu$m; □—□, 50 $\mu$m; ■—■, 25 $\mu$m; △—△, 12.5 $\mu$m; ▲—▲, 6.25 $\mu$m.

FIG. 5. Combination rates of a 3.41 $\mu$m Hp solution previously mixed with 6.5 $\mu$m solution of $\alpha_{HMB}$ chains and reacted with Hb A and $\beta_{HMB}$. All concentrations are before mixing. Temperature 22°. Solutions in 0.1 M potassium phosphate buffer, pH 7. O——O, combination rate of Hp 3.41 $\mu$m with a 13.7 $\mu$m solution of HbA; △——△, combination rate of Hp + 6.5 $\mu$m $\alpha_{HMB}$ with 13.7 $\mu$m oxyhemoglobin A (in heme); ■—■, combination rate of Hp + 6.5 $\mu$m $\alpha_{HMB}$ with 13.7 $\mu$m $\beta_{HMB}$.

system of DeSa and Gibson (20) and were corrected for total voltage to render it directly comparable.

RESULTS

Reaction of Deoxyhemoglobin A with CO-saturated Solution of Haptoglobin—This series of experiments was designed to answer the question of the participation of the tetramer of hemoglobin in the binding to haptoglobin, and it was based on the observation that deoxyhemoglobin does not bind Hp in any appreciable amount (21).

The experiments consisted of mixing, in a stopped flow apparatus with a fluorescence attachment, a deoxyhemoglobin solution and a CO-saturated haptoglobin solution. When these solutions are mixed, the reaction of hemoglobin with CO is exceedingly fast (half-time $\approx 10$ msec) while the dissociation of Hb to dimers is considerably slower (half-time $\approx 1$ sec). If the liganded tetramer is capable of binding as such to Hp, the reaction will proceed without delay. On the other hand, if dissociation to dimers is a necessary preliminary to binding, a lag in fluorescence quenching will be observed.

A 2.5 $\times 10^{-6}$ M (heme basis) solution of human hemoglobin A, diluted in deoxygenated 0.1 M phosphate buffer, pH 7.0, and in the presence of a trace of dichromate, was placed in one of the syringes of the stopped flow apparatus. The other syringe contained a 1.26 $\times 10^{-6}$ M haptoglobin solution saturated with CO. Other experiments were done with Hb concentration of $5 \times 10^{-6}$ and $1 \times 10^{-6}$ M (heme basis).

Fig. 1 shows the results of these experiments. A marked lag in hemoglobin binding by haptoglobin was observed at the beginning of the reaction. The results were satisfactorily fitted by a scheme in which only the hemoglobin dimer binds haptoglobin with a dissociation constant of $1.3 \times 10^{-6}$ M (heme basis) for the hemoglobin tetramer-dimer reaction, 2 sec$^{-1}$ for the tetramer-dimer dissociation rate constant, and $5.4 \times 10^{5}$ M$^{-1}$ sec$^{-1}$ for the rate constant for the association of Hp and the dimer of Hb (Fig. 2, A, B, and C). The fitting was performed by the least squares gradient method with hybrid computation to adjust all three rate constants to their optimum values. Two other experiments yielded similar results.

When the first order rate constants calculated from the results of Fig. 2 and the first order constants from previous experiments (7) were plotted against the dimer concentration in each hemoglobin solution, calculated with $1.5 \times 10^{-6}$ M as the value of the dissociation constant (see Edelstein and Gibson (12)), a straight line was obtained corresponding to a rate constant of $5.7 \times 10^{6}$ M$^{-1}$ sec$^{-1}$ for the haptoglobin-hemoglobin dimer reaction (Fig. 3).

