INTERACTION OF HUMAN HEMOGLOBIN WITH HAPTOGLOBIN STUDIED BY THE METHOD OF OXYGEN SATURATION CURVES

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Two types of hemoglobin–haptoglobin complexes were studied which had been prepared by mixing together the two components in a different order. The oxygen saturation curves have shown that hemoglobin was bound in these complexes either in the form of tetramers or in the form of noncooperative dimers. The investigation of the interaction of deoxyhemoglobin with haptoglobin demonstrated that these two proteins do not bind to one another; the corresponding oxygen saturation curve was the same as the curve obtained with hemoglobin alone. In contrast, haptoglobin interfered with the saturation curves of deoxyhemoglobin containing an allosteric effector.

Hemoglobin (Hb*) is a protein of considerable physiological importance[1]. Since it is readily accessible and its structure has been described in detail it represents one of the proteins suitable for modeling of systems designed to cast light on the relation between the protein and its biological function. Such a model suitable for studies on protein–protein interactions is also the hemoglobin–haptoglobin complex[2]. Haptoglobin (Hp) is a blood protein from the group of α-glycoproteins which shows an outstanding ability to form very rigid complexes[3] with Hb. The complexes have different stoichiometric compositions corresponding to the molar ratios of the reactants. If Hp is in excess a so-called half-complex[3] is formed, the Hb to Hp ratio being 1 : 2; the subsequent addition of Hb gives rise to a Hb : Hp = 1 : 1 complex (refs[3–5]) and with HpII a complex[6–8] can be formed in which the ratio of Hb to Hp is 2 : 1. The physicochemical characteristics of the Hb–Hp complex depend on the order in which the reactants have been mixed together[5,7]. The complex formed by slow addition of HbO_2 to an excess of Hp ($HbO_2 \rightarrow Hp$) has other properties[9] than the complex prepared by mixing together the components in the reversed order ($Hp \rightarrow HbO_2$). The difference between the complexes of the same stoichiometric composition yet whose components have been mixed together in the reversed order increases with the increasing molar Hb content[8].

These problems are closely related to the problem of the form in which Hb binds to Hp and of the localization of the corresponding binding sites. The dissociation of Hb to dimers was considered a condition necessary for complex formation[10]. As proof of this postulate was regarded the inability of Hp to form a complex with deoxyHb which practically does not dissociate and thus exists mostly in tetramer form[1]. In the light of more recent studies, however,

* Abbreviations used: Hb — hemoglobin, HbO_2 — oxyhemoglobin, deoxyHb — deoxyhemoglobin, Hb–Hp — hemoglobin–haptoglobin complex, $HbO_2 \rightarrow Hp$ — complex obtained by successive addition of HbO_2 solution to Hp solution, $Hp \rightarrow HbO_2$ — complex obtained by successive addition of Hp solution to HbO_2 solution, IHP — inositolhexaphosphate.

the lack of complex formation between Hp and deoxyHb is rather due to differences in tertiary and quaternary structure[8] between deoxyHb and HbO_2. Indirect experimental evidence has been also obtained of the ability of HbO_2 tetramers to bind Hp[7,8].

The oxygen saturation curve[1] is the dependence of Hb saturation with oxygen on its partial pressure. The important characteristics of this curve are the pressure necessary for half-saturation, p_{50}, and Hill's coefficient, n, which is a measure of the allosteric cooperative effect between the subunits. The value of this coefficient reported mostly for Hb alone is 2·8.

The ability of Hb–Hp complexes to bind oxygen has been studied during the past few years. The Hb–Hp complex prepared without attention to the order of mixing the components together shows a higher affinity for oxygen than Hb itself. The corresponding saturation curve did not have a sigmoidal character[11]. An important allosteric effector[12] considerably decreasing the affinity of Hb for oxygen while the sigmoidal character of the saturation curve remains unaltered, is IHP.

The aim of this study has been to examine the oxygen saturation curves with complexes of the same stoichiometric composition, *i.e.* HbO_2 : HpII = 2 : 1, but of different quaternary structures and thus also of different physical characteristics. We endeavored to determine the form in which Hb reacts with Hp. The determination of the oxygen saturation curves of a system containing deoxyHb, Hp, and IHP (an allosteric effector was) bound to contribute to the final elucidation of interaction of Hb and Hp tetramers.

