Kinetics and Mechanism of Complex Formation between Hemoglobin and Haptoglobin*

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SUMMARY

The interaction between human hemoglobin and haptoglobin has been followed by measuring the quenching of haptoglobin fluorescence associated with complex formation. The effects of varying the concentration of the reactants suggest that hemoglobin tetramers do not react with haptoglobin, but that hemoglobin subunits are involved. Separated $\alpha$ chains of hemoglobin react with haptoglobin; although separated $\beta$ chains do not do so, they react rapidly with a previously incubated mixture of haptoglobin and $\alpha$ chains. It is suggested that the haptoglobin site binds $\alpha$ chains specifically, and that the normal reaction between hemoglobin and haptoglobin proceeds either by consecutive binding of $\alpha$ and $\beta$ monomers or by attachment of $\alpha\beta$ dimers through the $\alpha$ chain. The rate of the reaction is markedly dependent on conditions: the observed second order constant may reach $4 \times 10^8$ M$^{-1}$ sec$^{-1}$, and the true value is probably $< 7 \times 10^8$ M$^{-1}$ sec$^{-1}$.

The binding of hemoglobin by haptoglobin is believed to be stoichiometric and irreversible (1). The complex which is formed with the globin moiety of hemoglobin (2) probably involves the B chains of haptoglobin (3). Although the reaction is not species-specific, and human haptoglobin has been found to combine with a number of animal hemoglobins, two forms of human hemoglobin do not show significant binding: these are Hb$^\text{p}$, H (p4) and deoxyhemoglobin (4, 5).

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1 The abbreviations used are: Hb, hemoglobin; Hp, haptoglobin; $\alpha$ and $\beta$ chains refer to hemoglobin; A and B chains to haptoglobin. HMB, $p$-hydroxymercuribenzoate; hemoglobin treated with HMB is written Hb$^{\text{HMB}}$; and chains are similarly distinguished, e.g. $\alpha^{\text{HMB}}$ for $\alpha$ chains derived from hemoglobin treated with HMB.
The solutions of $\alpha^\text{HMB}$ and $\beta^\text{HMB}$ were then dialyzed against 0.1 M phosphate buffer, pH 7.0. The method of Ranney, Briehl, and Jacobs (15) was also used to prepare $\alpha$ chains free from mercury. Hemoglobin tetramers prepared from $\alpha$ chains with free $-\text{SH}$ groups and $\beta$ chains with $-\text{SH}$ groups blocked by HMB, ($\alpha_2\beta_2^\text{HMB}$) were obtained by incubating these chains together with $\beta^\text{HMB}$ chains obtained by the method of Bucci and Fronticelli (13) described above. This tetramer was subsequently isolated by starch block electrophoresis (14).

Tetramers containing $\alpha$ chains blocked by HMB and $\beta$ chains with the $-\text{SH}$ groups free ($\alpha_2^\text{HMB} \beta_2$) were obtained by incubating $\alpha^\text{HMB}$ chains prepared by the Bucci and Fronticelli method and Hb H ($\beta_4$). This tetramer was also isolated by starch block electrophoresis.

**Fluorescence Studies**—Static fluorescent measurements were performed in an Aminco-Bowman spectrophotometer with an X-Y recorder attached. A 10-mm cell was used. No corrections were made for the spectral response characteristic of the photomultiplier, but corrections were applied to allow for the change in excitation energy with wave length.

**Kinetic Studies**—Stopped flow kinetic measurements were made with the apparatus of Gibson and Milnes (16), with a fluorescence cell of the type described by Gibson et al. (17) attached. Excitation at 287 m$\mu$ was obtained with a direct current xenon arc and a Bausch and Lomb 250-mm grating monochromator. Stray light was removed with a 2-mm Corning glass filter No. 7-54. Protein fluorescence was observed with an EMI photomultiplier type 9525-B and a Corning glass filter No. 7-60.

**Electrophoresis**—Vertical starch gel electrophoresis was performed according to Smithies (18) and starch block electrophoresis following the method of Kunkel et al. (14) with minor modifications.

