A CONTRIBUTION TO THE STUDY OF TWO COMPLEXES OF HAEMOGLOBIN WITH HAPTOGLOBIN

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Two types of the haemoglobin–haptoglobin complex were prepared. From the results of gel chromatographic, polarographic, spectrophotometric and peroxidase activity measurements the authors concluded that the two complexes differ in their structure, which is discussed in this paper.

Haptoglobin (Hp) reacts specifically with haemoglobin (Hb) to form a stable complex[1], haemoglobin–haptoglobin (Hb–Hp). Earlier investigations of the stoichiometry and binding properties indicated that the interaction in the Hb–Hp complex is irreversible[2] and takes place at a stoichiometric ratio 1 : 1. On the other hand, Hamaguchi[3] and Kagiyama and coworkers[4] succeeded in isolating an intermediate complex where to every molecule of Hp there is one half of a Hb molecule. The problem of a half-complex was studied in detail by other authors[5,6] who found that the shape of the redox equilibrium curves of the Hb–Hp complex changes with the ratio of Hb : Hp. At molar ratio Hp : Hb 2 : 1 only one type of complex forms[6], while at the ratio Hp : Hb 1 : 1 many types of complexes are present in the reaction mixture. Nagel and Gibson[7] submitted the mechanism of Hb–Hp complexes formation to detailed study and they assume that subunits of Hb take part in the interaction with Hp. In their view, either Hb binds to Hp through $\alpha\beta$ dimers of Hb, or a β chain of Hb may bind on to an α chain of Hb already fixed on the molecule of Hp. Other authors[8,9] have already proved that both an isolated α chain and a β chain of Hb reacts with Hp. The affinity of the β chain to Hp is smaller than that of the α chain, however. Waks and coworkers[10] proved experimentally the existence of three different Hb–Hp complexes. Using potentiometry, low-angle X-ray diffraction, gel electrophoresis and measurements of the kinetics of peroxidase activity, these authors[10] found two different complexes of identical composition of Hb : Hp 1 : 1 and one complex with a 1 : 2 ratio of components Hb : Hp. The behaviour of these complexes depended in this case on the molar ratio of Hb and Hp present in the original reaction mixture.

In our previous paper we tried to explain the mechanism of the formation of the Hb with Hp II complex using peroxidase activity measurements, histidine determination, spectrophotometric titration and optical rotation dispersion titration[11]. We found that the structure of the Hb–Hp complex depends on the order in which the two reactants are mixed during preparation of the complex. On adding Hb in small portions to an excess of Hp a complex was formed which was labelled $Hb_2 \rightarrow Hp$. Reversed order of mixing of the reactants lead to complex $Hp \rightarrow Hb_2$. Following the balance of histidine content in both complexes gave grounds to assume that the complex $Hb_2 \rightarrow Hp$ probably forms in a reaction of four $\alpha\beta$ dimers of Hb with one Hp molecule, while the $Hp \rightarrow Hb_2$ complex is formed by two Hb tetramers joining one Hp. A more detailed

study of the interaction of Hb with Hp, using spectrophotometry and optical rotation dispersion, suggested that the formation of Hp–Hb complexes is a consecutive association process.

This paper should contribute to the clarification of some questions raised by Waks and coworkers[10] in their previous study of the Hp–Hb complex. They consider the most important problems concerning this complex to be the determination of the number of types of associations between Hb and Hp, the study of the character of the interaction in the complex and the following of the effect of the interaction of Hb with Hp on the functional behaviour of the heme group. In the present paper we study in detail the properties of the two complexes, $Hb_2 \rightarrow Hp$ and $Hp \rightarrow Hb_2$, prepared in a relatively great amount in pure state. Gel chromatography, spectrophotometry and measurements of peroxidase activity are used as a proof that the two complexes differ in their properties and therefore in their structure as well. Using these new results we try to confirm the structure of the complexes suggested in our previous paper[11].