EXPERIMENTAL

All the experiments were carried out with human HbO_2 prepared from fresh human blood in the Institute of Hematology and Blood Transfusion in Prague[13]. The HbO_2 solution could not be kept in frozen state since its oxygen binding ability decreased. The stock solution was therefore kept at +4°C during the measurement. The exact HbO_2 concentration was determined spectrophotometrically. Human HpII was isolated from Cohn fraction IV by the procedure developed in our laboratory[6] and modified according to Pintera[14].

The solution of the $(HbO_2)_2 \rightarrow$ Hp complex was prepared by adding slowly a certain volume of the HbO_2 solution (concentration $2{\cdot}5 \cdot 10^{-5}$ ml dm^{-3}, tetramer) to the same volume of the Hp solution whose concentration was $1{\cdot}25 \cdot 10^{-5}$ mol dm^{-3}. The HbO_2 solution was added stepwise by a micropipet with constant stirring. The Hp $\rightarrow (HbO_2)_2$ solution was prepared by an analogous procedure and the reactants were mixed together in the reversed order. The solutions were made in Sörensen buffer, pH 7·2, 0·1 mol dm^{-3}. The solution of the system containing deoxyHb was prepared by adding the concentrated Hp solution directly to the deoxy Hb solution in an evacuated tonometer to obtain a final deoxyHb : Hp ratio of 2 : 1. This system was modified during the subsequent measurement by the presence of the allosteric effector (IHP, concentration $2 \cdot 10^{-3}$ mol dm^{-3}) which was added to the deoxyHb solution before the addition of Hp. The deoxyHb solution was prepared by evacuating HbO_2 directly in the tonometer.

The oxygen saturation curves were measured by a modification of the discontinuous method reported by Vodrážka and coworkers[15]. The method records the HbO_2 and deoxyHb content on the basis of differences in their absorption spectra[1]. The solution is therefore placed in a special cylindrical vessel, the tonometer, which permits the spectrophotometric measurement to be carried out even with evacuated solutions. The tonometer is constructed to permit also supplementary additions of solutions of various compounds through a teflon stopper even after the tonometer with the mixture assayed has already been evacuated. For the deoxygenation of the

solutions the tonometer was evacuated first by an oil pump; the contents were mixed afterwards in a mechanical shaker and the whole procedure was repeated. The air volume passed gradually into the tonometer was measured by a buret with a moving mercury drop (marker). After each partial addition of air and after the saturation equilibrium had established the tonometer was placed in a modified cell compartment of Specord UV VIS (Carl Zeiss, Jena) spectrophotometer and the absorption spectrum was recorded over the range 500–625 nm. The whole procedure was carried out at 20°C. The oxygen saturation degree Y was calculated from absorbance values measured at 540 and 555 nm using the following equation[15]

$$Y = \frac{(A_{540} - A^{d}_{540}) + (A^{d}_{555} - A_{555})}{(A^{0}_{540} - A^{d}_{540}) + (A^{d}_{555} - A^{0}_{555})},$$

where A stands for the absorbance of incompletely saturated Hb and indexes d and 0 denote the absorbance of deoxygenated Hb (at the beginning of the assay) and of fully oxygenated Hb (at the end of the measurement), respectively. The partial oxygen pressure p (Pa) was calculated as in the work of Vodrážka and coworkers[15].

RESULTS

The oxygen saturation curve measured with the $(HbO_2)_2 \rightarrow$ Hp complex is shown in Fig. 1. The oxygen pressure necessary for half saturation of Hb, $p_{50} = 0{\cdot}35$ kPa, was read off from this curve, whose character is hyperbolic. This character indicates a very low cooperativity in the Hb molecule bound in the complex studied. This fact is also evidenced by the value of Hill's coefficient n calculated which is a measure

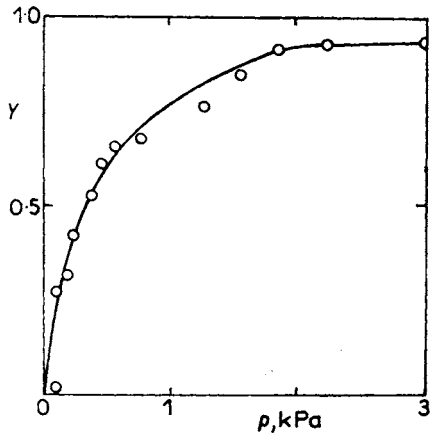

FIG. 1

Oxygen saturation curve of $(HbO_2)_2 \rightarrow$ Hp complex. Concentration: HbO_2 $1{\cdot}25 \cdot 10^{-5}$ mol dm^{-3}, Hp $0{\cdot}625 \cdot 10^{-5}$ mol dm^{-3}

