Interaction of Human Hemoglobin with Haptoglobin or Antihemoglobin Antibody*

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SUMMARY

The physicochemical and biochemical properties of hemoglobin associated with haptoglobin were compared with those of hemoglobin bound by antihemoglobin antibody. The mechanism of enhanced peroxidase activity of hemoglobin bound by haptoglobin was concluded not to be activation but stabilization of hemoglobin at acidic pH by haptoglobin. Haptoglobin protects hemoglobin from denaturation by acid, and moreover it can regenerate denatured hemoglobin at acidic pH. Specific antibody, on the other hand, does not enhance the peroxidase activity of hemoglobin, nor does it prevent the acid denaturation of hemoglobin. Hydrogen peroxide-peroxidase complex (Compound I), which has not yet been detected in the H2O2-hemoglobin system, was observed in the H2O2-hemoglobin-haptoglobin complex system, indicating that haptoglobin stabilized the H2O2-hemoglobin complex. Hemoglobin bound by antibody shows a higher affinity for oxygen than free hemoglobin (P50 = 2.0 mm Hg at pH 7.0, and P50 = 3.0 mm Hg at pH 7.4), a biphasic Hill plot, and slight preservation of heme-heme interaction (n = 1.6 at y/1 - y > 1.5, and n = 1.0 at y/1 - y < 1.5) and somewhat reduced Bohr effect (r = -0.37). These characteristic functions of hemoglobin bound by antibody are in striking contrast to those of hemoglobin bound by haptoglobin. Hemes of hemoglobin-antibody complex are not degraded by dithionite under aerobic conditions, whereas those of hemoglobin-haptoglobin complex are degraded, being consistent with the view that hemoglobin bound by antibody is tetrameric, whereas hemoglobin bound by haptoglobin is dissociated.

The binding site of hemoglobin for haptoglobin was investigated through immunological experiments. From the data on the immune precipitation reaction of antihemoglobin serum in gels with free hemoglobin, hemoglobin-haptoglobin (human) complex, and hemoglobin-haptoglobin (rabbit) complex, it was apparent that the antigenic determinants of hemoglobin were not modified nor masked and moreover new antigenic determinants of hemoglobin did not appear on complex formation with haptoglobin. These observations suggest that the binding site of hemoglobin for haptoglobin is quite different from that for antihemoglobin antibody.

The mechanism of enhanced peroxidase activity of Hb that follow complex formation with other macromolecules, such as haptoglobin or antihemoglobin antibody, have added to an understanding of protein surface-surface interactions as well as to clarification of the structure and function of Hb. Although similarities in structure between Hb and immunoglobulin G are known (1), many differences in immunological reactions, binding sites, and binding mechanisms have also been described (2). Because Hb is considered as a kind of natural antibody to Hb (1) and does not precipitate when it forms a stable complex with Hb (2), Hb may be one of the most useful tools to investigate protein-protein interactions. In this connection, the physicochemical and biochemical properties of Hb-Hp complex and soluble Hb-Ab complex (Hb/Ab = 2/1) were examined.

The mechanism of enhanced peroxidase activity of Hb associated with Hp, by which Hp was originally discovered (3), was investigated, and then the Hb-Hp interaction and Hb-Ab interaction were compared. The enhanced peroxidase activity of Hb associated with Hp is not due to activation but to stabilization of Hb by Hp. Hp prevents the acid denaturation of Hb and regenerates denatured Hb at acidic pH. Ab, on the other hand, does not protect Hb from denaturation by acid and has no noteworthy effects on the peroxidase activity of Hb. Hydrogen peroxide-peroxidase complex (Compound I) which is not seen in the H2O2-Hb system (4) does exist in the H2O2-Hb-Hp complex system, indicating that Hp stabilizes the H2O2-Hb compound. The oxygen equilibrium of Hb bound by antibody was investigated and its behavior was in striking contrast to that of Hb bound by Hp. Further, the reactivity of the hemes of the Hb-Ab complex

* The abbreviations used are: Hb, hemoglobin; Hp, haptoglobin; Ab, antihemoglobin antibody; IgG, immunoglobulin G.

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to dithionite was examined and compared with those of the Hb-Hp complex on the basis of their structures. The binding sites of Hb for Hp and for Ab are not fully understood, although there is considerable information about them (2, 5, 6). The binding site of Hb for Hp was investigated through immunological experiments. In order to determine whether or not the binding site of Hb for Hp is different from that for Ab, the Ouchterlony double immunodiffusion method (7) was used to investigate the possibility that the antigenic determinants of Hb may or may not be modified by complex formation with Hp. Moreover, the possibility of the appearance of new antigens in Hb associated with Hp was examined by comparing antisera against Hb, Hb-Hp (human) complex (Hb-Hp\textsuperscript{human} complex), and Hb-Hp (rabbit) complex (Hb-Hp\textsuperscript{rabbit} complex).

