134. Studies on Haptoglobin. V

Effects of Haptoglobin on Resistance and Regeneration of Hemoglobin to and from Acid Denaturation

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Introduction. In the preceding report,¹⁾ from the data of absorption spectra it was reported that haptoglobin (Hp) prevents the denaturation of hemoglobin (Hb) by acid, and that the enhanced peroxidase activity of Hb-Hp complex depends mainly on this preventing effect of Hp.

In the present studies, the effect of Hp on resistance of Hb to acid denaturation was further investigated and another effect of Hp on regeneration of Hb from acid denaturation was clarified. And further it was suggested that generalized concept that Hp enhances peroxidase activity of Hb needs some modifications in its details.

Materials and methods. Preparation procedures of human adult ferrihemoglobin (Hb^+A) and Hb^+A -Hp 2-2 complex were described previously.¹⁾

Heparinized ox blood were obtained in the slaughterhouse and ox Hb was prepared by the method of Drabkin.²⁾ And ox Hb was oxidized into ox Hb^+ by the same way described previously.¹⁾

Circular dichroism spectra measurements were performed at 25° using a Jouan Dichrograph II, the slit being controlled manually to maintain a good spectra resolution. The half-intensity bandwidths for the spectral region of 240–450 $\text{m}\mu$ and 450–610 $\text{m}\mu$ were 2–15 $\text{m}\mu$ and 6–10 $\text{m}\mu$, respectively. Molar ellipticities (θ) are given on a heme basis.

Denaturation experiments with spectrophotometer were performed as follows. Two ml of each 0.2% Hb^+A , Hb^+A -Hp 2-2 complex, and ox Hb^+ were mixed with the equal volume of citrate phosphate buffer at pH 4.0, ionic strength 0.65 in a cuvet having a light pass of 1 cm, and the acid denaturation was followed by measuring the increase in optical density at 540 $\text{m}\mu$ at 25°.

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Regeneration of Hb^+ from the acid denaturation at pH 4.5, 25° was followed spectrophotometrically. The final spectra of acid denatured Hb^+ was obtained in a cuvet having 5 mm pathway, and then equal volume of equivalent Hp 2-2 solution, which was previously adjusted to pH 4.5 by gelfiltration, was added to the acid denatured Hb^+ . The regeneration spectra were obtained in a cuvet having 10 mm pathway.

Peroxidase activities of Hb^+A , $\text{Hb}^+\text{A-Hp}$ 2-2 complex, ox Hb^+ , and ox $\text{Hb}^+\text{-Hp}$ complex were measured by the method of Smithies³⁾ modified in that reactions were carried out at room temperature ($25 \pm 0.5^\circ$).

Results and discussion. The final absorption spectra of acid denatured Hb^+ at pH 4.0 were already reported in the preceding report.¹⁾ The data obtained here by the CD measurements also clarify the effect of Hp on resistance of Hb^+ to acid denaturation.

Fig. 1. Visible CD spectra of Hb^+ and $\text{Hb}^+\text{-Hp}$ complex in citrate phosphate buffer, pH 4.0, ionic strength 0.325, at 25° .

Fig. 1 shows the visible CD spectra of free Hb^+A and $\text{Hb}^+\text{A-Hp}$ 2-2 complex in the citrate phosphate buffer at pH 4.0, ionic strength 0.325. CD spectra of Hb^+ at pH 4.0 are the spectra of acid denatured Hb^+ . Although at neutral pH marked differences in CD spectra between Hb^+ and $\text{Hb}^+\text{-Hp}$ 2-2 complex are not observed, great differences in CD spectra between them are obviously shown at pH 4.0.

Fig. 2. CD spectra in Soret and UV region of Hb^+ and $\text{Hb}^+\text{-Hp}$ complex in citrate phosphate buffer, pH 4.0, ionic strength 0.325, at 25° .

Fig. 3. An effect of Hp on renaturation of Hb^+ , in citrate phosphate buffer, pH 4.5, ionic strength 0.325, at 25° .

