THE HEMOGLOBIN BINDING AND ACTIVATION BY HAPTOGLOBINS : SPECTROPHOTOMETRIC AND POTENTIOMETRIC MEASUREMENTS

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Haptoglobins (Hp) are known to bind Hemoglobin (Hb). This binding implies a considerable increase of the peroxydase activity of Hemoglobin (Polonovski et Jayle 1938). The binding site of Hp on Hb is located on the globin moiety (Van Royen 1950, Nyman 1959), although the increased peroxydase activity of Hb is related to the configuration of the haem group.

In this study evidence has been obtained that the carbomonoxyhemoglobin-haptoglobin association is accompanied by :

1) Spectral modifications of HbCO in the visible range, which are related to the binding of HbCO with Hp : presence of charge transfer bands (CTB) characteristic of high spin derivatives (Brill and Williams 1961).

2) Release of protons H^+ measured during the association, which is closely related to the activating ability of Hp.

Material and methods : Pure Hp of genetic type 1-1 and 2-2 used in these studies was prepared as described elsewhere (Waks and Alfsen 1966). Horse carbomonoxyhemoglobin was prepared ac-

cording to Cann (1964). All reagent solutions were 0.3 M in KCl saturated with CO (99.98 %).

Difference spectra were recorded on a Bausch and Lomb Spectronic 505 recording spectrophotometer with a thermostated cell compartment ; some measurements were performed on a Cary 14. The wavelength range studied was from 270 to 700 mμ.

Potentiometric measurements were carried out according to the technique for determination of protein-protein association constants described in detail by Lebowitz and Laskowski (1962). The pH meter used was a model 33B Vibron Electrometer associated with the C 33B pH measurement unit (Electronic Instruments - Richmond - England), equipped with Leeds and Northrup miniature pH electrode assembly. The instrument was tested with revised Standard Values (Bates 1961). The sample, usually 2 ml, was placed in a thermostated microcell and HbCO was added by successive increments of 0.1 ml with a 2 ml Gilmont micrometer buret (R. Gilmont - Vineland - New Jersey). KOH 0.01 M was added with an Agla micrometer syringe outfit (Burroughs Welc. C° London).

Results : The spectral changes of HbCO in the visible range of the spectrum (fig. 1) are characterized by a new absorption band at about 500 mμ and a second band between 600 and 640 mμ. Furthermore, the intensity of the α and β bands (570 and 540 mμ) decreases with the Hp binding. The isobestic points at 473 mμ, 526 mμ and 592 mμ, support the evidence of a complex formation.

The spectral modifications appeared even when haptoglobins have lost their activating properties after heating at 40°C, for 30 min. pH 4.50 ; it must follow therefore that these

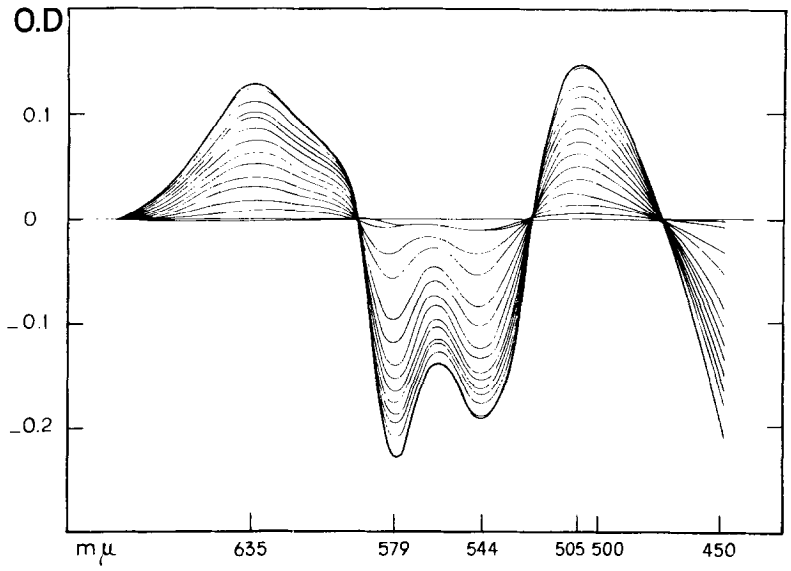

Fig. 1 Difference spectrum of HbCO - Hp_{1-1} : 0.3 M KCl is added in the reference cell and Hp in the sample cell, to HbCO by successive increments of 0.1 ml. The reaction is considered as complete when further additions of Hp do not produce any spectral change. Spectra of HbCO - Hp_{2-2} are identical.

spectral characteristics are related to the binding of Hp with Hb, as verified by polyacrylamide gel electrophoresis and ultracentrifuge experiments.