EXPERIMENTAL

Isolation of two Hb–Hp *complexes.* Human Hp II was isolated from the Cohn fraction IV using a method devised in our laboratory[12]. Bovine Hb, a commercial product of Serva (Heidelberg) was in the oxyhaemoglobin form. Pure oxygen was passed through the solutions of this Hb before use. The $Hb_2 \rightarrow Hp$ complex (called K-I in this paper) was prepared by slowly adding a 0·4% solution of Hb to a 0·6% solution of Hp under constant stirring at room temperature. Both Hb and Hp were dissolved in Sörensen phosphate buffer, pH 7·0, I 0·15. The Hb solution was added in slight excess over the actual stoichiometric ratio Hb : Hp 2 : 1. The $Hp \rightarrow Hb_2$ complex (K-II) was prepared in an analogous way, only the order of mixing the stock solutions of reactants being reversed. Solutions of both complexes were dialysed and then freeze-dried. The complexes were finally purified from any small excess of either reactant by preparative column chromatography on Sephadex G-200 in Sörensen phosphate buffer. Solutions of pure complexes were then dialysed and freeze-dried.

Gel chromatography for analytical purposes was performed on a Sephadex G-150 column (1·5 . 28 cm) equilibrated with Sörensen phosphate buffer or with a phosphate buffer containing $2 . 10^{-4}$M sodium dodecyl sulphate (SDS). Flow-rate was regulated by a micropump attached to an automatic fraction collector. The protein content in the fractions was determined on a universal Zeiss spectrophotometer at 406 nm.

Reaction of the complexes with Zn^{2+} *ions.* Solutions of complexes K-I and K-II in acetate buffer pH 5·0M, 0·01 were prepared. These solutions, of protein concentration 0·33 mg/ml, were filtered using a Millipore filter (0·45 μ) before measurements. To 3 ml of solution of a complex in a 1 cm cuvette, 10 μl of 1M zinc acetate were added and the change in absorption in time was followed spectrophotometrically at 406 nm. To accelerate the reaction the cuvettes with solutions of complexes were illuminated by a 200 Watt bulb from a 50 cm distance.

Polarographic experiments were carried out on a PO 4 polarograph (Radiometer. Copenhagen). The capillary flow rate was 3·8 mg/s, drop time 3 s. The composition of the supporting electrolyte, Brdička cobaltic solution, was: 0·001M-$Co(NH_3)_6Cl_3$, 0·1M-NH_4Cl, 1M-NH_4OH. All experiments were carried out in Kalousek's polarographic vessel with a saturated calomel electrode as the

reference electrode. Each of the two complexes was first dissolved in 8M urea; after 30 mins a 0·5 ml sample was taken and added to 9·5 ml of Brdička solution, and then the polarographic curve was recorded. The height of the second catalytic maximum of the protein double wave was then plotted in the graph as catalytic current in time $t = 0$ (without reduction by β-mercaptoethanol). After that β-mercaptoethanol was added to the reaction mixture, up to a final concentration of 0·013M. Samples of 0·5 ml of the reaction mixture were taken at regular intervals and polarographed in 9·5 ml of Brdička solution. The heights of the second catalytic maxima were plotted in the graph against time.

Absorption and differential spectra of the complexes were measured on a universal Zeiss spectrophotometer, type VSU-1. Measurements were carried out in 1 cm cuvettes at 20°C. Concentration of the solutions of complexes for absorption measurements was 3 mg/ml. For measuring the differential spectra, concentrations of 3 mg/ml (350—440 nm) and 5 mg/ml (450—650 nm) were used. A solution of K-I complex was used as reference in this case. In all cases Sörensen phosphate buffer was used as solvent. In order to exclude scattering effects the solutions were filtered through Millipore filters (0·45 μl) before measurements.