Some of the data presented here have appeared in preliminary communications (8–11).

**EXPERIMENTAL PROCEDURE**

Preparation procedures for human adult Hb (Hb A), Hb-Hp 1-1, 2-1, and 2-2 complexes were described previously (2), and were modified so that the hemolysate was first filtered through Sephadex G-100 equilibrated with 0.01 M sodium phosphate buffer, pH 8.4, in order to exclude the catalase present in the hemolysate. Purification of Hb A-Hp\textsuperscript{rabbit} complex was carried out in the same way.

Antisera against Hb A were obtained from immunized rabbits by the method described by Sasazuki (2), and specific antibody (Ab) against Hb A was separated from the serum proteins using an immunoadsorbent. Soluble Hb-Ab complex was separated from excess free Hb on a Sepharose 6B column. Details of all these procedures have been described elsewhere (2). Antisera against Hb A-Hp\textsuperscript{human} complex or Hb A-Hp\textsuperscript{rabbit} complex were obtained from the immunized rabbits in the same way.

Bovine blood was obtained from a slaughterhouse and bovine hemolysate was prepared by the method of Drabkin (12). A 1% solution of oxygenated Hb was oxidized to ferric Hb (Hb\textsuperscript{ferric}) with 5 eq per tetramer of dissolved ferriyanide followed by gel filtration through a Sephadex G-25 column (2.5 x 25 cm) equilibrated with 0.05 M sodium chloride solution. Hb-Ab complex, bovine Hb, bovine Hb-Hp complex, and Hb-Ab complex were oxidized in the same way.

Absorption spectra of Hb, Hb-Ab complex, and Hb-Hp complex were measured using a Hitachi recording spectrophotometer model EPS-3T.

Denaturation experiments, followed spectrophotometrically, were performed as described. Two milliliters each of 0.1 mM (as heme) Hb\textsuperscript{ferric}, Hb\textsuperscript{ferric}-Hb complex or Hb-Ab complex were obtained from the hemolysate by the same procedures (16). Absorption spectra of Hb, Hb-Hp complex, and Hb-Ab complex were measured after each experiment and found to be negligible in Hb A and 19% in Hb A-Ab complex.

The Ouchterlony double immunodiffusion method (7) was used to investigate the possibility that the antigenic determinants of Hb A may or may not be modified by complex formation with both rabbit or human Hp.

**RESULTS**

Absorption Spectra of Hb, Hb-Hp, and Hb-Ab Complexes, and Effect of Dithionite on These Hemoproteins—Absorption spectra of Hb, Hb-Hp 2-1, and Hb-Ab complexes are shown in Fig. 1. Absorption spectra of the oxygenated forms of these three substances are quite similar both in the Soret and the visible regions. However, the millimolar extinction of deoxygenated Hb-Hp complex at its Soret peak is somewhat less than that of Hb as is that of Hb-Hp complex. Differences in absorption spectra of CO derivatives were not observed among these substances. These findings justify all the following comparisons using spectrophotometric measurements.

Deoxygenation with sodium dithionite in air does not cause degradation of hemes derived from either free Hb or Hb-Ab complex, whereas the hemes of Hb-Hp complex are degraded using the same procedures (16).

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Peroxidase Activities and Acid Denaturation of Hb\textsuperscript{ferric} and Effects Thereon of Hp and Ab—Fig. 2 shows the peroxidase activity of Hb\textsuperscript{ferric} and the effect thereon of Hp 2-1. Hb\textsuperscript{ferric} has peroxidase activity at acid pH, showing an optimum pH at 5.3. At pH 4.0, Hb\textsuperscript{ferric} has only slight peroxidase activity. On the other hand, the Hb-Hp 2-1 complex has its greatest activity at pH 4.0, which declines linearly from pH 4.0 to pH 6.0. In the region below pH 4.0, peroxidase activity of both the Hb\textsuperscript{ferric} and Hb-Hp complexes cannot be measured by this method because the final reaction product, tetrahydroxalochine, with which the enzyme activity was determined, is destroyed below pH 4.0. The measurement of the peroxidase activity below pH 4.0 with leucomalachite green as the substrate showed lower activity than at pH 4.0. In any case, it is noteworthy that Hb\textsuperscript{ferric}-Hp 2-1 complex shows the great-
PHb-Hp

Wavelength (nm)

PHb-Hp

Absorbance

0 2 4 6

Absorbance

Hb

Hb-Hp

ΔA 540 × 10⁻²

Time (minutes)

0 20 40 60

Fig. 2 (left). Peroxidase activity of Hb⁺ and effects thereon of Hp and Ab. Peroxidase activities of human Hb⁺ (□—□), human Hb⁺-Hp 2-1 complex (■—■), human Hb⁺-Ab complex (△—△), bovine Hb⁺ (○—○), and bovine Hb⁺-Hp complex (●—●) were measured by the method of Connell and Smithies (13), modified in that the reactions were carried out at room temperature (25 ± 0.5°C).

