186. Studies on Human Haptoglobins. IV

Peroxidase-like Activity and Oxygen-equilibria of the Intermediate and Saturated Forms of Hemoglobin-Haptoglobin 1-1 Complex

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Introduction. In the previous communications of this series,^{1),2)} two different hemoglobin-haptoglobin (Hb-Hp) 1-1 complexes were demonstrated on acrylamide gel electrophoresis of Hp partially saturated with Hb. From the physicochemical and immunological characterization of these complexes, the authors postulated that the intermediate and saturated forms of Hb-Hp 1-1 complex would be Hp 1-1 bound to an $\alpha\beta$ -subunit and two $\alpha\beta$ -subunits of Hb A, respectively.^{1),2)}

The present communication is concerned with the peroxidase-like activity and the oxygen-equilibria of these two complexes to gain further insight into the binding mechanism of Hb-Hp complex.

Materials and methods. *Purification of Hb-Hp 1-1 complexes.* The intermediate and saturated forms were isolated from crude Hp 1-1 undersaturated with Hb, and these preparations were ascertained to be homogeneous since each of them showed a single band on starch and acrylamide gel electrophoresis at alkaline pH, and gave a single symmetrical peak on the ultracentrifugal diagram as reported previously.^{1),2)}

The peroxidase-like activity of Hb-Hp 1-1 complexes. The peroxidase-like activity was determined using the guaiacol, hydrogen peroxide system according to the method of Connell and Smithies³⁾ with some modifications.⁴⁾ The reaction mixture for the standard estimation procedure consisted of (1) 5 ml of 0.03 M guaiacol used as electron donor in 0.1 M acetic acid adjusted to pH 4.0 with 1 M sodium hydroxide, (2) 0.5 ml of the intermediate or saturated form (5 mg/dl Hb concentration) converted to Met-Hb form with potassium ferricyanide, and (3) 1.0 ml of 0.05 M hydrogen peroxide used as oxidizing substrate prepared immediately before use.

The oxygen-equilibria of Hb-Hp 1-1 complexes. The continuous oxygen-equilibrium curves were recorded at 20°C automatically according to the method of Imai *et al.*⁵⁾ The intermediate and saturated

complexes in their oxygenated forms were dialysed against 0.1 M phosphate buffer pH 7.0 for 24 hours and adjusted to 0.01 per cent Hb concentration with the same buffer used for dialysis. The oxygen pressure in the samples was determined with a Beckman 39065 polarographic oxygen sensor and the per cent saturation of Hb with oxygen was measured spectrophotometrically at $430\text{ m}\mu$

Fig. 1. Time plot of the peroxidase-like activity of Hb-Hp 1-1 complexes and free Hb by the standard estimation procedure.

Results. *The peroxidase-like activity of Hb-Hp 1-1 complexes.* Fig. 1 gives a time plot of the peroxidase-like activity of the intermediate and saturated forms obtained by the standard estimation procedure. The curves of the intermediate and saturated form showed rapid and linear increases during the initial 3 minutes and reached their maxima within 7 minutes. The initial reaction rate and the maximum color development of these two complexes were approximately 10 and 20-fold higher than those of free Hb at identical heme concentration, but no significant difference was disclosed in the time course of the intermediate and saturated forms.

As shown in Fig. 2, pH of the reaction mixture was varied between 3.8 and 5.6. It will be noted that the optimum pH for free Hb is shifted to acidic in the presence of Hp, and pH optimum for the initial reaction rate and the maximum color development were 3.8 and 4.6, respectively, either for the intermediate form or for the saturated form.

The effect of hydrogen peroxide concentration on the peroxidase-like activities of Hb-Hp 1-1 complexes is given in Fig. 3. The initial reaction rate of both of the intermediate and saturated forms in-

Fig. 2. Effect of pH on the peroxidase-like activity of Hb-Hp 1-1 complexes and free Hb.

creased in a similar fashion with increasing concentration of hydrogen peroxide between 12.5 and 400 mM, and the maximum color development of the two complexes was slightly decreased at the higher concentration of peroxide. Fig. 4 shows the influence of guaiacol concentration on the peroxidase-like activity of Hb-Hp 1-1 complexes. The activity of the intermediate and saturated forms increased sharply up to 10 mM and then decreased gradually either in the initial reaction rate or in the maximum color development. In contrast, the activity of free Hb decreased sharply with increasing concentration of guaiacol over 2.5 mM.

The oxygen-equilibria of Hb-Hp 1-1 complexes. The oxygen dissociation curves of Hb-Hp 1-1 complexes and free Hb were studied under identical conditions. The absorption spectra of these samples were checked between 350 and 650 mμ before and after measurements using a Beckman DK-2 recording spectrophotometer. Methemoglobin formation during the measurement seemed negligible, judging from changes in the absorption spectra. The absorption maxima for these two complexes, deoxygenated by addition of sodium dithionite after the measurements, were 428–430 mμ in good agreement with that of free Hb.