Peroxidase activity measurements were performed according to Connell and Smithies[13]. Absorbance of tetraguaiacol at 470 nm, recorded one minute after addition of 1% H_2O_2, was used as a measure of relative peroxidase activity.

RESULTS

Gel chromatography of complexes K-I *and* K-II. Our previous experiments[14] have already shown that the Hp–Hb complex separates during gel chromatography when SDS is present. We therefore studied both our complexes, K-I and K-II, using gel chromatography on Sephadex G-150 equilibrated with phosphate buffer and con-

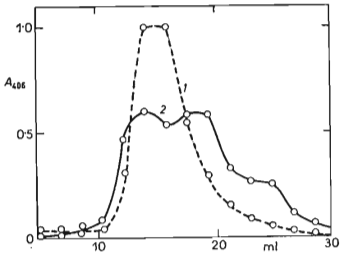


FIG 1

Gel Chromatography of Complexes K—I (1) and K—II (2) on Sephadex G—150
Equilibration with phosphate buffer pH 7, *I* 0·15, 2 . 10^{-4}M-SDS. Flow rate 7 ml/hour.

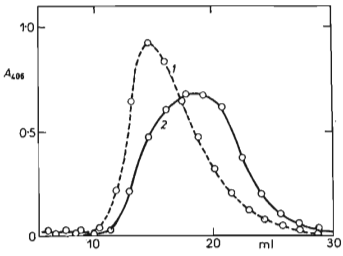


FIG. 2

Gel Chromatography of Complexes K—I (1) and K—II (2) on Sephadex G—150
Phosphate buffer pH 7, *I* 0·15. Flow rate 7 ml/hour.

taining SDS (Fig. 1). The complexes were also separated in pure phosphate buffer under otherwise identical conditions (Fig. 2). Figs 1 and 2 indicate that in both cases complex K-I behaved as a single component. Complex K-II in the presence of $2 \cdot 10^{-4}$M-SDS separated into three components each of which contained Hb. The elution velocity of the component with the smallest elution volume corresponded to that of complex K-I. In a medium devoid of SDS the K-II complex behaved as a single component, but compared to K-I, the elution maximum was broader and the elution volume larger.

Reaction of the complexes with Zn^{2+} *ions.* Cann[15] has already shown that Zn^{2+} ions affect Hb and myoglobin by reducing the intensity of the Soret band. The rate determining step in this case is the change of conformation caused by disruption of the bond between iron and imidazole residue where the Zn^{2+} ions bind instead. The rate of this comformation change depends[15] on the strength of the bond between iron and imidazole and is accelerated by light. In view of these facts we evaluated the properties of haemoglobins in complexes K-I and K-II. Solutions of the complexes in acetate buffer with Zn^{2+} ions were irradiated by white light and the change in absorption at 406 nm in time was recorded. While the absorbance of complex K-I increased during the experiment, the absorbance of complex K-II, after a brief increase, started decreasing similarly to that of Hb in reaction with Zn^{2+} ions (Fig. 3).

Reduction cleavage of the complexes K-I *and* K-II, *followed by polarography.* Complex K-II exhibited a marked increase of the catalytic polarographic wave after a very short time of reduction by β-mercaptoethanol compared with the polarographic effect of this complex without the reducing agent (Fig. 4). After further

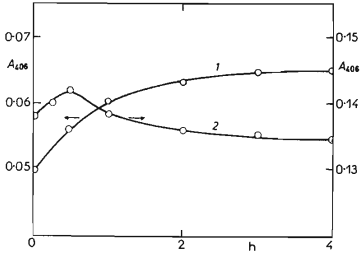


FIG. 3

The Effect of Zn^{2+} Ions on Complexes K—I (*1*) and K—II (*2*) as a Function of Time, Followed by Spectrophotometry

