Biophysical Chemistry 4 (1976) 223–228

THE INFLUENCE OF ORGANIC PHOSPHATES ON THE BOHR EFFECT OF HUMAN HEMOGLOBIN VALENCY HYBRIDS

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Received 9 October 1975
Revised manuscript received 26 January 1976

The Bohr effect of hemoglobin and that of the aquomet and cyanomet valency hybrids was measured in the presence and the absence of IHP (inositol hexaphosphate) and DPG (2,3-diphosphoglycerate). In the absence of these organic phosphates the four hybrids show similar, but suppressed Bohr effects as compared to hemoglobin. Addition of IHP and DPG results in all cases in an increase of the Bohr effect. The additional phosphate induced Bohr effect of the hybrids with the α chain in the oxidized form is almost identical to that of hemoglobin, while this effect of the hybrids with oxidized β chains is slightly lower than that of hemoglobin. The results suggest (a) that the Bohr effect is correlated to the ligation state of the hemoglobin molecule rather than to its quaternary structure, (b) that the additional phosphate induced Bohr effect is related to the change in quaternary structure of the tetramer, and (c) that with respect to the Bohr effect of the hybrids there is no difference between high and low spin species.

1. Introduction

Since the discovery of the Bohr effect by Bohr et al. [1] much work has been done to elucidate the molecular mechanism of this effect (for a review, see ref. [2]). It has been well established that the so called alkaline Bohr groups Val 1α and His 146β account for approximately 70% of the alkaline Bohr effect. As a candidate for the remaining 30% of the effect Perutz has suggested His 122α [3]. Kilmartin and Rossi-Bernardi, however, hypothesized that this remaining part of the Bohr effect could be based on a difference in chloride ion binding between oxy- and deoxyhemoglobin [2]. Recently we have presented evidence for the validity of this hypothesis [4,5].

The change in pK of the Bohr groups must be caused by a change in structure of the protein upon ligand binding. According to the stereochemical model of Perutz [6] this structural change involves both the tertiary structure of the subunits and the quaternary structure of the whole tetramer, the latter going upon ligation from the deoxystructure or T state to the oxystructure or R state. At intermediate stages of ligation intermediate structures exist in which the subunits are in the liganded or unliganded tertiary structure while the whole tetramer is in the R or T state.

If the Bohr effect is correlated with a change in tertiary structure of the subunits, one expects a gradual release of Bohr protons as the molecule is saturated with ligand. This gradual release should be absent in case the Bohr effect is correlated with a change in quaternary structure. In CO recombination studies a linear relationship between the release of Bohr proton and the rate of ligand saturation has been found [7–9] suggesting a correlation with the tertiary structure. On the other hand the properties of chemically modified and mutant hemoglobins in which one of the two quaternary structures is destabilized indicate a linkage of the Bohr effect to the quaternary structure [2,10–12].

In this respect the properties of artificial intermediates are of great interest, because these molecules have two chains frozen in the liganded state while the other two are free to bind ligands. Banerjee and Casso studying oxygenation equilibria have shown [13,14] that the two aquomet hybrids have a suppressed alkaline Bohr effect, although the degree of suppression is quite different for both hybrids. The oxygenation studies of Brunori et al. [15] indicate, however, that

the cyanomet hybrids possess a suppressed but equal Bohr effect. The suppression of the Bohr effect observed for the valency hybrids may be understood in terms of tertiary structural changes, since upon ligation only two of the four subunits change their tertiary structure. Relating the Bohr effect to the T–R transition the suppressed Bohr effect of the hybrids can also be explained by assuming that of the intermediates in the unliganded form not all molecules possess the quaternary T-structure. In the latter case, however, the full Bohr effect should be restored by addition of an effector such as IHP or DPG, which are known to stabilize the T-structure [16].

To obtain more information on this subject we measured the Bohr effect of the artificial aquomet and cyanomet intermediates in the presence and absence of IHP and DPG. The results are compared with those obtained for hemoglobin.

