A PHYSICO-CHEMICAL STUDY OF THE INTERACTION BETWEEN HUMAN HEMOGLOBIN AND BENZENE

Pavel SEDLÁČEK, Zdeněk PAVLÍČEK and Vítěz KALOUS

Department of Physical Chemistry,
Charles University, 128 40 Prague 2

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The interaction between human hemoglobin and benzene produced difference spectra (in the visible region) changing with time. In addition to these changes we studied the dependence of the interaction on temperature and on concentration of the two components. The interaction of hemoglobin and benzene was also followed by means of CD spectra. These spectra in the region 200–260 nm demonstrate that the secondary structure of hemoglobin was not affected by the interaction. By contrast, in the region 270–300 nm the CD spectra show a change in the quaternary structure of hemoglobin, which goes from the *R* state to the *T* state, and after a period it returns to the original state.

Hemoglobin (Hb)* has long been the subject of study by many authors[1–3]. The protein has a great physiological importance, is readily accessible, and the detailed knowledge of its structure makes it very suited for the modelling of such systems as might throw some light on the relation between conformation of a protein molecule and its biological function in the organism. At present attention is paid mainly to the molecular mechanism of the cooperative effect in the molecule of Hb, and the consequential changes in the heme and the globin moieties. In studying the interactions of Hb with ligands most interest has been focussed on the binding of oxygen and on the changes in conformation and other physico-chemical properties of Hb produced by this interaction. Perutz[3] proposed a stereochemical interpretation of the cooperative binding of oxygen to Hb in terms of Monod's symbols[4], describing the process as a transformation of deoxyHb in the *T* state (a tense form, with a low affinity to oxygen) to HbO_2 in the *R* state (a relaxed form, with a high affinity to oxygen). The $T \rightarrow R$ transition affects the individual heme groups, as well as the tertiary and quaternary structures of globin[5]. The atom of iron in each heme of deoxyHb has five coordination bonds and occurs in the high-spin state[6]. In the octahedral complex of HbO_2 the iron is in the low-spin state[6].

In addition to the interaction with oxygen, the binding of low-molecular-weight allosteric effectors 2,3-diphosphoglycerate[2] (DPG), inositol hexasulphate[7] and inositol hexaphosphate[8], has also been studied. From the effect of IHP on the structure of metHb Perutz[9,10] infers that the $R \rightarrow T$ transition of metHb is characterized by increased intensities of the absorption bands at 500 and 630 nm, a decreased intensity of the band at 540 nm and a shift to lower wave lengths in the Soret band region.

* Abbreviations used: Hb hemoglobin; HbO_2 oxyhemoglobin; deoxy Hb deoxyhemoglobin metHb methemoglobin; DPG 2,3-diphosphoglycerate; IHP inositol hexaphosphate.

A good indicator of the influence of the effectors on the *R–T* equilibrium proved to be the CD spectra in the region 270—300 nm. All the ligand forms of Hb in the *R* state exhibit a low specific positive ellipticity with two minima, at 285 and 290 nm. DeoxyHb, which is quite stable in the *T* form, has a marked negative minimum[11] at 290 nm.

The interaction of Hb with low-molecular-weight aromatic hydrocarbons has been studied mainly *in vivo*[12–14]. Most of the authors agree that benzene, toluene, phenol and derivatives of these compounds very strongly enhance the formation of metHb in blood[15,16]. In a benzene-saturated Hb the affinity to oxygen and the heme–heme interactions are suppressed and the linkage of globin to the heme moiety is not so firm. Lampe[17] believes that these changes in the properties of Hb are caused by "insertion" of benzene between porphyrin and globin. A similar conclusion was reached by Cann[18,19] in studying the interaction of benzene and myoglobin. The heme normally occurs in a hydrophobic "pocket" close to the surface of the myoglobin molecule[20], where it interacts with the aromatic rings Phe 1 CD and Phe 15 H. In the presence of benzene this π–π interaction is interrupted and the benzene binds to porphyrin[21]. Kiehs, Hansch and Moore[22] studied the interaction of bovine Hb with benzene and other eighteen aromatic hydrocarbons. In their view it is more likely that hydrocarbons bind in the space between the four subunits of Hb, rather than they should incorporate into the chains.

The objective of the present work was to study the system Hb–benzene spectrophotometrically and by the method of circular dichroism, and to ascertain whether the interaction leads to conformational changes. The results should contribute generally to the problem of protein–hydrocarbon interaction, and give some insight into the molecular mechanism by which pollution of the ecosystem affects a living organism.