Reaction of $\alpha_{HMB}$ Chains with Haptoglobin—An experiment on the reaction between Hp and $\alpha_{HMB}$ at different concentrations is shown in Fig. 4. The haptoglobin solution was 3.41 $\mu$m and the $\alpha_{HMB}$ chain concentration ranged from 100 to 6.25 $\mu$m (in heme).
The combination rate of Hp 3.41 μM Hp solution previously mixed with 0.8 μM solution of Hb A and reacted with Hb A, αHMB, and βHMB. All concentrations are before mixing. Temperature 22°C. Solutions in 0.1 M potassium phosphate buffer, pH 7.0. □—□, combination rate of Hp + 6.8 μM Hb A with 13.7 μM Hb A; ○—○, combination rate of Hp + 3.41 μM with 13.7 μM Hb A; ●—●, combination rate of Hp + 6.8 μM Hb A with 13.7 μM αHMB; Δ—Δ, combination rate of Hp + 8.8 μM Hb A with 13.7 μM βHMB.

The quenching observed was approximately half of that observed with a 13.7 μM Hb A solution (in heme) at each concentration of α chains.

Reaction of Hp Previously Mixed with αHMB Chains Toward βHMB Chains and Hb A—These experiments were designed to examine the specificity and interaction of the binding sites in the haptoglobin molecule.

Haptoglobin (3.41 μM) was mixed with 6.5 μM solution of αHMB chains (capable of only half-saturating the haptoglobin solution) and then the reactions of this mixture with βHMB chains and Hb A were followed by a stopped flow apparatus. A striking difference was observed (Fig. 5); while a very slow reaction occurs between previously mixed Hp and Hb A, a rapid reaction occurs between the βHMB chains.

Reaction of Hp Previously Mixed with Half-Saturating Amounts of Hb A toward αHMB and βHMB—These experiments were undertaken to examine the possibility of interaction between the sites involved in dimer binding.

When half-saturating amounts of hemoglobin are added to haptoglobin three species are formed: Hpaβ, Hpaβ, and Hpα, in proportions apparently fitting a binomial distribution (22). When this mixture is reacted with isolated chains we are actually examining the behavior of only Hpaβ and Hpα, because Hpaβ is already fully saturated. Free Hb is known to bind βHMB chains with a very low affinity (23) and αHMB chains initially only to half-saturation. It was of interest then, to observe the reactivity of the Hpaβ component of the mixture.

The results are shown in Figure 6. The αHMB chains are capable of satisfying about half of the remaining binding capacity, but βHMB exhibits a very low affinity for the uncomplexed components of the mixture.

The reaction of α chains and Hp and the reactivity of Hp half-dimer as first suggested by Laurell and Gronvall (24). The data presented here fully support this contention and furthermore eliminate the hemoglobin tetramer as a form capable of rapid binding to haptoglobin. Although kinetic experiments are inherently unable to provide chemical identification of the reaction species involved, information already available about haptoglobin dissociation into subunits provides a plausible explanation of the results obtained on mixing deoxyhemoglobin with CO-saturated haptoglobin. Edelstein and Gibson (12) have found that deoxyhemoglobin has a dissociation constant of about 1 × 10^{-4} M under the conditions of the experiments, and thus would be at least 95% tetrameric. After ligand binding, the dissociation constant becomes about 1.5 × 10^{-6} M and much dimer will be expected to form. The approach to the new equilibrium is not instantaneous but requires a second, depending of course on haptoglobin concentration, for half-completion. Similar conclusions have recently been reached, from quite different evidence by Kellett and Gutfreund (25). If Hp binds indifferently to α and β chains, no lag will be expected; if the tetramer is incompetent, a lag of the order of a second will be expected, to allow for the formation of dimer and this, indeed, was observed (Fig. 1). Furthermore, the dissociation constant of 1.3 × 10^{-6} M obtained by fitting our data with a scheme in which only the hemoglobin dimer was competent to bind, is in excellent agreement with the value obtained by Edelstein and Gibson (12).