Fig. 3 (center). Final absorption spectra of Soret (A) and visible regions (B) for human Hb⁺ and human Hb⁺-Hp 2-1 complex.

Fig. 4 (right). Denaturation velocity of Hb⁺, Hb⁺-Hp 2-1 complex, and bovine Hb⁺ in citrate-phosphate buffer at pH 4.0. Two milliliters each of 0.1 mM (as heme) human Hb⁺ (□—□), human Hb⁺-Hp 2-1 complex (●—●), and bovine Hb⁺ (○—○) were mixed with an equal volume of citrate-phosphate buffer at pH 4.0, ionic strength 0.2, in a cuvette having a light path of 10 mm, and the acid denaturation was followed by measuring the increase in optical density at 540 nm at 25°C.

Fig. 5. Acid denaturation of Hb⁺ and effects thereon of Hp 2-1 and Ab. Percentage of denatured Hb⁺ in initial 5 s in citrate-phosphate buffer, ionic strength 0.2, 25°C, was measured spectrophotometrically at each pH as a decrease in the Soret band.

Hb⁺ to acid denaturation between Hp and Ab (Fig. 5): 50% of free Hb⁺ was denatured at pH 4.0. Fifty per cent of Hb⁺ associated with Hp is still native at pH 2.0 in the first 5 s at room temperature. Hb⁺ associated with Ab is not as resistant to acid denaturation as Hb⁺-Hp complex. At pH 3.4, over 50% of Hb⁺ associated with Ab was denatured.

Fig. 6 shows the effect of Hp on the denatured Hb at pH 4.5. Addition of Hp, adjusted to pH 4.5 previously, to the Hb which was denatured by acid at pH 4.5, regenerates the Hb from its denatured state. The decrease of the Soret peak at pH 4.5 is reversed and returned to its maximum intensity. The effect of additional Hp on the denaturation of Hb-Hp complex in the lower pH range was also examined. The denaturation velocity of the Hb⁺-Hp complex decreases when an increased amount of Hp is added into the reaction cuvette. When a 30-fold excess of Hp (as Hb-binding capacity (milligrams of hemoglobin with which 100 ml of haptoglobin solution can combine)) was added to Hb⁺-Hp complex at pH 2.8, no acid denaturation of the Hb⁺-Hp complex was observed.

Fig. 7A compares the absorption spectrum of the Hb⁺-Hp complex compared with those of the Hb⁺ and Hb⁺-Hp complexes. Ab does not enhance the peroxidase activity of Hb⁺ above pH 5.0; moreover, there is only slight enhancement below pH 4.5.

There were a large difference in the effect on the resistance of
Fig. 6. Effect of Hp 2-1 on the regeneration of Hb+ from acid denaturation. Regeneration of 5.5 μM (as heme) Hb+ from the acid denaturation at pH 4.5, 25°, was followed spectroscopically. The final spectrum of acid-denatured Hb+ in citrate-phosphate buffer, pH 4.5, and ionic strength 0.2, at 25°, was obtained in a cuvette having a 5-mm path, and then an equal volume of 1.5 μM Hp 2-1 solution, equivalent to Hb+, which was previously adjusted to pH 4.5 with the same buffer by gel filtration, was added to the acid-denatured Hb+. The regeneration spectra were obtained in a cuvette having a 10-mm path, at 10-min intervals after the addition of Hp.

Fig. 7. A, Soret absorption spectra of Hb7-Hp complex in the absence and presence of hydrogen peroxide in 0.05 M citrate-phosphate buffer, pH 5.3. Immediately after the addition of 0.3 ml of 50 μM hydrogen peroxide aqueous solution to 3.0 ml of 5.0 μM (as heme) Hb7-Hp complex in 0.05 M citrate-phosphate buffer, pH 5.3, the absorption spectrum was measured in the Soret region. B, thirty seconds after the addition of 0.3 ml of 50 μM hydrogen peroxide aqueous solution to 3.0 ml of 5.0 μM (as heme) Hb7-Hp complex in 0.05 M citrate-phosphate buffer, pH 5.3, 0.3 ml of 25 or 50 μM ferrocyanide solution was added and the changes in absorption spectra were measured at room temperature.