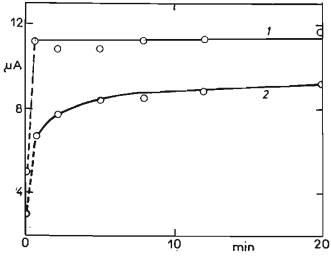


FIG. 4

The Time Dependence of Reduction Cleavage of Complexes K—I (*2*) and K—II (*1*) Followed by Polarography

TABLE I

Concentration Dependence of the Peroxidase Activity of Complexes K-I, K-II and the Dependence of their Peroxidase Activity on the Amount of Hp Added

Solution of complex[a] µl	A_{470} K-I	A_{470} K-II	Solution of Hp[b] µl	A_{470} K-I	A_{470} K-II
5	0·09	0·16	0	0·13	0·26
7·5	0·15	0·26	10	0·17	0·34
10	0·21	0·35	20	0·18	0·36
15	0·34	0·54	30	0·20	0·36
20	0·49	0·75	40	0·22	0·37
25	0·62	0·94	50	0·22	0·37

[a] Appropriate number of µl of 4% solution of a complex was mixed in the spectrophotometric cuvette with 3 ml of the guaiacol reagens. After adding 100 µl of 1% H_2O_2 the peroxidase activity was measured (A_{470}). [b] A 2% solution of Hp was added to 100 µl of 0·34% solution of the appropriate complex and the peroxidase activity was measured (A_{470}).

TABLE II

Properties of Complexes K-I and K-II

Method	Behaviour and properties	
	K-I	K-II
Gel chromatography	no splitting	splitting
Reaction with Zn^{2+} ions	haemoglobin properties lost	haemoglobin properties retained
Polarography	slow kinetics of reduction cleavage	very fast kinetics of reduction cleavage
Absorption spectrophotometry	Soret band with lower absorbance	Soret band with higher absorbance
Differential spectrophotometry	lower proportion of high-spin complex	higher proportion of high-spin complex
Peroxidase activity	lower activity	higher activity
Binding with excess of haptoglobin	two-step increase in peroxidase activity	one-step increase in peroxidase activity

reduction cleavage the catalytic effect of complex K-II remained practically unchanged. The catalytic effect of complex K-I, on the other hand, increased gradually, indicating that the reduction cleavage took place at a lower rate, possibly as the result of a more compact structure of the molecule.

Differential spectra. Differential spectra were recorded in the visible region where the heme group exhibits characteristic spectral behaviour. Fig. 5*a* shows the differential spectrum of complex K-II in the 350–440 nm wavelength range where there are two maxima, at 390 nm and 415 nm. The differential spectrum of complex K-II in the range 450–650 nm is given in Fig. 5*b*. In this spectrum there are three maxima, at 500, 540 and 625 nm. Comparison with the spectrum of oxyhaemoglobin indicates that the β band at 540 nm remained while the α band at 570 nm disappeared. The absorption band around 500 nm is typical for high-spin charge transfer complexes[16]. The band at 625 nm also appears in spectra of high-spin complexes[16,17].

Peroxidase activity of the complexes K-I *and* K-II. The increase of peroxidase activity in Hb after binding on to Hp is a very important characteristic of Hb molecule, widely used for analytical determinations[1] of Hp. Peroxidase activity can also be used for estimating the extent to which the native structure of the complex remains intact during a certain process[18]. In our case measurement of peroxidase activity was used to study the differences in structure of the two complexes. For these reasons, peroxidase activity of the complexes was measured after their reaction with pure Hp. In the range of concentrations studied, the relative peroxidase activity depends linearly on concentration, for both complexes. However, peroxidase activity of complex K-II is higher than that of K-I (Table I). The dependence of peroxidase

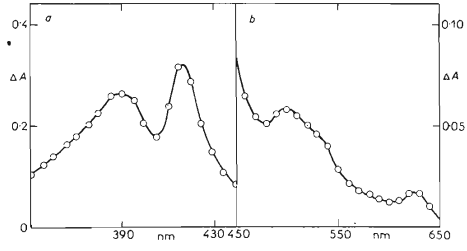


FIG. 5

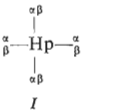
Differential Absorption Spectrum of Complex K–II in the Range of 350–440 nm (*a*) and 450–650 nm (*b*)