2. Materials and methods

Hemoglobin was prepared according to the toluene method of Drabkin [17]. The hemoglobin solutions were freed from organic phosphates by passage over a mixed bed ion-exchange column (Amberlite IRA 400 and IR 120). For the preparation of α and β chains carbon monoxide hemoglobin was reacted with p-chloromercuribenzoate [18]. The chains were separated on DEAE-Sephadex (start buffer, 0.1 M Tris-HCl, pH 8.0; limit buffer, 0.1 M Tris-HCl, pH 8.0, 0.4 M NaCl). The regeneration of the SH-groups of the α and β chains was achieved by a β-mercaptoethanol treatment on G25-Sephadex [19]. After this treatment the α chains contained 1.0 free SH-groups as judged from a Boyer titration [20]. A number of 2.0 free SH-groups for the β chains was found after they had been incubated for approximately 12 h with a 5-fold excess of dithiothreitol, which was removed on G25-Sephadex.

To check whether this procedure yielded native α^{SH} and β^{SH} chains, the chains were recombined to form hemoglobin. The hydrogen ion titration curve of the recombined α and β chains was within the experimental accuracy identical with the titration curve obtained with freshly prepared oxyhemoglobin. For the sedimentation coefficient of the recombined hemoglobin an apparent value of 4.1 S (20°C, 0.1 M KCl, pH 7.3) was found, identical to the value we found for oxyhemoglobin.

After replacement of the bound CO by O_2 (see below) the chains were oxidized by adding a stoechiometric amount of $K_3Fe(CN)_6$ in 0.2 M phosphate buffer pH 6.6 [21]. Immediately after chain oxidation the hybrids were prepared by adding the other chain having CO bound. If required a small excess of KCN was added to obtain the cyanomet hybrids.

Measurements of the apparent sedimentation coefficient of the hybrids yielded a value of 4.5 S (25°C, 0.05 M KCl, 0.05 M bis–tris buffer, pH 7.0). Electrophoresis showed that no single chains were present. The percentage of oxidized heme groups was determined by optical spectroscopy; samples which showed a deviation of more than 5% from the theoretical value were discarded. ^{13}C-NMR spectra of the hybrids reacted with ^{13}CO (Stohler Isotope Chemicals), recorded one week after preparation, showed one single resonance characteristic for the reduced chain [22,23] indicating that heme exchange from one chain to the other did not occur. Nevertheless all experiments were carried out within four days after chain recombination.

Ultracentrifugation experiments were performed with a model E Spinco ultracentrifuge at a speed of 67 770 rpm.

The ^{13}C-NMR spectra were obtained at 25.2 MHz on a Varian XL-100 spectrometer equipped with a Varian 620/L computer using the pulse Fourier transform technique.

Electrophoresis was performed with the Gelman Sepratek electrophoresis system.

DPG (Calbiochem), obtained as the pentacyclohexylammonium salt, was converted to the acid form by passage through Amberlite IR 120. The concentration of the DPG stock solution was determined by titration. The DPG solutions were neutralized with NaOH.

The concentration of the IHP (Sigma) solutions was determined by weight.

The Bohr curves in the presence and the absence of IHP and DPG were measured at 25°C with a pH-stat equipment constructed for this type of experiments [24]. After the hybrids were freed from the phosphate buffer, removal of CO was achieved in a rotating tonometer, passing oxygen over the solution under constant illumination. The tonometer was cooled by ice to 0°C. Subsequently the hybrids were deoxygenated under a constant flow of argon. From the tonometer a known volume was transferred anaerobically to the

titration vessel of the pH-stat equipment. Deoxygenation was checked for completeness by withdrawing anaerobically a small amount of the solution from the titration vessel followed by measurement of the optical spectrum. For the measurement of the Bohr effect of hemoglobin the same procedure was followed.