## RESULTS

### Static Titration

The fluorescence spectra of haptoglobin showed a maximum of excitation at 287 m$\mu$ and maximum emission at 350 m$\mu$.
Fig. 1 gives the results of the titration of 2 ml of a $7 \times 10^{-7} M$ (in terms of hemoglobin-binding capacity) haptoglobin solution by a $3.1 \times 10^{-4} M$ hemoglobin solution (calculated as tetramer). The same haptoglobin solution was titrated also with $\beta_{HMB}$ chains (which by electrophoretic methods do not bind haptoglobin) to estimate the contribution of optical screening to the titration curve. The difference between the two curves is inserted at the bottom of the figure and shows that the amount of hemoglobin required to give maximal quenching of haptoglobin was 0.040 ml, a figure that compares well with the 0.045 ml expected for a reaction with 1:1 stoichiometry. The "quenching" due to optical screening by the $\beta_{HMB}$ chains is consistent with a rough estimate made by assuming that the fluorescence is concentrated in the center of the cell. In this case the exciting light and the fluorescent light will be diminished by the factors of $10^{-1d}$ and $10^{-fe}$, respectively, where $c_1$ is the absorption coefficient for the exciting light, and $c_2$ that for the fluorescent light; $c$ is the concentration and $d$ the distance from the center of the cell, in this case 0.5 cm. The expected optical screening would cause a loss of about 40% of the emitted light, which is in acceptable agreement with the experimental results.

**Reaction of Hb A with Haptoglobin**

1. Experiments were performed in which Hb A and haptoglobin were mixed in equivalent concentrations and both reactants systematically diluted. The results of one experiment covering a 32-fold range of concentration are given in Fig. 2, and Fig. 3 is a plot of the apparent second order reaction rate (initial rate) against concentration.

2. The concentration of haptoglobin was held constant and the concentration of hemoglobin was varied (Fig. 4, A and B). When the concentration of hemoglobin significantly exceeded that of haptoglobin, the course of the reaction became approximately first order. The observed first order rate did not increase as rapidly as did the hemoglobin concentration (Fig. 5).
FIG. 6. Combination of a 2.5 X 10^{-4} M (tetramer) Hb A solution with Hp 1:1 solutions of different concentrations, as measured by the quenching of fluorescence. X---X, [Hpb] 5 X 10^{-7} M; O---O, [Hp] 2.5 X 10^{-7} M; ▲---▲, [Hpb] 1.25 X 10^{-7} M; ■ ■ ■, [Hp] 6.10 X 10^{-8} M; ● ● ●, [Hp] 3.0 X 10^{-8} M. Concentrations after mixing. Conditions as for Fig. 2.

Fig. 7. Combination of a 1.06 X 10^{-4} M (tetramer) solution of Hb A with a 1.06 X 10^{-4} M solution of Hp 1:1, in 0.1 M sodium phosphate buffer, pH 7.0 (O---O); in 0.1 M buffer with 2 M NaCl (O---O). Other conditions as for Fig. 2.

3. In another series of experiments the concentration of hemoglobin was held constant and haptoglobin concentration was varied. Fig. 6 illustrates the results of these experiments. The rate constant shows relatively little variation over the range examined; however, hemoglobin was always in considerable excess over haptoglobin.

4. The effect of temperature on the reaction of haptoglobin with hemoglobin was studied with [Hpb] = 1.2 X 10^{-7} M and [Hb] = 6 X 10^{-7} M, yielding a temperature coefficient (Q10) of 1.5 per 10°; with the low concentration of hemoglobin utilized, a substantial portion of the total hemoglobin would be expected to be dissociated.

5. The effect of changing the ligand bound to the hemoglobin was nil. Methemoglobin, carboxyhemoglobin, and oxyhemoglobin all reacted indistinguishably with haptoglobin under comparable conditions; (a) fixed haptoglobin concentration of 2.5 X 10^{-7} M following mixing, and (b) variable hemoglobin concentrations over a range similar to that in Fig. 4. Experiments with labeled hemoglobin have shown that deoxyhemoglobin does not bind haptoglobin, but the absence of binding of deoxyhemoglobin could not be checked by the fluorometric method because of technical difficulties. If dithionite were employed to remove O2, it would interfere with the fluorescence method, while without dithionite, if deoxygenation were not quite complete, the great increase of oxygen affinity on formation of the HpHb complex would cause scavenging of traces of O2 and invalidate the results. Further, as observed by Benerseh, MacDuff, and Benerseh (19) significant oxidation to methemoglobin might occur during the process of deoxygenation of the dilute solution, again invalidating the results.

6. The effect of 2 M salt was investigated, comparing the behavior of hemoglobin and haptoglobin diluted in 0.1 M phosphate buffer, pH 7.0, with the same solutions diluted in 2 M sodium chloride. The results, shown in Fig. 7, indicated that the binding rates were closely similar.