Further support to these considerations is given by the excellent agreement of previous and present experiments, when the first order rate constants are plotted against the α/β dimer concentration (utilizing a dissociation constant of 1.5 × 10^{-4} M) instead of the haptoglobin concentration. The result is a straight line (Fig. 3) from which a rate constant of 5.7 × 10^{4} M^{-1} sec^{-1} for the haptoglobin-hemoglobin dimer reaction can be calculated. This is in excellent agreement with the figure 5.5 × 10^{4} M^{-1} sec^{-1} obtained by curve fitting.

There remains to be explained the molecular basis of the incompetence of deoxyhemoglobin to bind Hp (12). Even if Hp dissociates less extensively at the concentrations used for these studies, this difference per se, is incapable of explaining the absence of binding to Hp when the reaction is followed for extensive periods. Only if deoxyhemoglobin is shown to be entirely, or almost entirely, incapable of dimerizing, can the lack of binding be attributed to unavailability of dimers. One attractive alternative is that the α/β dimer arising from deoxyhemoglobin is conformationally different from the oxyhemoglobin α/β dimer and incompetent to bind Hp. The existence of two distinct α/β dimers has been previously suggested (12).

In relation to the number, specificity, and interaction of the binding sites in haptoglobin, a number of conclusions can be drawn from the data presented here.

The experiments in which the reactivity of Hp previously mixed with half-saturating amounts of Hb A towards isolated chains was examined failed to indicate any difference between the form Hp α/β and free Hp (Fig. 6).

It seems to follow that although the haptoglobin molecule is bivalent towards the hemoglobin dimer, the site or sites that react with each dimer are independent and noninteracting.

The reaction may be written formally as

\[
\frac{1}{2} \text{Hp} + \alpha \beta \rightarrow \frac{1}{2} \text{Hp} \alpha \beta
\]

with a rate constant of about 5.5 × 10^{4} M^{-1} sec^{-1}.

In the light of the results shown in Figs. 4 and 5, pertaining to the reaction of α chains and Hp and the reactivity of Hp half-
saturated by α chains towards β chains and Hb A, the following conclusions seem justified.

1. α Chains, in the range of concentration studied, are capable of saturating only half of the haptoglobin binding sites.

2. Once α chains have bound half of the haptoglobin sites, β-specific sites are created in the molecule which exhibit very high affinity toward isolated βHMβ chains.

3. When haptoglobin half-saturated with α chains is mixed with Hb A, a very slow reaction occurs. The time needed for this reaction suggests that it might involve previous dissociation of the tetramer to monomers (reputedly a slow process) and subsequent binding of the liberated β chains to the β-specific binding sites.

We can conclude then, on the basis of the data presented here, that the haptoglobin molecule probably contains four binding sites, two for each hemoglobin dimer. These two pairs of sites are independent and noninteracting. Within each pair nevertheless, a strong interaction is observed. Initially only a single α specific binding site orients upon saturation of this site a β-specific binding site.

In the absence of isolated chains the sequence of events may be as follows. The haptoglobin molecule binds an αβ dimer in its α-specific binding site. Upon binding of this dimer a β-specific binding site is created. Then either the β chain forming part of the dimer dissociates (because of the conformational changes induced in the α chains by the binding to haptoglobin) and is then free to bind the β specific site; or the binding of the αβ dimer by the α portion to the α-specific site is followed by a rearrangement of this dimer (without actual dissociation) and the β chain then becomes capable of occupying the newly formed β-specific binding site. The second mechanism would imply that the α and β-binding sites are in reasonable proximity in the molecule. The data presently available do not serve to distinguish between the two possibilities.

It is probably of significance to notice certain general similarities between this proposed mechanism and the binding of antigens by immunoglobulins, particularly its bivalence. This is interesting in view of the homology recently postulated by Black and Dixon (26) between the light chains of the immunoglobulins and the αβ chains of haptoglobin.

Acknowledgments—We are greatly indebted to Dr. Helen M. Hanney for encouragement, assistance, and advice. The technical assistance of Seymour Garte is gratefully acknowledged.

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