With a molecular weight chosen as 85,000 for Hp 1-1 and for Hp 2-2 (Waks and Alfsen 1966), 64,500 for Hb (Perutz 1965), the molar ratio Hb/Hp was 0.9 at pH 7.00.

The association of Hp with Hb is accompanied by a release of protons between pH 4.50 to 6.00. The maximum number of protons is released at pH 4.50, decreasing continuously until pH 6.00. The plot of the number of moles of H^+ liberated versus the number of moles of protein added is shown in fig.2.

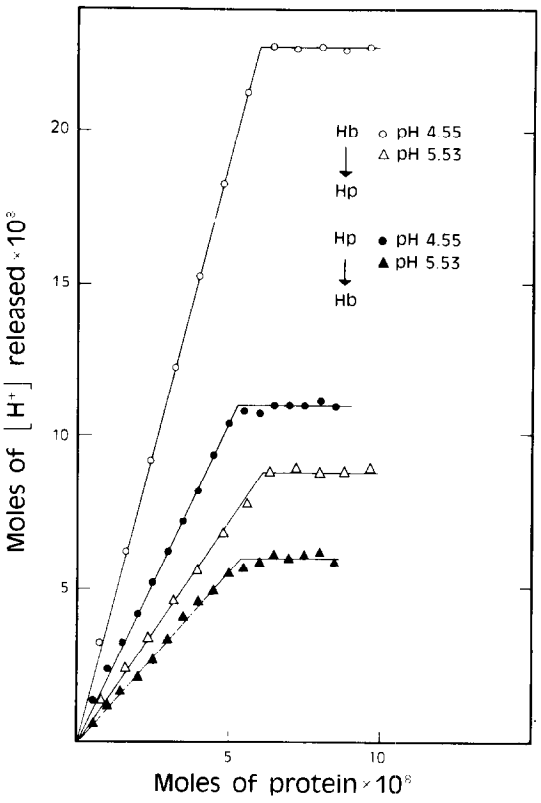

Fig. 2

The association of HbCO with Hp2-2
Vo = 2.10^{-3} liters
C_{Hp} = 5.10^{-5} moles/liter
mHbCO : 6.5 10^{-8} moles

The association of Hp 1-1 with HbCO
Vo 2.10^{-3} liters
C_{HbCO} 2.65 10^{-5} moles/liter
mHp 6.7 10^{-8} moles.

The number of H^+ released by addition of Hb to Hp is higher at a given pH than for Hp added to Hb. At any concentration of proteins the molar ratio Hb/Hp is about 0.80 for Hp 1-1 and 0.65 for Hp 2-2. This ratio is constant in the range of pH studied. The proton release is closely related to the activating properties of Hp measured by the method of Connell and Smithies (1959). After heating at 40°C, for 30 min. pH 4.50, Hp has lost its activating properties and no proton release is observed although the binding properties remained.

Discussion : Using Molecular Orbital and Ligand Field theory (George and al. 1961, Offenhartz 1965) to discuss the spectral modifications obtained, it is known that HbCO is a low spin

complex and the changes described above are characteristic of the formation of a certain amount of a high spin derivative. The carbomonoxyhemoglobin, low spin component, is not auto-oxydable, contrarily to the high spin derivatives (Williams 1961). The only addition of Hp does not lead to HbCO oxydation since, when the reaction is complete, further addition of Hp produces no more spectral change ; addition of Ferricyanide on the contrary transforms all the HbCO present into MetHb.

According to the extinction coefficients of pure high spin and pure low spin components (George and al. 1961), the final result of the association is a mixture of two different spin forms, which can be compared to the ferrihemoglobin hydroxides. The characteristics of the spectrum in U.V. and Soret region (to be published) are in agreement with these findings.

The protons released may be due either to the association of Hp to the globin moïety of Hb or to the ionization of some groups which are not located on the binding sites but may become accessible to the solvent by a conformational change of Hb induced by the association with Hp. In the conditions of potentiometric experiments described above, the dissociation equilibrium of Hb is known to be displaced towards the dimers. It is especially remarkable that the maximum of protons released during the association Hb-Hp occurs at pH 4.50 where the dimer form of Hb is predominant and the pH of the peroxydase activity of Hb-Hp is optimum. The difference between the molar ratio Hb/Hp 1-1 and Hb/Hp 2-2 is consistent with the hypothesis of a common subunit undergoing different association equilibrium

in Hp 1-1 and Hp 2-2. This equilibrium is probably involved in the higher number of H^+ released when Hb is added to Hp, instead of Hp to Hb.

It should be emphasized that all hemoproteins which react rapidly with peroxydes are high spin derivatives (Williams 1961), that the transformation of HbCO into a high spin component after the binding to Hp seems to be necessary but not sufficient to activate the HbCO peroxydase, and that the release of protons which is a proof of the HbCO activation is dependent on the Hp conformation.

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