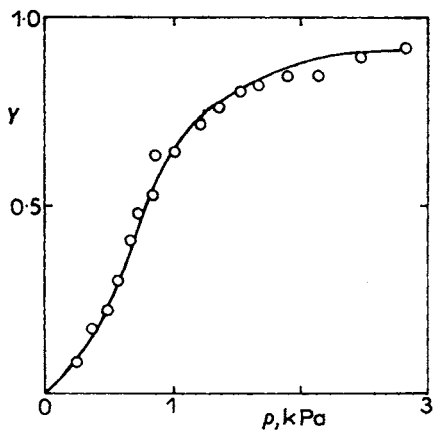

FIG. 2

Oxygen saturation curve of Hp $\rightarrow (HbO_2)_2$ complex. Concentration: HbO_2 $1{\cdot}25 \cdot 10^{-5}$ mol dm^{-3}, Hp $0{\cdot}625$ mol dm^{-3}

of the heme–heme interaction ($n = 1{\cdot}02$). Unlike with the $(HbO_2)_2 \rightarrow Hp$ complex the oxygen saturation curve of the $Hp \rightarrow (HbO_2)_2$ complex showed a sigmoidal character (Fig. 2) similar to that observed with the saturation curves of hemoglobin itself. The heme–heme interaction in this complex was considerably higher ($n = 1{\cdot}92$) and likewise the affinity of oxygen binding was lower ($p_{50} = 0{\cdot}78$ kPa). Fig. 3 shows the oxygen saturation curve obtained with deoxyHb placed in the evacuated tonometer to which a single addition of the Hb solution had been made in order that the final molar deoxyHb to Hp ratio might equal 2 : 1. The curve obtained showed a sigmoidal character and was very similar to the curve obtained with Hb itself. When, however, the Hp solution was added to the deoxyHb solution containing IHP as an allosteric effector, the character of the oxygen saturation curve was hyperbolic (Fig. 4). This IHP-containing system was able to bind IHP more difficultly than the system lacking IHP ($p_{50} = 1{\cdot}27$ kPa).

DISCUSSION

It has been shown in earlier experiments[7] that the order in which the solutions of Hb and Hp are mixed together plays an important role in the structure[9] of the Hb–Hp complexes formed. The investigation of difference absorption spectra of complexes of the Hb → Hp and Hp → Hb type has shown that after these complexes

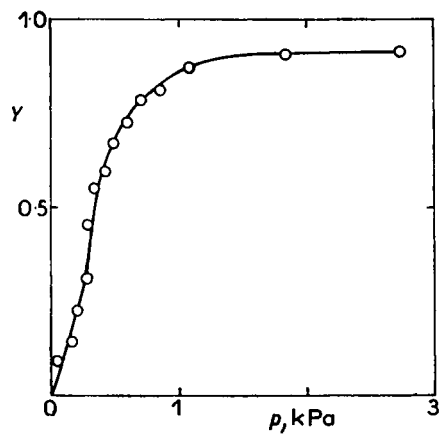

FIG. 3

Oxygen saturation curve of deoxyHb + Hp system. Concentration: deoxyHb $1{\cdot}25 \cdot 10^{-5}$ mol dm^{-3}, Hp $0{\cdot}625 \cdot 10^{-5}$ mol $\cdot dm^{-3}$

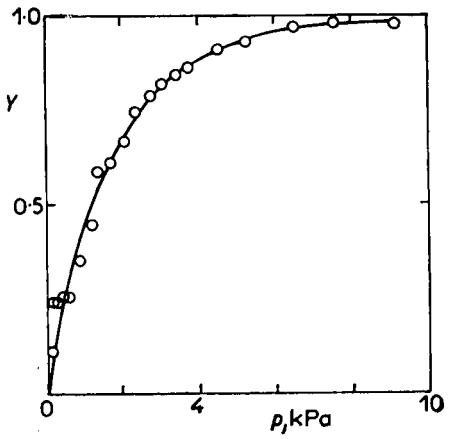

FIG. 4

Oxygen saturation curve measured with deoxyHb in presence of IHP and Hp. Concentration: deoxyHb $1{\cdot}25 \cdot 10^{-5}$ mol $\cdot dm^{-3}$, IHP $2 \cdot 10^{-3}$ mol dm^{-3}, Hp $0{\cdot}625 \cdot 10^{-5}$ mol dm^{-3}