3. Results and discussion

Fig. 1 shows the Bohr effect of hemoglobin in the absence and the presence of a 6-fold excess of IHP. The curves shown are very similar to those presented by Kilmartin [21]. The additional IHP induced Bohr effect observed (i.e., the Bohr effect in the presence of IHP minus the Bohr effect in the absence of IHP) is due to a difference in interaction of IHP with oxy- and deoxyhemoglobin.

Figs. 2 and 3 show the Bohr effect of the aquomet

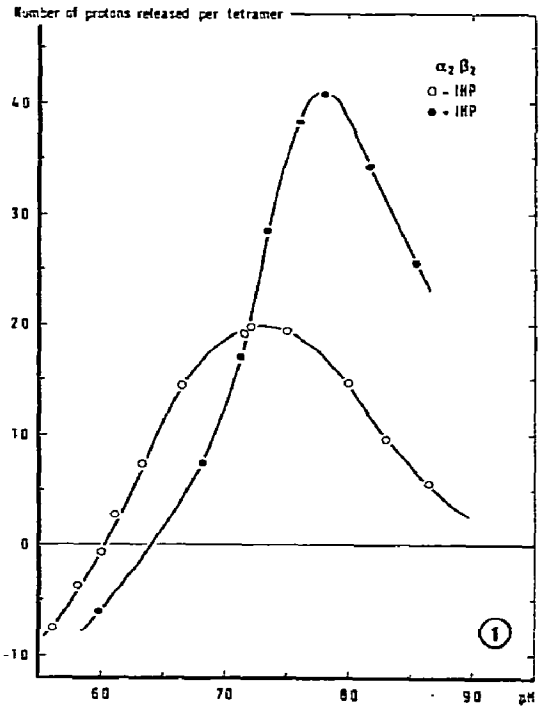

Fig. 1. The Bohr effect of hemoglobin (measured as the number of protons released per tetramer upon ligation) in the presence (●) and the absence (○) of IHP; hemoglobin concentration, 1.7 × 10^{-4} M on tetramer basis; IHP concentration, 1.0 × 10^{-3} M; 0.1 M KCl; 25°C.

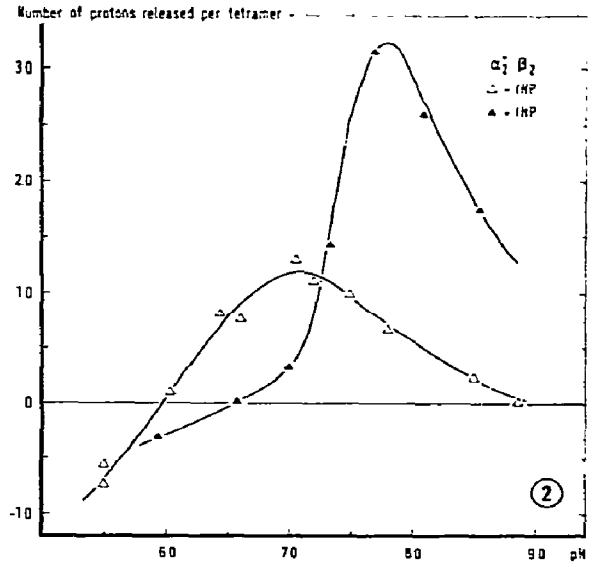

Fig. 2. The Bohr effect of $\alpha_2^+\beta_2$ (measured as the number of protons released per tetramer upon ligation) in the presence (▲) and the absence (△) of IHP; hybrid concentration, 1.7 × 10^{-4} M on tetramer basis; IHP concentration, 1.0 × 10^{-3} M; 0.1 M KCl; 25°C.

