THE CONFORMATIONAL CHANGES OF HAEMOGLOBIN ON ITS BINDING TO HAPTOGLOBIN

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The binding of human haemoglobin to human haptoglobin has been found to alter the conformation of haemoglobin. Spectrophotometric measurements, measuring of peroxidase activity, thin-layer gel chromatography and modelling on an analogue computer led to the conclusion that the binding of haemoglobin to haptoglobin was associated with a change in the quaternary structure of haemoglobin, with a transition from the R state to the T state. The kinetics of the conformational changes had an autocatalytic character.

The binding of oxygen to haemoglobin** is still being studied by a number of authors[1-3]. At present the attention is focused on the molecular mechanism of the cooperative effect in a molecule of Hb, and the associated changes in both the haem and the globin parts of the molecule.

Even in an early study[4] of the binding of oxygen to deoxyHb, conformational changes of the individual chains of Hb and of the whole tetrameric molecule were observed. The atom of iron in every haem of deoxyHb forms five coordinate bonds, and is capable of binding another ligand (O_2, CN^-, CO), with the formation of an octahedral complex[4] with the coordination number six. In deoxyHb the atom of iron is present in a high-spin state, and therefore protrudes from the plane of the porphyrin ring in the direction to the proximal histidine[5]. By contrast, in the octahedral complexes of HbO_2 the iron atom is coplanar with the nitrogen atoms and is in a low-spin state[5]. Perutz[1] advanced a stereochemical interpretation of the coordinate bond of oxygen to Hb and, using Monod's symbols[6], described the considered change as a transition from the T state (a strained form with a low affinity to oxygen) to the R state (a relaxed form with a high affinity to oxygen). The T→R transition involves the individual haem groups, as well as the tertiary and the quaternary structures of globin[4].

Several authors[2,7-9] studied the action of some low-molecular-weight effectors, which, like oxygen, bind to Hb and thus induce the T→R transition or *vice versa*. An allosteric effector which

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** Abbreviations used: Hb-haemoglobin, Hp-haptoglobin, HbO_2-oxyhaemoglobin, metHb-methaemoglobin, deoxyHb-deoxyhaemoglobin, Hb–Hp — complex of haemoglobin with haptoglobin, Hb→Hp — complex obtained by gradual addition of a solution of Hb to a solution of Hp, Hp→Hb — complex obtained by gradual addition of a solution of Hp to a solution of Hb.

is present in red cells and promotes the liberation of oxygen from HbO_2 is 2,3-diglycerol phosphate[2]. Its binding fixes the T state of Hb, thus facilitating the release of oxygen in tissues. Benesch and coworkers[7] found that an effector very similar to 2,3-diglycerol phosphate, in its action on Hb, was hexasulphoinositol. Perutz[8,9] used P_6-inositol as an allosteric effector and studied its action on metHb's of various vertebrates. The R→T transition produced by P_6-inositol manifested itself in the spectra by a shift to shorter wave lengths in the Sorret band region, by greater intensities of the bands at 500 and 630 nm, and a lower intensity of the band at 540 nm.

Changes of the physico-chemical properties of Hb associated with its binding to a blood α_2-glycoprotein, haptoglobin, were studied too[10–13]. The properties of the complex depend not only on the starting molar ratio of Hb to Hp, but also on the order of mixing the two reactants[11,14]. Waks and Alfsen[11] isolated and characterized three types of complex of horse Hb with human Hp, depending on the starting molar ratio of Hb to Hp. Pavlíček and Jaenicke[14], working with bovine Hb, observed different physico-chemical properties of complexes prepared in different ways of mixing. Makinen and coworkers[15] compared the behaviour of bovine, horse and human Hb. Human Hb always formed the same Hb–Hp complex, irrespective of the way the constituents were mixed. The other two Hb's formed different complexes, depending on the order of mixing.

The Hb–Hp complexes exhibit substantially changed oxygen equilibria, their affinity to oxygen being up to thirty times higher than that of Hb itself[15,16]. Unlike the binding of oxygen to Hb, the binding curve of oxygen on a Hb–Hp complex is not sigmoidal[13,16], as a consequence of the absent haem–haem interaction. In the spectrophotometric study of a Hb–Hp complex changes were observed in the visible region of the spectrum[10]. These were characterized by new absorption bands at 500 nm and in the region 600—630 nm. The intensities of the α and β bands of Hb in the complex were lower than those of Hb alone.

The present paper deals with complexes of human Hb with human Hp, prepared in different ways of mixing, and with alterations in a molecule of Hb caused by Hp as a high-molecular-weight allosteric effector.