FIG. 8. Precipitin lines which were seen in the agarose plate by Ouchterlony double immunodiffusion technique between (A) anti-Hb A serum and three test antigens (Hb A, Hb A-Hpαα complex, and Hb A-Hpβα complex), between (B) anti-(Hb A-Hpαα) complex serum and three test antigens, 1, anti-Hb A serum; 2, anti-(Hb A-Hpαα) complex serum from which antibodies specific for human Hp were excluded; 3, anti-(Hb A-Hpβα) complex serum. a, Hb A; b, Hb A-Hpαα complex; c, Hb A-Hpβα complex.

Discussion

Studies on the interaction of Hb with other macromolecules, such as Hp and Ab, may yield some important information about protein surface-surface interaction.

Hp associated with Hb is reported to dissociate into αβ dimers (18-20); however, Hb associated with Ab (soluble Hb-Ab complex) is tetrameric (2), and this is further supported from the observation that the hemes of the Hb-Ab complex are stable against reduction with dithionite under aerobic conditions. Only the hemes of dissociated Hb are susceptible to dithionite.2 These two complexes, Hb-Hp and Hb-Ab complexes, are (αβ)2Hp-Ab and (αβ)2Ab-αβHb, respectively. So far, physio-

2 T. Sasazuki, unpublished observation.
The enzyme-like activity of Hb, and the effects thereon of Hp. The absorption spectra of oxygenated derivatives of the Hb-Ab and Hb-Hp complexes are quite similar. The peak extinction coefficient of the deoxygenated derivative of the Hb-Ab complex in the Soret region is lower than that of deoxygenated Hb, indicating lack of cooperativity, as mentioned later.

One of the outstanding changes observed in Hb when forming the complex with Hp was the enhancement of peroxidase activity. Since Polonovski and Jayle (3) discovered that Hp enhanced the peroxidase activity of Hb, other investigators have described this enzyme-like activity of Hb, and the effects thereon of Hp (13, 21–29).

In the case of human Hb, Hp actually seems to enhance the peroxidase activity at acidic pH, but not at neutral pH (Fig. 2). This suggests that there are greater conformational differences between the Hb-Hp complex and Hb alone at pH 4.0 than at neutral pH with respect to the environment of the hemes. The great difference in conformation between the Hb-Hp complex and Hb was clearly shown in Fig. 3. The protective effect of Hp against acid denaturation of Hb was described briefly by Roy et al. (30) in their simple method for the quantitative determination of serum Hp. Detailed studies of the acid denaturation of Hb were made by Steinhardt and his co-workers (17, 31–35). The acid denaturation of Hb+ is reported to involve the liberation of acid binding groups, especially the imidazoles of the histidine residues to be protonated (17). The molecular mechanism of the stabilization of Hb by Hp at acid pH is not known. However, this effect of Hp on the resistance of Hb+ to acid denaturation probably accounts for the mechanism of enhancement of peroxidase activity of Hb+, and is supported by the fact that bovine Hb+, an acid-stable Hb+, has the same high peroxidase activity as the Hb+/Hp 2–1 complex (Fig. 2), and that Hp does not enhance the peroxidase activity of bovine Hb+ (Fig. 2).

From the results obtained here, it is apparent that the generalized concept that Hp enhances peroxidase activity of Hb requires some modification. It should be said that Hp prevents denaturation of Hb+ by acid and by this effect Hb+ in the Hb+/Hb complex can maintain full or most of its peroxidase activity at acid pH. In pH regions where Hb+ does not undergo acidic denaturation or in the case of acid-stable Hb+, such as bovine Hb+, Hp does not enhance the peroxidase activity of Hb+. In other words, Hp only indirectly affects the peroxidase activity of Hb+ by its stabilization of Hb+ at acidic pH. Consequently, it would appear that the “enhanced” peroxidase activity of the Hb+/Hp complex is probably meaningless with reference to its activity in vivo.

The peroxidase activity of Hb+ has not been thought to be “true peroxidase activity” because Compound I, the H2O2-peroxidase complex, has not been detected yet in H2O2–Hb+ systems. In association with Hb, the peroxidase activity of Hb seems to be changed qualitatively as well as quantitatively. Hydrogen peroxide–Hb-Hp complex, Compound I, is detected in absorption spectra (Fig. 7A) and recovery of free enzyme, Hb+–Hp complex, is seen in Fig. 7B. These observations indicate that Hp stabilizes H2O2–Hb+ complex, as well as Hb+ in acid solution.