And in the Soret region main positive CD band seen in every derivatives of Hb at neutral pH disappears in the free Hb^+ at pH 4.0 suggesting the great changes in environmental structure around heme (Fig.2). On the contrary, even at pH 4.0 $\text{Hb}^+\text{-Hp}$ 2-2 complex shows almost similar CD band at Soret region as those at pH 6.0. The intensity of the positive CD band around 260 $\text{m}\mu$ declines in the free Hb^+ whereas that of $\text{Hb}^+\text{-Hp}$ 2-2 complex are not affected at acidic pH.

Fig. 4. Acid denaturation of Hb^+A , $\text{Hb}^+\text{A-Hb}$ complex, and ox Hb^+ , in citrate phosphate buffer, pH 4.0, ionic strength 0.325, at 25° .

Further, not only prevents denaturation of Hb^+ by acid, Hp acts as a regenerator in renaturation of Hb^+ from acid denaturation. When Hp solution was added to the acid denatured Hb^+ , renaturation of Hb^+ from acid denatured form was observed (Fig. 3). Hb^+ undergoes acid denaturation at pH 4.5, ionic strength 0.325 at 25° , showing the isosbestic points at 376 $m\mu$ and 419 $m\mu$. When Hp previously adjusted to pH 4.5 was added to this denatured Hb^+ , renaturation was observed in the absorption spectra pointing the same isosbestic points. Recently Makinen and Kon⁴) observed that the binding of Hp prevents denaturation of Hb by guaiacol. Relationship between resistance of Hb to denaturation by acid and resistance of Hb to denaturation by guaiacol are under study.

Effects of Hp on the peroxidase activity of Hb^+ were also further studied. Ox Hb^+ , which is highly resistant to acid denaturation just as human $\text{Hb}^+\text{-Hp}$ complex (Fig. 4), has higher peroxidase activity at acidic pH (Fig. 5). This linearly increasing activity of ox Hb^+ at acidic pH is as same as that of human $\text{Hb}^+\text{-Hp}$ complex (Fig. 5). And then the effects of Hp on this ox Hb^+ are interesting problems. In the case of ox Hb^+ , Hp rather decreases the peroxidase activity at all pH range so far examined (Fig. 6). These facts may suggest that Hp enhances the peroxidase activity by preventing the acid denaturation of Hb^+ at acidic pH, but that the peroxidase activity of acid stable Hb^+

Fig. 5. Peroxidase activity of Hb^+A , $\text{Hb}^+\text{A-Hp}$ complex, and ox Hb^+ .

just as ox Hb^+ is rather decreased by association with Hp. The similar phenomena are also observed in human Hb^+ , that is, peroxidase activities of free Hb^+ are rather higher than those of $\text{Hb}^+\text{-Hp}$ complex at neutral pH at which human Hb^+ are quite stable. Hb moieties of Hb-Hp complex are reported to dissociate to $\alpha\beta$ dimer⁵⁾ and many differences in environmental structure around heme between free Hb and Hb-Hp complex at neutral pH are reported and the higher peroxidase-activities of Hb-Hp complex are often thought to depend on these altered heme environments in the complex. But data obtained here are not consistent with those concepts. At neutral pH these altered heme environments in the complex are not the cause of enhanced peroxidase activity of $\text{Hb}^+\text{-Hp}$ complex but may be the cause of decreased activity of $\text{Hb}^+\text{-Hp}$ complex. And there seems to need some modification in the generalized concept that the Hp enhances the peroxidase activity of Hb. It should be said that Hp prevents the denaturation of Hb^+ by acid and by this effect $\text{Hb}^+\text{-Hp}$ complex can maintain a part of peroxidase activity of Hb^+ at acidic

Fig. 6. Peroxidase activity of ox Hb^+ , and effect thereupon of Hp.

pH, and in the regions Hb^+ does not undergo acidic denaturation or in the case of acid stable Hb^+ such as ox Hb^+ , Hp rather decreases the peroxidase activity of Hb^+ which Hb^+ primarily possesses.

Summary. The effect of Hp on resistance of Hb to acid denaturation is further confirmed by the CD spectra measurements and the CD spectra of acid denatured human Hb were presented.

Another effect of Hp on regeneration of Hb^+ from acid denaturation was also clarified spectrophotometrically.

And from the facts that Hp decreases the peroxidase activity of acid stable Hb^+ such as ox Hb^+ or decreases that of human Hb^+ in neutral pH region, some modifications are proposed against the generally accepted concept that Hp enhances the peroxidase activity of Hb.

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