Solution of K-I complex was as reference sample.

activity in both complexes on the amount of Hp added is also given in Table I Again the two complexes differ in their behaviour. While in the case of complex K-II the peroxidase activity practically reached a limit after addition of 2 mol of Hp, in the case of K-I the peroxidase activity increased in two equally high steps, corresponding to 2 and 4 mol of Hp respectively. Table II gives a survey of the differing properties of complexes K-I and K-II.

DISCUSSION

In a previous paper[11], studying the accessibility of histidyl residues in a Hb–Hp complex, the hypothesis of a different structure of complexes K-I and K-II was put forward. A study of both saturation processes (Hb → Hp and Hp → Hb) by spectrophotometry and optical rotation dispersion also indicated a different structure for the two complexes. In the case of complex K-I, formed by adding Hb to an excess of Hp II, an interaction of four αβ Hb dimers with one Hp molecule was considered (*I*)



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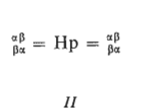

II

In the case of K-II complex, formed by adding Hp to an excess of Hb, two Hb tetramers interact with one molecule of Hp (II).

The conditions of formation of K-I complex allow the assumption that because of relatively great dilution of the Hb solution the molecule of Hb dissociates into dimers. Such a dissociation of Hb in very low concentrations has been already studied by sedimentation[19] and gel chromatography[20]. Though the problem of Hb dissociation is not yet solved in detail, the so-called dimer hypothesis is successfully used for interpreting reactions of Hb under conditions where dissociation takes place[21]. An alternative explanation of the presence of dimers on the surface of the Hp molecule has been offered[11], which considered a secondary cleavage of bonded Hb tetramers by an excess of Hp. To what extent this phenomenon takes place, or whether it is quite impossible, could not be decided on the basis of existing experimental evidence. On the other hand, when Hp interacts with an excess of Hb the concentration of Hb is high enough for the molecule to remain in a tetramer form[22]. The tetramer can bind to Hp through one of its dimers. (The fact that deoxyhaemoglobin is incapable of forming a complex with Hp (ref.[23]) seems to be due to its different conformation, compared with oxyhaemoglobin, rather than to *a priori* incapability of the tetramer forming a bond with Hp.) Under conditions of K-II complex formation, however, Hp II may dissociate.

Waks and Alfsen[2] studied the dissociation of Hp in detail and concluded that in low concentration the molecule of haptoglobin dissociates reversibly. It is very probable that during the Hp → Hb reaction Hp also dissociates and that the actual reaction partners of Hb are subunits of Hp. When the solutions are concentrated by freeze-drying an association process seems to take place, which leads to formation of K-II molecules (formula *II*). The K-II complex, however, retains its capability to dissociate, as can be seen from Figs 1 and 2. In the presence of SDS during gel filtration an additional stabilization[24] of the K-II molecule occurs (*viz* the first peak with smallest elution volume on Fig. 1). The behaviour of the K-I complex during gel filtration indicates a much greater molecular weight of this complex compared to complex K-II. This fact leads to assumption that the αβ dimers on the surface of the Hp molecule stabilize the K-I molecule to such an extent that dissociation occurs only to a very small degree, if at all. It is possible that the αβ dimers on the surface of Hp actually shield those parts of the molecule where the dissociation process might take place and thus stabilize the molecule of the complex.