EXPERIMENTAL

Preparation of Hb–Hp *complexes.* Human Hp II was isolated from the Cohn fraction IV according to Pintera[17]. Human Hb was isolated from blood[18] in the Institute of Haematology and Blood Transfusion, Prague 2. The solution of a Hb→Hp complex was prepared by adding a solution of HbO_2, of a concentration of $2{\cdot}5 \cdot 10^{-5}$ mol dm^{-3} (tetramer), to a solution of Hp, whose concentration was $5 \cdot 10^{-5}$ mol dm^{-3}. The solution of HbO_2 was added dropwise from a micropipette under constant stirring. The solution of a Hp→Hb complex was prepared analogously, the constituents being mixed in the reverse way. In the resulting complexes the ratio of Hb to Hp was 1 : 1. The medium in which the starting reactants (Hb and Hp) were prepared was the Sörensen phosphate buffer (pH 7·2, 0·1 mol dm^{-3}).

Peroxidase activity of the Hb–Hp *complexes* was measured according to Connel and Smithies[19]. The time dependence of the absorbance of tetraquajacol at 470 nm was recorded with a spectrophotometer Specord UV, VIS (Carl Zeiss, Jena). The absorbances were read 2·5 min after the addition of 1% H_2O_2.

Thin-layer gel chromatography was carried out in a TLG apparatus (Pharmacia, Uppsala using Sephadex G-150 (superfine). The chromatography ran 3 h at an angle of 15° and a temperature of 20°C in the medium of the Sörensen phosphate buffer (pH 7·2, 0·1 mol dm^{-3}).

Spectrophotometric measurements were performed with the apparatus Specord UV, VIS. To measure the absolute and the difference spectra we used a two-cell arrangement, the cell thickness being 1 cm. The difference spectra were recorded in a supplementary recorder EZ 2 (Laboratorní přístroje, Prague). In evaluating the difference spectra of the Hb–Hp complexes we read the difference between the heights of two adjoining peaks, a positive one at 500 nm and a negative one at 540 nm. All measurements refer to 20°C.

Modelling on an analogue computer. The kinetics of the protein reactions were modelled on an analogue computer MEDA 80 T (Tesla, Prague). The computer was connected to a recorded Bryans-XY Recorder 26000 A3, in which the modelled reaction courses were recorded. Different computer connexions were chosen, depending on the reaction schemes proposed. By varying the parameter characterizing the slope of the straight part of the line (and thus the average reaction rate) the best possible agreement with experimental data was found.

RESULTS

Spectrophotometry of HbO_2–Hp *Complexes in the Visible Region*

The difference spectra of the HbO_2–Hp complexes in relation to time were measured in the region 454·5 to 713 nm, the solution of HbO_2 being used as the reference sample. The intensities of the bands at 500 and 630 nm increased with time, while those of the bands at 540 and 570 nm decreased (Fig. 1). After 30 h the spectral

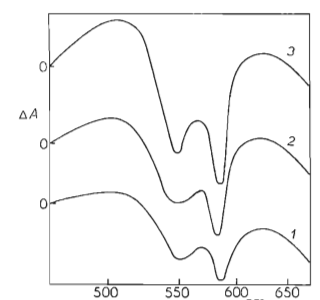

FIG. 1

Difference Absorption Spectrum (ΔA) of a HbO_2→Hp Complex in the Region 470 to 700 nm and Its Change with Time h
1 3, *2* 4, *3* 5 hours.

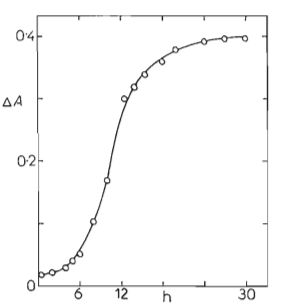

FIG. 2

Difference Absorbance (ΔA) of a Hp→HbO_2 Complex in Relation to Time (h)

changes had reached the limit state. The amplitude of the difference spectrum of a HbO_2–Hp complex as a function of time took a sigmoidal course (Fig. 2), which in the first hours depended on the order of mixing the reactants in preparing the complex (Fig. 3).

To a solution of HbO_2, of a concentration of $0{\cdot}33 \cdot 10^{-5}$ mol dm^{-3}, and to a solution of a HbO_2–Hp complex of the same concentration, was added a slight amount of crystalline sodium dithionite. The difference spectrum was then measured against the solution of HbO_2 in the region 454·5 to 713 nm. The character of the difference spectrum was the same as in the "aging" of the HbO_2–Hp complex (Fig. 4).

We further examined whether HbO_2 bound in a complex with Hp retained its oxygen in the course of the changes observed. The absolute spectrum of the complex measured after 30 h had marked α and β bands, characteristic of oxyHb. It was only after the addition of dithionite to the complex that the α and β bands vanished and a spectrum characteristic of deoxyHb appeared.