EXPERIMENTAL

All experiments were carried out with HbO_2 prepared from fresh human blood withdrawn into the citrate buffer. The red blood cells, obtained by centrifugation, were washed in 0·9% NaCl and hemolysed overnight in a two-fold volume of distilled water. The hemolysate was stirred for 2 h with a freshly prepared gel of aluminium hydroxide. The gel and the stroma were removed by centrifugation and the supernatant was dialysed against distilled water. In the experiments the solution of HbO_2 was diluted with a phosphate buffer (c = 0·1 mol dm^{-3}, pH 7·20). In measuring the CD spectra the concentration of Hb was 1·4 . 10^{-5} mol dm^{-3}. The exact concentration of HbO_2 was checked spectrophotometrically[1]. To prepare solutions of HbO_2 with benzene the buffer was presaturated with benzene at 20°C. The benzene concentration was also determined spectrophotometrically[23–29].

Spectrophotometry was carried out using an apparatus Perkin–Elmer Hitachi 340 with a supplementary microcomputer. Quartz glass cells Hellma of one series, optical path 0·5 and 1 cm, with ground-glass lids were employed; their temperature was thermostatically controlled. In view of the exacting demands on precision and sensitivity of the record a long time of registration was chosen (6—12 min). In evaluating the difference spectra we read the amplitude ΔA^*, representing the absorbance difference between the negative peak at 577 nm and the adjoining positive peak at 560 nm ($\Delta A^* = \Delta A_{560} - \Delta A_{577}$). The CD spectra were measured with a spectrophotometer Carry 61, the temperature of the cells being kept at 20°C. The cells were made of a special quartz glass and were equipped with ground-glass lids. The molar ellipticity [Θ] was related to the average molecular mass of the amino acid content of Hb. The concentration of HbO_2 was 1·40 . 10^{-5} mol dm^{-3}, the concentration of benzene 0·01 mol dm^{-3}.

Analysis of the experimental CD *spectra* was performed by a described method[30] on the assumption that the spectra were linearly additive for all the components. In the calculation we used the reference spectra of the α-helix, β-form and random coil, derived from circular dichroism of five proteins of known structures[31].

RESULTS

Immediately on mixing benzene with HbO_2 the difference spectrum in the range 450–650 nm was not pronounced, but gradually the bands at 528, 560 and 592 nm became stronger, whereas the intensities of the bands at 541 and 577 nm decreased (Fig. 1). Further we followed the amplitude ΔA^* of a Hb–benzene complex in relation to temperature (Fig. 2). The change in ΔA^* was fast during the first 10 h; after this time, if the temperature was low, the value of ΔA^* was practically constant.

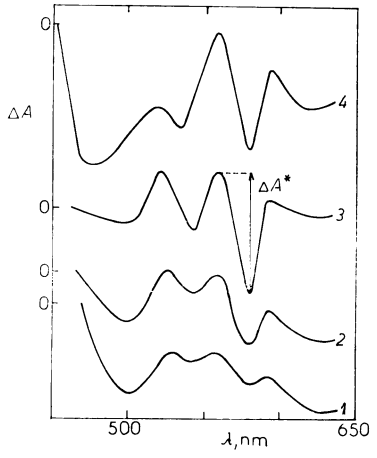

FIG. 1

Difference spectra of deoxyHb and Hb–benzene complexes in the region 470–700 nm recorded after different times from mixing the two components at 20°C. *1* 1 h, *2* 5 h, *3* 10 h; *4* deoxyHb. ΔA^* amplitude of a difference spectrum between 560 and 577 nm. Concentrations: HbO_2 or deoxyHb $1{\cdot}4 \cdot 10^{-5}$ mol dm^{-3}, benzene $1 \cdot 10^{-2}$ mol $\cdot dm^{-3}$. The reference sample was the solution of HbO_2

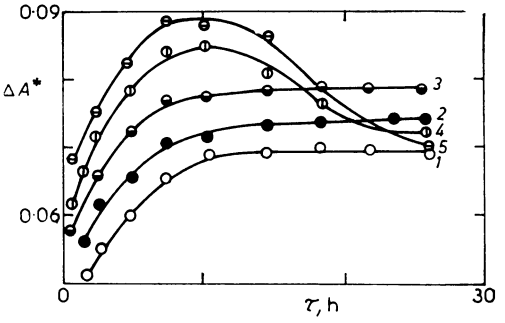

FIG. 2

Amplitude of difference absorbance, ΔA^*, of a Hb–benzene complex plotted *vs* time at temperatures 20°C *1*, 25°C *2*, 30°C *3*, 35°C *4* and 40°C *5*

At temperatures higher than 30°C and at $\tau > 10$ h the absorbance slowly decreased. Under these conditions we observed aggregation of Hb, caused by the presence of benzene. The ratio of ΔA^* to $\Delta\tau$ also increased with elevating temperature in the first 10 h of the HbO_2–benzene interaction.