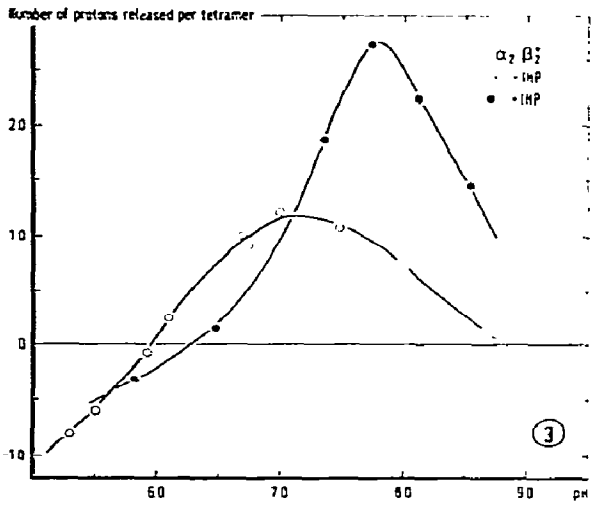

Fig. 3. The Bohr effect of $\alpha_2\beta_2^+$ (measured as the number of protons released per tetramer upon ligation) in the presence (●) and the absence (○) of IHP; hybrid concentration, 1.7 × 10^{-4} M on tetramer basis; IHP concentration, 1.0 × 10^{-3} M; 0.1 M KCl; 25°C.

hybrids with and without IHP. In the absence of IHP both aquomet hybrids show a Bohr effect which is about half the Bohr effect of hemoglobin. This result seems to contradict the observation of Banerjee and Cassoly [13,14], that the hybrid with the β chain in the oxidized form has a Bohr effect twice as large as that of the other hybrid. In our opinion this apparent discrepancy can be accounted for by a difference in solvent conditions.

If the Bohr effect is related to the T–R transition, the observed suppression of the Bohr effect for the hybrids would suggest that in the deoxygenated form about half of the molecules possess the R quaternary structure. In this event it is likely that addition of IHP will result in a Bohr effect comparable to that found for hemoglobin in the presence of IHP. Figs. 2 and 3 show that this is definitely not so. The maximum value for the Bohr effect measured in the presence of IHP is significantly lower than the value measured for hemoglobin in the presence of IHP.

The influence of IHP on the Bohr effect of the cyanomet hybrids is shown in the figs. 4 and 5. In the absence of IHP a decreased Bohr effect is observed of about half the effect of normal hemoglobin. It should

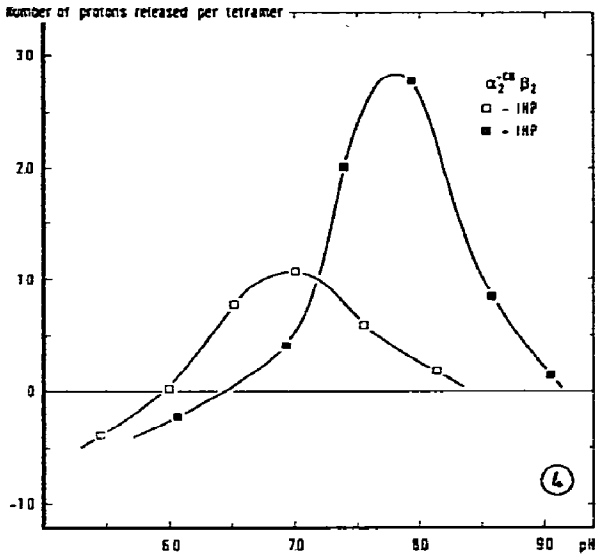

Fig. 4. The Bohr effect of $\alpha_2^{+CN}\beta_2$ (measured as the number of protons released per tetramer upon ligation) in the presence (■) and the absence (□) of IHP; hybrid concentration, 1.7×10^{-4} M on tetramer basis; IHP concentration, 1.0×10^{-3} M; 0.1 M KCl; 25°C.