Experiments with Zn^{2+} ions also indicate a different structure of Hb bound in complexes K-I and K-II. The decreased intensity of the Soret band caused by Zn^{2+} ions, which is characteristic for pure Hb (ref.[15]), was found only in K-II (Fig. 3). We can therefore conclude that in relation to Zn^{2+} ions the structure of Hb in K-II is much closer to the structure of free Hb than the structure of Hb in K-I. Cann[15] assumes that the rate of the Hb reaction with Zn^{2+} depends on the stability of the bond between iron and histidine. As has been previously found[25], the bond between iron and histidine in Hb becomes firmer after a Hb–Hp bond forms. The reaction with Zn^{2+} ions indicates that the strength of the Fe–His bond is greater in complex K-I than in K-II. The suggested structure of the two complexes seems to fit these facts. Thus in K-I all Hb dimers are in close contact with the surface of the Hp molecule, while in K-II the outer dimers of both Hb tetramers are available for a reaction with Zn^{2+} ions.

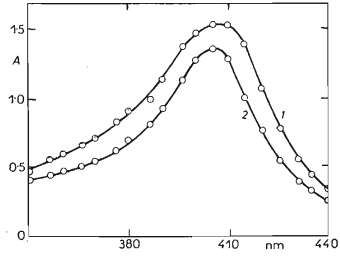

FIG. 6

Absorption Spectrum of Complexes K-I (*2*) and K-II (*1*) in the Soret Band Region

That the accessibility of the Hp molecule differs in complexes K-I and K-II, and therefore that the structure of bound Hb differs, is indicated by the course of reduction cleavage as well as by gel chromatography and Zn^{2+} ions. In the case of the K-II complex the height of the catalytic polarographic wave, which indicates the number of —S—S— and SH— groups in a protein molecule[26], reaches maximum immediately at the beginning of reactions with urea and mercaptoethanol. The K-I complex, on the other hand, splits at a lower rate and the SH— groups accessible for the catalytic polarographic reaction appear gradually (Fig. 4).

When the absorption and differential spectra in the visible region were measured, different behaviour was observed in each complex. The Soret band of complex K-I was lower and its maximum shifted to shorter wavelengths compared to K-II (Fig. 6). Contemporary knowledge shows that the configuration state of a protein influences the character of the chromophore at the electron level[27]. In our case the Soret band can be used as an indicator of interaction between a molecule of the complex and a heme group. This interaction is different for both complexes. Lower absorbance of complex K-I agrees with the results of Uchida and coworkers[20], who found higher absorbance in Hb tetramers than in Hb fragments. The differential spectrum of the complexes in the Soret band region (Fig. 5*a*) exhibits two maxima which also point to the different interaction of Hb and Hp in complexes K-I and K-II. We can exclude the possibility of the differential spectra arising as a result of different concentrations of the protein because the complexes were prepared from the same stock solutions of reactants and the difference in the yields of both complexes was less than 4%. The differential spectrum in the 440–650 nm region showed that the K-II complex is of a more high-spin character than complex K-I. This fact is indicated by the absorption bands at 500 and 630 nm (Fig. 5*b*). Waks and Alfsen[28] have already observed the formation of the 500 and 630 nm absorption bands, which are of a charge transfer complex nature[16], working only with a Hb–Hp complex prepared by adding Hb to Hp. According to Williams[17], all hemoproteins which react rapidly with peroxides are high-spin compounds. It can therefore be assumed that the complex K-II, where the spin equilibrium is shifted towards the high-spin state, should be a better peroxidase than complex K-I. This fact was confirmed by measuring the concentration dependence of peroxidase activity of both complexes (Table I).

The dependence of peroxidase activity of the complexes on the amount of free Hp added also indicated the different reactivity of bound Hb (Table I). The sharp increase in peroxidase activity in K-II after the addition of two mol of Hp proves the formation of new contacts of Hp with outer αβ dimers of the bound Hb tetramers. On the other hand, the dependence of peroxidase activity in K-I indicates that in this case only additional, possibly weaker, binding of added Hp took place with the surface bound dimers of Hb. The two-step increase in peroxidase activity points to the fact that the binding characteristics of the dimer couples are different.

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