By measuring the amplitude ΔA^* of the difference spectrum in relation to concentration of the two components we found that in the given concentration ranges the increase in ΔA^* was directly proportional to the amount of benzene. The same was observed in raising the concentration of HbO_2 (at a constant concentration of benzene, 10^{-2} mol dm^{-3}). Evaluation of the CD spectra of a solution of HbO_2 and a solution of Hb–benzene (at 20°C) in the region 200–260 nm in relation to the interaction time showed that only the α-helix represented an orderly secondary structure of Hb and the interaction with benzene did not affect it. The results were more complex in measuring CD spectra in this region at temperatures above 30°C. Benzene produced a visible aggregation of Hb; the macroscopic aggregates caused light scattering. Despite a small deformation, the CD spectra retained the shape characteristic of the α-helix. Mathematical analysis of these spectra[30] showed that the position of the random coil in the secondary structure was increased. With a temperature increase from 30 to 40°C the presence of benzene raised the content of the random coil in hemoglobin from 10 to 35%. The α-helix partially decomposed at the higher temperatures.

The CD spectra had two minima, at 285 and 290 nm (Fig. 3), which during the first 5 h of the HbO_2–benzene interaction showed an increase in negative ellipticity. However, with further time the negative ellipticity decreased, so that the CD spectra returned to the original values (Fig. 4).

In the region 510–600 nm (*i.e.* in the region of the α, β bands) the interaction HbO_2–benzene intensified the characteristic band at 541 nm in the first 4 hours. However, after 10 h it returned to its original state and went on changing (Fig. 5*a*). Fig. 5*b* shows the CD spectra of HbO_2 and deoxyHb in the region 510–600 nm.

DISCUSSION

The character of the time-dependent difference spectrum of a Hb–benzene complex was very similar to that of deoxyHb (Fig. 1). This suggests that the interaction between HbO_2 and benzene leads to structural alterations similar to those associated with the transition $HbO_2 \rightarrow$ deoxyHb[24], *i.e.* the $R \rightarrow T$ transition[9,10]. This conclusion is supported by the fact that the difference spectrum of HbO_2, stabilized in the *T* state by IHP[8], strongly resembled the difference spectra of deoxyHb and the studied Hb–benzene complex.

The relation of the amplitude of the difference absorbance, ΔA^*, to concentration of benzene for the complex Hb–benzene was found to be linear. In view of this

fact the magnitude of the spectral effect in the visible region was taken as a measure of the extent of the benzene–HbO_2 interaction. The dependence of the amplitude ΔA^* on temperature shows that with elevating temperature the interaction of benzene with HbO_2 increases (Fig. 2). The more extensive spectral changes suggest that

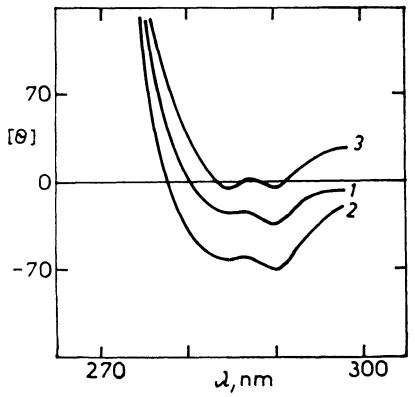

FIG. 3

CD spectra of some Hb–benzene complexes in the region 270—300 nm at 20°C and their change after 2 h *1*, 5 h *2* and 30 h *3* from mixing the components. Concentrations: Hb $1{\cdot}40 \cdot 10^{-5}$ mol dm^{-3}, benzene $1{\cdot}0 \cdot 10^{-2}$ mol dm^{-3}