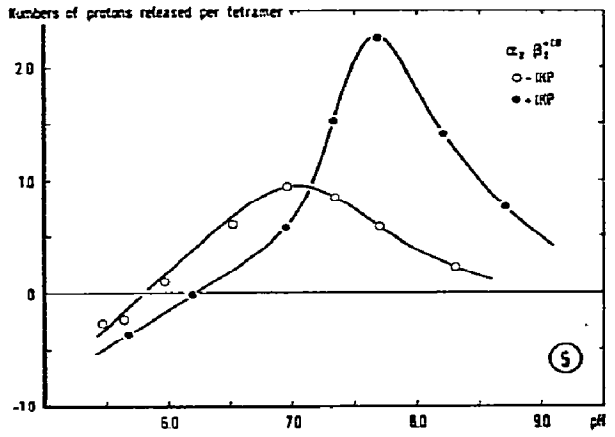

Fig. 5. The Bohr effect of $\alpha_2\beta_2^{+CN}$ (measured as the number of protons released per tetramer upon ligation) in the presence (●) and the absence (○) of IHP; hybrid concentration, 1.7×10^{-4} M on tetramer basis; IHP concentration, 1.0×10^{-3} M KCl; 25°C.

be stressed here that this Bohr effect is observed while it is known from NMR studies [25] that in the absence of phosphates the deoxy cyanomet hybrids are for the greater part in the R quaternary state. As is seen the effect of IHP on the Bohr effect of the cyanomet hybrids

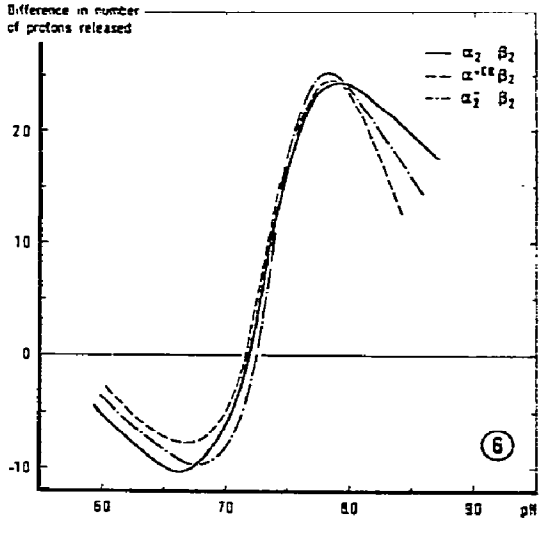

Fig. 6. The additional IHP induced Bohr effect (i.e. the Bohr effect with IHP minus the Bohr effect without IHP) of $\alpha_2^{+}\beta_2$ (–·–), $\alpha_2^{+CN}\beta_2$ (– – –) and hemoglobin (——). Experimental conditions as in figs. 1, 2, and 4.

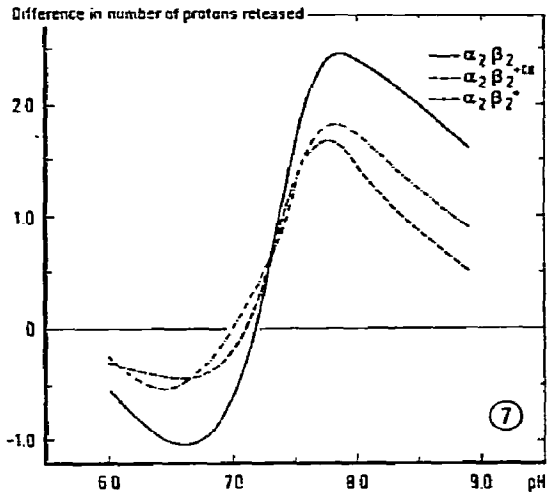

Fig. 7. The additional IHP induced Bohr effect of $\alpha_2\beta_2^+$ (–·–), $\alpha_2\beta_2^{+CN}$ (– – –) and hemoglobin (——). Experimental conditions as in figs. 1, 3 and 5.