Reactivity of Isolated Hemoglobin Chains with Haptoglobin

Isolated chains of hemoglobin, prepared by the HMB method (13) and with —SH groups still blocked by HMB, were mixed with haptoglobin separately, together, and sequentially, according to the scheme of Table I, with the results shown in Fig. 8, A and B. The total fluorescence quenching brought about by the different combinations was significantly different. The reaction of a mixture of α and β chains with haptoglobin resulted in a total quenching which was 30% lower than that with Hb A in equivalent concentration. When α chains alone were used, a total quenching, which was about 25% less than with a mixture of α and β chains, was observed. On the other hand, with isolated β chains, only a very small quenching was noted, which can be interpreted as reflecting the inability of isolated β chains to bind haptoglobin. These results were in good agreement with electrophoretic data on the binding of βHMB chains and previous data on Hp H (βH). When β chains were added to haptoglobin mixed previously with α chains, the change in fluorescence was of smaller amplitude but progressed at a faster rate than that observed during the reaction of haptoglobin with α chains. Finally, when the reaction of α chains with a mixture of haptoglobin was nil. Methemoglobin, carboxyhemoglobin, and oxyhemoglobin all reacted indistinguishably with haptoglobin under comparable conditions; (a) fixed haptoglobin concentration of 2.5 X 10^{-7} M following mixing, and (b) variable hemoglobin concentrations over a range similar to that in Fig. 4. Experiments with labeled hemoglobin have shown that deoxyhemoglobin does not bind haptoglobin, but the absence of binding of deoxyhemoglobin could not be checked by the fluorometric method because of technical difficulties. If dithionite were employed to remove O2, it would interfere with the fluorescence method, while without dithionite, if deoxygenation were not quite complete, the great increase of oxygen affinity on formation of the HpHb complex would cause scavenging of traces of O2 and invalidate the results. Further, as observed by Benerseh, MacDuff, and Benerseh (19) significant oxidation to methemoglobin might occur during the process of deoxygenation of the dilute solution, again invalidating the results.

Table I

<table>
<thead>
<tr>
<th></th>
<th>1. Syringe 1</th>
<th>2. Syringe 2</th>
<th>3. Observed fluorescence quenching</th>
</tr>
</thead>
<tbody>
<tr>
<td>α (2 eq) + β (2 eq)</td>
<td>Hp (1 eq)</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>α (4 eq)</td>
<td>Hp (1 eq)</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>β (4 eq)</td>
<td>Hp (1 eq)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>α (2 eq)</td>
<td>Hp (1 eq) + β (2 eq)</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>β (2 eq)</td>
<td>Hp (1 eq) + α (2 eq)</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

The concentrations of the reagents and the other conditions of the experiments were the same as those given in the legend to Fig. 8. Column 3 gives the change in fluorescence upon mixing the reagents specified in Columns 1 and 2 in arbitrary units.
Kinetics of Hemoglobin-Haptoglobin Reaction

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Fig. 8. A (left), combination of isolated hemoglobin polypeptide chains with Hp 1-1. Haptoglobin concentration constant = 1 \times 10^{-4} \text{ M} after mixing. O---O, combination of previously mixed \( \alpha^{\text{HMB}} + \beta^{\text{HMB}} \) chains (total concentration = 1 \times 10^{-6} \text{ M}; 4 hemes) with haptoglobin. \( \Delta \longrightarrow \Delta \), combination of \( \alpha^{\text{HMB}} \) chains (1 \times 10^{-4} \text{ M}; 4 hemes) with Hp 1-1 (1 \times 10^{-8} \text{ M}). Temperature, 20°; solution in 0.1 M potassium phosphate buffer, pH 7.0. B (right), combination of isolated haptoglobin polypeptide chains with Hp 1-1. O---O, combination of previously mixed \( \alpha^{\text{HMB}} + \beta^{\text{HMB}} \) chains (total concentration = 1 \times 10^{-6} \text{ M}; 4 hemes) with Hp 1-1. \( \bullet --- \bullet \), combination rate of previously mixed Hp 1-1 (1 \times 10^{-4} \text{ M}) and \( \beta^{\text{HMB}} \) chains (0.5 \times 10^{-5} \text{ M}; 4 hemes) with \( \alpha^{\text{HMB}} \) chains (0.5 \times 10^{-6} \text{ M}; 4 hemes). Conditions as for A.

globin and \( \beta \) chains was followed, the quenching was of about the same extent as that observed on mixing haptoglobin with premixed \( \alpha \) and \( \beta \) chains, but developed somewhat less rapidly (Fig. 8B).