To 1000 µl of the solution of HbO_2, $2{\cdot}5 \cdot 10^{-5}$ mol dm^{-3}, was added 125, 250, 375, 500, 750 and 1000 µl of a solution of Hp, having the same concentration, and the volumes of the samples were made up to 2 ml with the Sörensen phosphate buffer. The solutions were left standing for 24 h at 20°C, then their difference spectra against the reference solution of HbO_2 were measured. The amplitudes of the spectra

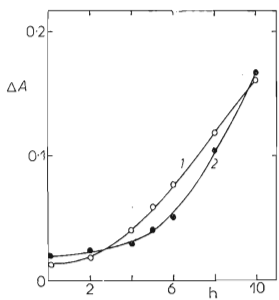

FIG. 3

Difference Absorbance (ΔA) of HbO_2–Hp Complexes within 10 h of Mixing the Constituents

1 Complex $HbO_2 \rightarrow Hp$, *2* complex $Hp \rightarrow HbO_2$.

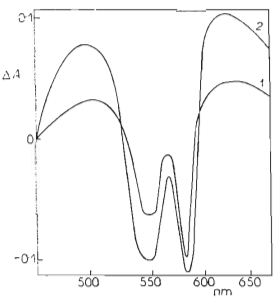

FIG. 4

Difference Spectra (ΔA) of a Solution of HbO_2 ($0{\cdot}33 \cdot 10^{-5}$ mol dm^{-3}) with an Admixture of Dithionite *1* and a Solution of HbO_2–Hp Complex of the Same Conc. of HbO_2 with an Admixture of Dithionite *2* in the Region 370—700 nm

were directly proportional to the concentrations of Hp. The dependence of the amplitudes on the amount of the Hp added was linear for either way of mixing the reactants, but the slopes of the two lines were different.

Measurement of Peroxidase Activity and Thin-Layer Gel Chromatography

These two methods revealed that a complex is formed immediately on mixing the reactants, and not in the whole course of 30 h. Further it was found that during these 30 h, when the spectral properties of the complex exhibited changes, no disintegration or denaturation of the complex occurred.

Modelling the Reaction Schemes on the Analogue Computer

The character of the difference spectra of the HbO_2–Hp complexes, and the time dependence of their amplitudes, allow of drawing the conclusion that the observed changes in the difference spectra result from the changing structure of the complexes. The sigmoidal form of the dependence of the amplitude of a difference spectrum on time suggested a consecutive or autocatalytic course of the reaction. This led us to propound several reaction schemes. After modelling on the analogue computer only two autocatalytic schemes fitted the experimental dependence of ΔA on t:

$$2\ K_R \rightarrow 2\ K_T \quad (K_R \text{ denotes a complex with Hb in the R state}) \qquad (1)$$

$$K_R \rightarrow K_T \quad (K_T \text{ denotes a complex with Hb in the T state.}) \qquad (2)$$

The autocatalytic character of the reaction was verified experimentally by an addition of the 30 hours' old complex to a fresh reaction mixture. As can be seen from Fig. 5, the amplitude of the spectra of systems catalysed in this way increased much faster than in a non-catalysed reaction.

The time dependences of amplitudes of the difference spectra, characterizing the conformational changes in the complexes studied, were modelled on the analogue computer according to the reaction schemes proposed. The obtained curves were confronted with experimental data and found to fit them very well. An example is given in Fig. 6, showing both the calculated reaction curve and the experimental points, describing the changes of the complex with time.

DISCUSSION

In the present study we concentrated on the optical properties of a complex HbO_2–Hp. In agreement with the paper by Waks and Alfsen[10], the complex studied exhibited a difference spectrum in the region 454–713 nm. A new finding was that the difference spectrum changed with time; the intensities of the bands at 500 and 630 nm increased, while those of the bands at 540 and 570 nm decreased (Fig. 1). A detailed

measurement of these difference spectra in relation to time revealed that the amplitudes of the spectra increased with time, the dependence having a sigmoidal character (Fig. 2).

The appearance of spectral bands in a difference spectrum had been observed before[2,7,20], in studying the effect of low-molecular-weight effectors on the binding of oxygen to Hb. The bands at 500 and 630 nm were called "high-spin" bands, those at 540 and 570 nm "low-spin" bands. An increase or decrease in intensity of these bands was regarded as indicating the operation of one spin state or the other[8]. Later on it was demonstrated that the appearance of the difference bands at 500 and 630 nm might not always be caused by a change in the spin properties, although a change in the spin state of the haem iron was invariably accompanied by a change of the difference bands considered[8].

According to Perutz[8,9] the presence of bands at 500 and 630 nm is characteristic of the transition of a molecule of Hb from the quaternary R structure to the T structure. This transition of Hb probably occurs even in the Hb–Hp complex, whose spectral properties change with time.

The R→T transition occurs[1] in HbO_2 alone in the liberation of oxygen and the formation of deoxyHb. This process was effected *in vitro* by the addition of sodium dithionite to HbO_2 and to a freshly prepared complex HbO_2–Hp. In either case the difference spectra in the visible region (Fig. 4) had the character of the spectrum exhibited by the HbO_2–Hp complex after several hours.