The oxygen-equilibrium curves of these two complexes were markedly different from that of free Hb which showed a typical sigmoidicity. Fig. 5 shows the oxygen dissociation curves plotted according to the logarithmic form of Hill's equation. Both of the intermediate and saturated forms had a high affinity for oxygen even at low oxygen pressure and the curves of these two complexes closely resemble each other. The value for $P_{1/2}$ observed in free

Fig. 3. Effect of concentration of hydrogen peroxide on the peroxidase-like activity of Hb-Hp 1-1 complexes and free Hb.

Fig. 4. Effect of concentration of guaiacol on the peroxidase-like activity of Hb-Hp 1-1 complexes and free Hb.

Hb was 8.6 mmHg and the value of n was 2.8. In contrast, the values of $P_{1/2}$ were 1.2 mmHg for the intermediate form and 1.4 mmHg for the saturated form, and n values of the intermediate and saturated forms were calculated to be 1.1 and 1.2, respectively.

Discussion. Recently Waks *et al.*⁶⁾ have proposed that the addition of an excess of human Hp 1-1 to horse Hb leads to the mixture of three components; free Hp, a complex of 1 mole of Hp and one Hb dimer and a complex of 1 mole of Hp and four Hb chains. These data,

Fig. 5. Oxygen-equilibria of Hb-Hp 1-1 complexes and free Hb at pH 7.0, 20°C, plotted according to the Hill's equation.

considered together, are consistent with our previous postulation that the intermediate and saturated forms would be Hp 1-1 bound of an $\alpha\beta$ -subunit and two $\alpha\beta$ -subunits of Hb A, and symmetrical splitting (into $\alpha\beta$ -subunits) of Hb A would occur by complex formation.^{1),2)}

The present studies showed no difference in the time course of the peroxidase-like activity of the intermediate and saturated forms. Concerning the activation mechanism of the peroxidase-like activity of Hb, Waks and Alfsen⁷⁾ demonstrated that the maximum proton release during the association of Hp and Hb, which parallels the rise in the peroxidase-like activity, occurs at pH 4.5. Connell and Smithies³⁾ reported that Hp increases the peroxidase-like activity by protecting Hb from denaturation at low pH and peroxide. In the present studies, the optimum pH for both complexes was shifted to acidic pH and the effect of pH variation of the reaction mixture was almost identical in the intermediate and saturated forms. The inhibition of the peroxidase-like activity of Hb-Hp complex and free Hb is said to be highly dependent on the concentration of hydrogen peroxide or guaiacol.^{3),4)} Under the conditions studied here, the inhibition by various concentration of hydrogen peroxide or guaiacol was similar in these two complexes. These findings may be reasonably explained by the structural similarity of the Hb moiety within the intermediate and saturated forms.

Nagel *et al.*⁸⁾ found that human Hb-Hp 1-1 complex, corresponding to the saturated complex designated by the authors, has a high affinity for oxygen, the n value of approximately 1 indicating absence of heme-heme interaction, and they⁹⁾ postulated that in Hb-Hp 1-1 complex Hb can no longer undergo conformational change with ligand binding, and its structure is constrained in such a way that the heme groups can react only with a high ligand affinity.

The oxygen-equilibria of Hb-Hp 1-1 complexes obtained here

indicate that the intermediate complex also has a high affinity for oxygen, the n value of approximately 1 is almost identical with that of the saturated complex. These results may reflect the configurational changes of Hb moiety in these two complexes which make impossible the structural alteration underlying heme-heme interaction.

The functional changes, peroxidase-like activity and oxygen binding capacity, of the Hb moiety bound to Hp 1-1 demonstrated in the present studies are closely related to the configurational changes at heme-iron level induced by the binding of Hb to Hp. Therefore, it will be pertinent to consider that these results seem to explain adequately the similarity in the structural changes occurring in the Hb moiety of the intermediate and saturated complexes.

Summary. The peroxidase-like activity of the intermediate and saturated forms of human hemoglobin-haptoglobin 1-1 complex was markedly higher than that of free hemoglobin, but no difference was seen in the activity of these two complexes under the various experimental conditions studied. Both the intermediate and saturated forms have a high affinity for oxygen with values of n approximately 1 indicating the absence of heme-heme interaction.

From these findings and the data previously reported, it may be said that the configurational changes in the hemoglobin moiety induced by the association with haptoglobin 1-1 and thus the binding mechanism between haptoglobin 1-1 and hemoglobin closely related each other in the intermediate and saturated complexes.

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