It is also important to elucidate the effect of specific antibody on the peroxidase activity of Hb+. Of particular interest are the structural similarities reported between Hp and IgG (1), and the finding that catalase is stabilized by specific antibody at alkaline pH (36). From Fig. 2, it appears that specific antibody against Hb has no noteworthy effect on the peroxidase activity of Hb+. From the assumption concerning the “true” effect of Hp on the peroxidase activity of Hb+, the effect of antibody on the acid denaturation of Hb+ might be even less apparent than that of Hp. The results shown in Fig. 5 seem to confirm this presumption. Stated in another way, it is curious that the similar, but larger protein (specific IgG) does not protect Hb+ from acid denaturation. A possible explanation for this phenomena might be that Hb-Hp complex does not dissociate into free Hb and free Hp over a wide pH range (37) and that antigen-antibody complex (Hb-Ab complex) easily dissociates into antigen and antibody below pH 4.0.

The effectiveness of Hp in the regeneration of Hb from acid denaturation and in the inhibition of acid denaturation of Hb associated with Hp at lower pH, however, suggests the following equilibrium:

$$Hb\cdot Hp\ complex \leftrightarrow free\ Hb + free\ Hp$$

From the data of Fig. 5, it is suggested that dissociation of Hb-Hp complex largely occurs at pH 2.0, although the dissociation of Hb-Hp complex into free Hb and free Hp in lower pH regions has not been described.

Oxygen equilibria for the Hb-Ab complex are somewhat unusual. The Hill plot for the Hb-Ab complex is characteristically biphasic. Reichlin et al. (20) have reported a biphasic Hill plot for Hb bound to papain-digested specific antibody (Hb/Fab < 1/4) explaining that the large proportion of oxidized Hb in their complex and a heterogeneous antibody population were responsible for this biphasic Hill plot. An example of Hb with a biphasic Hill plot was also reported for a human homolysate containing an abnormal Hb, Hb Bethesda (β-His 145) (38), or Hb Hiroshima (β-Asp 146) (39, 40); the mixture of normal Hb and high affinity Hb was considered to account for this phenomena. Also in our case, a heterogeneous antibody population might account for the biphasic Hill plot. The preservation of the Bohr effect and heme-heme interaction in the Hb-Ab complex shows a striking contrast to their absence in the Hb-Ip complex. Thus, the ingenious molecular mechanisms which enable Hb to transfer oxygen and which distinguish Hb from many other hemoproteins, are completely destroyed by complex formation with Hp, but are at least partially preserved in the Hb-Ab complex. The differences in environmental structure of the hemes between the Hb-Ab complex and the Hb-Hp complex suggested by Sasazuki and Isomoto (21) from data on circular dichroism spectra may have some bearing on these functional differences between the Hb-Ab complex and the Hb Hp complex. A dimeric structure of Hb in the Hb-Hp complex (18–20) and a tetrameric structure of Hb in the Hb-Ab complex (2) also explain the characteristic oxygen affinity of these two complexes, since it is reported that dimeric Hb does not have the cooperativity (41) of tetrameric Hb. A millimolar extinction coefficient for the Soret band in the Hb-Ab complex with a peak lower than that for deoxygenated Hb, observed here, which reflects little or no cooperativity in Hb, similar to that reported for Hb Bart’s (42) and some other hemoglobinins (43–47), is not inconsistent with the data reported here.

From the data on Ouchterlony double immunodiffusion, it was shown that antigenic determinants of Hb are not modified nor masked by the complex formation with Hp, although Hb bound to Hp dissociates to αβ dimers (18–20) and it was shown that the
conformation of Hb bound to Hp is changed as evidenced by circular dichroism spectra (21, 48-50) and absorption spectra (45). Further, there was no evidence for “the appearance of neoantigens” (51) in Hb on complex formation with Hp. Thus, Hp which causes great conformational changes in Hb does not have any effect on the antigenic determinants of Hb. In other words, the binding site of Hp for Hb is quite different from that for antibody. Further, it might be concluded that the binding site of Hb for Hp is that portion where there is no difference in amino acid sequence between human Hb and rabbit Hb. During the preparation of this manuscript, similar results were reported independently by Cohen-Dix et al. (52).

Further detailed investigations of the interaction between Hb and Hp or Ab, carried out in our laboratory, may throw further light on the important role of protein surface-surface interactions in biological function.

REFERENCES

44. Benesch, R., Gibson, Q. H., and Benesch, R. E. (1964) J. Biol. Chem. 239, PC1065-PC1069
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