have been formed they undergo very slow conformational changes of autocatalytic character[16]. Hb was converted during these changes, involving both the tertiary and the quaternary structure, from *R*-state into *T*-state. Both from the knowledge of different physicochemical characteristics of the Hb–Hp complexes and from the results of studies on the peroxidase activity of the complexes formed gradually a hypothesis[8] has been proposed postulating that four Hb dimers are bound to the Hp molecule in the HbO_2–Hp complex and that two Hb tetramers are bound to Hp in the Hp–HbO_2 complex. The idea that not only Hb dimers but also Hb tetramers can bind to the Hp molecule is a novel one. The dissociation of Hb to dimers has namely been considered in literature the necessary condition[10] of Hb interaction with Hp. As evidence supporting this postulate has been regarded the inability of Hp to bind to deoxyHb which unlike HbO_2 dissociates to dimers considerably less and therefore exists predominantly in tetramer form[1] even in dilute solutions. In contrast, it is our opinion that the inability of deoxyHb to bind to Hp is due rather to differences in tertiary and quaternary structure existing between deoxyHb and HbO_2. Direct experimental proof showing that both Hb dimers and Hb tetramers can react with Hp has been lacking until now and is presented for the first time in this study based on the investigation of the oxygen saturation curves. As can be seen in Fig. 1 the character of the oxygen saturation curve in the $(HbO_2)_2 \rightarrow$ $\rightarrow$ Hp complex is hyperbolic. This finding indicates that Hb bound to Hp in the complex lost the ability of heme–heme interaction and thus also the corresponding cooperative effect. The absence of the heme–heme interaction is also evidenced by the value of Hill's coefficient approaching 1 ($n = 1{\cdot}02$). These results are in agreement with the idea that noncooperative Hb dimers exist in the $(HbO_2)_2 \rightarrow$ Hp complex according to earlier predictions[7,9]. In contrast, the sigmoidal character of the Hp $\rightarrow (HbO_2)_2$ complex shows that Hb retains its cooperative effect in this complex; the existence of the heme–heme interaction was confirmed by the value of Hill's coefficient, $n = 1{\cdot}92$. This experimental finding corresponds to the idea that Hb tetramers retaining the ability of interaction between heme groups are bound to Hp in the Hp $\rightarrow (HbO_2)_2$ complex. The oxygen saturation curve of Hb to which a single portion of Hp has been added after its deoxygenation and hence after its conversion into deoxyHb has a sigmoidal character similar to that shown in Fig. 1. These results and also the method of saturation curves have shown that Hp does not bind to deoxyHb and therefore does not affect its saturation with oxygen, either.

Interesting data, however, also yielded the measurement of the oxygen saturation curve of Hb to which in deoxygenated state IHP as an allosteric effector and subsequently a single dose of Hp solution had been added. Unlike with deoxyHb without the allosteric effector (Fig. 3) a saturation curve of hyperbolic character was obtained (Fig. 4). This result can be explained by the following hypothesis: deoxyHb binds to the allosteric effector IHP thus stabilizing the molecular form of deoxyHb, *i.e.*

the tetramer and the *T*-structure of the Hb molecule. After the binding of the first two oxygen molecules, which is more difficult with the *T*-state, the latter state of the dimer is converted into the *R*-state. Hp can bind to the dimer at this very instant. Hp most likely prevents IHP from dissociation and thus from stabilization of the *T*-structure of the remaining dimer of the Hb molecule. Subsequently two additional oxygen molecules are bound to the dimer with the same affinity as the first two molecules, *i.e.* without the cooperative effect. The result is a saturation curve of hyperbolic character. The stabilization of the deoxy form and of the Hb tetramer occurs also with Hb lacking Hp yet in the presence of the allosteric effector IHP. Hence, the affinity of oxygen binding is lower (and p_{50} therefore higher) yet the sigmoidal shape of the saturation curve is retained since the quaternary structure of Hb is changed from the *T*-state to the *R*-state at the moment of oxygenation of the first dimer of the tetrameric Hb molecule and the allosteric effector dissociates off. Hence the binding of oxygen to the second dimer proceeds with a higher affinity.

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