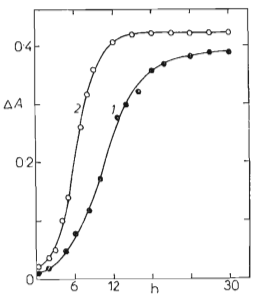

FIG. 5

Difference Absorbances (ΔA) of a HbO_2→Hp Complex *1* and the Same Complex with a Catalyst Added *2* in Relation to Time (h)

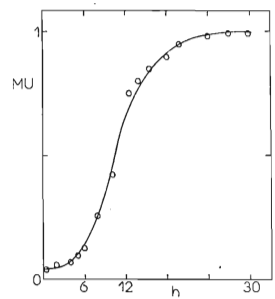

FIG. 6

Curve Simulating the Reaction Course (MU) for a Hp→HbO_2 Complex Modelled according to Equations (*2*)

The experimental points are given for the sake of comparison.

As a Hb–Hp complex has a much higher affinity to oxygen than Hb alone[16], the R→T transition of a HbO_2–Hp complex is probably not accompanied by the liberation of oxygen. Still, we considered the possibility that the difference spectrum of the HbO_2–Hp complex was primarily produced by a slow liberation of oxygen from the haem group of HbO_2. However, even after 30 h the absolute spectrum of the HbO_2–Hp complex exhibited strong α and β bands, typical of HbO_2. It was only after the addition of dithionite that the oxygen was removed from the complex, with the appearance of a blunt peak in the region 540 to 570 nm, typical of deoxy Hb.

The question arises whether the R→T transition of HbO_2 in the complex is accompanied by a change in the spin state of iron. In HbO_2 the central atom of iron occurs in a low-spin state, has a coordinate number 6 and is placed in the plane of the porphyrin ring[5]. In deoxyHb, by contrast, the central atom of iron is in a high-spin state and protrudes from the porphyrin plane towards the proximal histidine[5]. This motion of iron is a significant feature associated with the change of the spin state. In our opinion the binding of Hp to HbO_2 is followed by a slow alteration of the tertiary and the quaternary structures of the globin part of HbO_2. This change of globin conformation may produce a change in the haem structure, which, in turn, would be responsible for the change of the spin state of iron.

In our case the change in the haem structure might originate as follows: a change in conformation of globin might set helix F in motion; this, in turn, would induce a motion of the proximal histidine, to which the central atom of iron is bound. Since the bond of iron to the proximal histidine is very strong and its lengths in the R and the T states are nearly the same[8,9], the atom of iron follows the motion of the histidine, thus protruding from the plane of the porphyrin ring. In contrast, the bonds of iron to the porphyrin nitrogens are not so firm, so that they can be stretched[8,9]. This apparently gives rise to the high-spin complex of the iron atom.

The dependence of the difference absorbance on time had a character resembling the course of an autocatalytic reaction. The autocatalytic character of the observed gradual change of the HbO_2–Hp complex was confirmed by an experiment in which a small amount of the old complex (30 h incubation) was added to a fresh one. The spectral changes had a much faster course in this case (Fig. 5).

The autocatalytic character of the conformational change of the complex was further confirmed by simulation of the reaction on the analogue computer. Of all the reaction schemes considered only two, corresponding to autocatalytic reactions, proved satisfactory: $2\ K_R \rightarrow 2\ K_T$ and $K_R \rightarrow K_T$. However, the experiments with the analogue computer have not allowed us to decide between them.

The results described may cast some light on the nature of the bond between HbO_2 and Hp. The binding of Hp to HbO_2 appears to affect the conformation of the latter like the binding of a low-molecular-weight allosteric effector. However, the mechanism of action is probably different than with, *e.g.*, 2,3-diglycerol phosphate. This effector interacts preferably with the T form of Hb, stabilizes it and shifts

the R⇌T equilibrium in favour of the T structure. By contrast, the binding of Hp to Hb induces the R→T conversion through intramolecular effects. In the latter case, consequently, the decisive role is played by conformational changes of the globin chains, caused by the binding of Hp. This is also indicated by the different rates of the autocatalytic changes in the complexes Hb→Hp and Hp→Hb (Fig. 3). In these complexes, depending on which reactant was added to the other, different subunits of Hb are supposed to interact with Hp and form bonds of different types and strengths.

Two types of bond, and thus different properties of Hb–Hp complexes, were demonstrated with bovine and horse Hb (ref.[11,14,15]). As for human Hb the authors[14,15] believe that only one type of bond is formed in the complex, irrespective of which reactant is added to the other. The results described herein show that even in the case of human Hb the way in which a Hb–Hp complex is prepared is decisive for its physico-chemical properties.

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