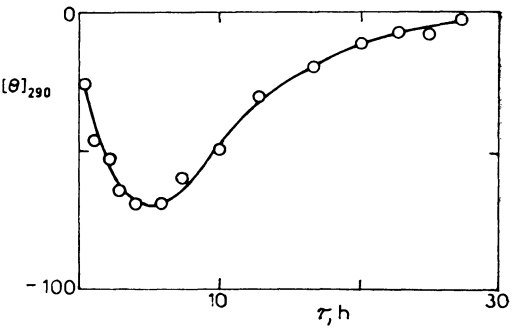

FIG. 4

Time dependence of molar ellipticity $[\Theta]_{290}$ of a Hb–benzene complex

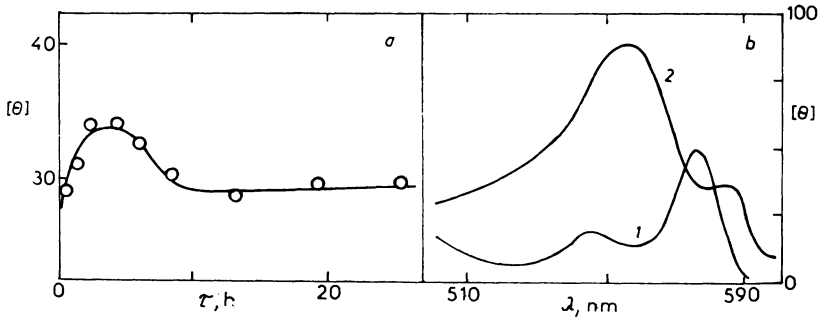

FIG. 5

Molar ellipticity $[\Theta]_{541}$ of a Hb–benzene complex. *a*) time dependence, *b*) CD spectrum of HbO_2 *1* and deoxyHb *2* in the region 510—600 nm. Concentration of both HbO_2 and deoxyHb was $1{\cdot}40 \cdot 10^{-5}$ mol dm^{-3}

a greater number of benzene molecules get bound at a higher temperature. This endothermic character of the temperature dependence testifies to the interaction being hydrophobic[25,26]. At temperatures below 30°C, in the time range from the 10th to the 30th hour of the interaction, no more changes in the magnitude of ΔA^* occurred. At temperatures above 30°C after 10 h a moderate decrease of ΔA^* was observed. The binding of more benzene destabilized the Hb–benzene complex to such a degree that in prolonged experiments at higher temperatures it began to disintegrate slowly. A partial denaturation of the protein by benzene at the higher temperatures must also have occurred and played a role. This fact manifested itself macroscopically by the appearance of a fine cloudiness.

An interpretation of the CD spectra can be based on the results published by Perutz and coworkers[11], who showed a relationship between the CD signal of Hb at 287 nm and the proportion of the conformational states *R* and *T*. Analysis of the CD spectra of the complex Hb–benzene at 290 nm has shown unequivocally that benzene acts as an allosteric effector on the *R*–*T* equilibrium of HbO_2. In the first hours the action of benzene increased the portion of HbO_2 in the *T* state, but later HbO_2 returned to the *R* state (Figs 3 and 4).

The changes in intensity of the band at 541 nm in the CD spectrum can again be interpreted as due to the $R \rightarrow T$ transition of HbO_2, followed by return to the *R* state. This statement is based on the band intensities at 541 nm of the pure components. Pure deoxyHb in the *T* state exhibits a high CD signal (Fig. 5*b*, curve 2), whereas pure HbO_2 in the *R* state only a low one (Fig. 5*b*, curve 1). The CD spectra in the region 270–300 nm, which reveal the conformational changes of globin, allow us to draw some conclusions on the course of the Hb–benzene interaction. Molecules of benzene bind to the globin moiety of HbO_2 and, by an intermolecular mechanism, produce the $R \rightarrow T$ conversion of the Hb molecule. It seems justifiable to suppose that a motion of the helical segment F will induce a motion of the proximal histidine[10], to which an atom of Fe is firmly bound. This deflects the iron atom from the plane of porphyrine and creates a space for the binding of benzene in the region of the heme group. Benzene may bind by $\pi-\pi$ interactions to the porphyrin ring, but hydrophobic interaction to non-polar amino acid residues of globin in the neighbourhood of heme is also possible. For most of the non-polar groups project inwards the molecule of HbO_2, whereas the polar groups are on its surface[27,28], so that the heme is located in a hydrophobic pocket[6]. The considered binding of benzene to heme and its close environs might account for the loosening the heme-globin bond and the moderation of the heme–heme interaction, observed previously by Lampe[17]. It remains to be explained, however, why (after about 5 hours' interaction) Hb returns to the *R* state.

According to what has hitherto been found out, by other authors and ourselves, the following hypothesis seems possible: the binding of benzene to the globin moiety produces the transition of Hb from the *R* state to the *T* state. The latter form starts

binding benzene in the heme region, which alters the molecule in such a fashion as leads (judging by the CD spectra) to restoring the *R* state. The benzene bound in the heme region counteracts the effect of heme, bound to globin, and puts the Fe atom back to the plane of porphyrin. As a result, the helical segments are shifted to the configuration characteristic of the *R* state. In the considered Hb–benzene interaction the low-molecular-weight ligand binds to both the globin and the heme moieties of Hb. With a high-molecular-weight ligand (haptoglobin) some similar changes in the quaternary structure of Hb were observed ($R \rightarrow T$ transition). In this case, however, the binding affected only the globin part of the molecule. The possibility of a $T \rightarrow R$ back transition of the complex Hb–haptoglobin will be the subject of further study.

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