Electrophoretic Studies

Fig. 9 shows the starch gel electrophoresis of mixtures of Hp 1-1 with \( \alpha^{\text{HMB}}, \beta^{\text{HMB}}, \alpha^{\text{HMB}} + \beta^{\text{HMB}}, \) and Hb A. No significant binding of \( \beta^{\text{HMB}} \) chains can be demonstrated. There is partial binding of \( \alpha^{\text{HMB}} \) chains, but the haptoglobin does not become saturated even in the presence of a large excess of \( \alpha^{\text{HMB}} \) chains. A mixture of \( \alpha^{\text{HMB}} + \beta^{\text{HMB}} \) chains does bind Hp 1-1 to the same extent as does Hb A. It should be pointed out that such a mixture does not reconstitute Hb A in the absence of haptoglobin, as judged from starch gel electrophoresis. It may be noted that the HpHb complex formed with HMB chains has a slightly faster migration rate on electrophoresis than the complex formed with Hp A, probably due to the negative charges added by the \( p \)-hydroxymercurobenzoate. Fig. 10 shows the binding of \( \alpha_2\beta_2^{\text{HMB}} \) with Hp 2-1. The binding of this hybrid tetramer is qualitatively comparable to the binding of Hb A by Hp 1-1. The same result was obtained with \( \alpha_2^{\text{HMB}}\beta_2 \).

DISCUSSION

Stoichiometrically the binding of hemoglobin to Hp 1-1 appears to be the reaction between 1 molecule of Hp 1-1 (mol wt 80,000) and 1 molecule of hemoglobin (mol wt 64,500) (1).

Qualitatively, the results of the experiments presented suggest that the reaction between haptoglobin and hemoglobin may proceed via a hemoglobin subunit. Thus, the rate of reaction does not increase in a linear manner with hemoglobin concentration and becomes relatively slower at higher hemoglobin concentrations (Fig. 5). A similar conclusion may be drawn from the results of Fig. 3, where, if initial rates alone are considered, the apparent second order rate constant decreases 5-fold as the hemoglobin concentration is increased 32-fold. These results suggest that the reaction of haptoglobin with hemoglobin may involve either the dimer or proceed via isolated hemoglobin chains.

The possibility that isolated polypeptide chains may be involved in the reaction under study was tested in the series of experiments summarized in Fig. 8, A and B, and Table 1. These results may be explained if it is supposed that \( \alpha \) chains alone interact with haptoglobin, whereas \( \beta \) chains do so only to a very limited extent. Once binding of \( \alpha \) chains has occurred, however, rapid binding of \( \beta \) chains can follow, as shown by the experiment in which \( \beta \) chains were added to a previously incubated mixture of haptoglobin plus \( \alpha \) chains. In these experiments there was an initial rapid increase in fluorescence quenching when the \( \beta \) chains were added.

When haptoglobin was first incubated with \( \beta \) chains, the reaction which accompanied the subsequent addition of \( \alpha \) chains exhibited a rate similar to that observed when \( \alpha \) chains alone were added; this suggests that under these circumstances the isolated \( \alpha \) chains may react first and the \( \beta \) chains follow.

The addition of haptoglobin to a mixture of \( \alpha \) and \( \beta \) chains gave a biphasic reaction with an initially somewhat faster rate than that obtained on addition of \( \alpha \) chains to haptoglobin previously incubated with \( \beta \) chains. These differences suggest an interaction between the \( \alpha \) and \( \beta \) chains during prior incubation, giving rise to a form, perhaps the \( \alpha\beta \) dimer, which in turn gives rise to quenching faster than the \( \alpha \) chains alone (Fig. 8B).

The kinetic results are in general agreement with previous and
present electrophoretic data. They are consistent with the slight binding of haptoglobin to Hb H and \( \beta^{HM} \) chains. Early experiments (4) with \( \alpha \) chains prepared in dilute solution (without HMB) did not clearly demonstrate binding between \( \alpha \) chains and haptoglobin. However, when \( \alpha \) chains were prepared in a more concentrated solution (by the HMB method), limited binding with haptoglobin was observed electrophoretically (Fig. 10). Also Chiancone et al. (20) have observed the binding of \( \alpha \) chains with haptoglobin, with the use of ultracentrifuge analysis to follow the reaction.

The above experiments appear to be consistent with a stepwise reaction of the type

\[
Hp + \alpha^A \rightarrow Hp \alpha^A
\]

\[
Hp \alpha^A + \beta^A \rightarrow Hp \alpha^A \beta^A
\]

**Fig. 9.** Vertical starch gel electrophoresis of mixture of Hp with \( \alpha^{HM} \), \( \beta^{HM} \), \( \alpha^{HM} + \beta^{HM} \), and Hb A. One-half of the gel (A) was stained with benzidine and the other half (B) with Amido black 10B. 1A, Hp (1 eq) + \( \beta^{HM} \) (2 eq); 2A, Hp (1 eq) + \( \alpha^{HM} \) (1 eq) + \( \beta^{HM} \) (1 eq); 3A, Hp (1 eq) + Hb A (1 eq); 4A, Hp (1 eq) + \( \alpha^{HM} \) (0.25 eq); 5A, Hp (1 eq) + \( \alpha^{HM} \) (0.5 eq); 6A, Hp (1 eq) + \( \alpha^{HM} \) (1 eq); 7A, Hp (1 eq) + \( \alpha^{HM} \) (2 eq); 8B, Hp. The different bands can be identified as: a, \( \alpha^{HM} \); b, \( \beta^{HM} \); c, HP; d, impurities of the haptoglobin tetramer or subunit; e, Hb; f, impurities of the haptoglobin separation in \( 5H \) and \( 8H \). In 1A and 2A superimposition of this band with one of the isomeric forms of \( \beta^{HM} \); e, \( \beta^{HM} \).

**Fig. 10.** Vertical starch gel electrophoresis, pH 8.6, of mixture of Tris-borate buffer. Hp 2-1 with \( \alpha^A \beta^{HM} \) and \( \alpha^A \beta^A \). 1, Hb A; 2, Hp 2-1 + Hb A; 3, Hp 2-1 + \( \alpha^A \beta^{HM} \); 4, \( \alpha^A \beta^{HM} \).

This reaction could be repeated to form the final complex \( Hp \alpha^A \beta^A \). An alternative scheme, which does not exclude the previous mechanism, may be written as follows:

\[
Hp + \alpha^A \beta^A \rightarrow Hp \alpha^A \beta^A
\]

\[
Hp \alpha^A \beta^A + \alpha^A \beta^A \rightarrow Hp \alpha^A \beta^A \alpha^A \beta^A
\]

The second scheme would be in accordance with the finding that reactants in 2 M salt exhibit rates of binding identical with the rate in solutions of low ionic strength. In 2 M salt, as has been shown (21), Hb A exists largely in the form of \( \alpha \beta \) dimers.

Either of these mechanisms could explain the presence of two bands of HbHp 1-1 on starch gel electrophoresis when amounts of hemoglobin insufficient to saturate the haptoglobin are used. Under these conditions Laurieff (22) observed, besides the usual band, a faster band which disappeared when saturating amounts or excess of hemoglobin was added. Due to the sieving effects of the starch gel, it is possible that the fast moving band could correspond to Hp \( \alpha^A \beta^A \) and the slower band to Hp \( \alpha^A \beta^A \alpha^A \beta^A \).

A point could be raised in relationship to the validity of using HMB chains instead of hemoglobin chains with the ---SH group free. The results in Fig. 10 indicate no gross difference in binding when a hemoglobin tetramer is used with the ---SH group of its \( \beta \) chains blocked by HMB. The reverse is also true, i.e. no difference can be observed in binding when the cysteine \( \alpha -104 \) is blocked. This agrees well with previous data (22, 23) which indicated no participation of the hemoglobin ---SH groups in the binding. It seems that the behavior of HMB chains is very similar to that of native hemoglobin polypeptide chains.

Several lines of evidence which have already been mentioned point to the composition of the final complex as Hp \( \alpha^A \beta^A \); the experiments described here do not yield information on this point. If this stoichiometry is accepted, however, since haptoglobin contains two \( \beta \) chains to which binding could occur (3), perhaps the simplest formulation of the reaction may be achieved by assuming two similar independent binding sites, so that the final complex would be written

\[
\beta^A \alpha^A \beta^A \alpha^A \beta^A
\]

where \( \alpha^A \) and \( \beta^A \) refer to the polypeptide chains of normal adult.
hemoglobin, and A and B represent the polypeptide chains of haptoglobin referred to by other authors as $\alpha_1^H$ and $\beta_1^H$. In this model, binding of haptoglobin and hemoglobin could occur directly via $\alpha_2$ dimers, or $\beta$ chains could bind to $\alpha$ chains which had already been bound by the haptoglobin molecule. The static titrations show a linear relation between the amount of hemoglobin added and the extent of quenching. This result is consistent with several possible mechanisms, but appears to exclude the case of random binding with the full quenching effect due to the first chain bound, since the quenching effect would not then be proportional to the amount of hemoglobin added.

The results reported here afford indirect evidence supporting the idea that quite extensive dissociation of hemoglobin occurs in dilute solution (24). Although the number of variables is too great to encourage systematic treatment of the results, it does seem that there is a change in the behavior of the hemoglobin in the concentration range expected (1 to 10 $\mu$m in heme) from the work cited above.

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