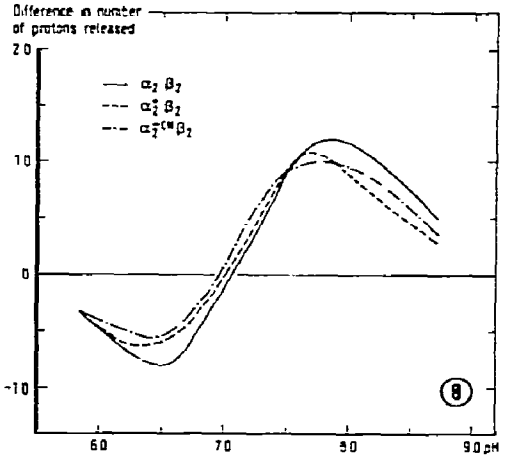

Fig. 8. The additional DPG induced Bohr effect of hem (——), $\alpha_2^+\beta_2$ (– – –) and $\alpha_2^{+CN}\beta_2$ (–·–); protein concen 1.3 × 10^{-4} M on tetramer basis; DPG concentration, 1. 10^{-3} M; 0.1 M KCl; 25°C.

is similar to that observed for the aquomet hybrids.

Figs. 6 and 7 show the additional IHP induced Bohr effect of hemoglobin and of the aquo- and cyanomet hybrids. The figures clearly demonstrate that the additional Bohr effect of the hybrids resembles very much that found for hemoglobin. This result suggests that in the presence of IHP the deoxy hybrids possess a quaternary structure very similar to that of deoxy hemoglobin. The fact, however, that the additional Bohr effect of the hybrids does not exceed that observed for hemoglobin indicates that upon addition of IHP the suppressed Bohr effect observed without IHP does not become restored. Otherwise the additional Bohr effect of the hybrids should have exceeded that of hemoglobin.

Figs. 8 and 9 show the additional DPG induced Bohr effect of hemoglobin and of the aquo- and cyanomet hybrids. It is seen that DPG has an influence on the Bohr effect of the hybrids analogous to the effect of IHP. From these data the same conclusions are reached as from the results obtained with IHP.

The results presented so far strongly suggest that the alkaline Bohr effect is related to the state of ligation of the subunits within the tetramer rather than to the change in quaternary structure of the hemoglobin tetramer. This conclusion is supported by the observation that the sum of the Bohr effects of the two cyanomet and the two aquomet spin state hybrids i equal to the Bohr effect of normal hemoglobin.

Our observations are in accordance with the l studies of the Bohr effect [7–9] and with the st chemical model presented by Perutz [6]. The ob tions of Olson and Gibson (9) that in case of n-b isocyanide binding the β chains contribute 20% Bohr effect and the α chains 80% cannot be reac

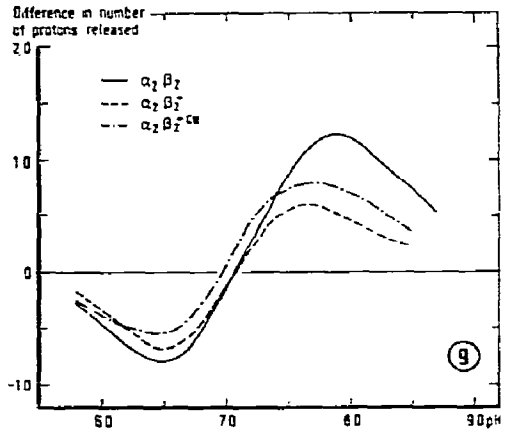

Fig. 9. The additional DPG induced Bohr effect of hem (——), $\alpha_2\beta_2^+$ (– – –) and $\alpha_2\beta_2^{+CN}$ (–·–); experimental c tions as in fig. 8.

understood unless there are ligand specific effects, because the four hybrids show similar Bohr effects, suggesting an equal contribution of both chains to this effect in case of oxygen binding.

In addition to the things we discussed some other features of the data presented need some further comment. First figs. 6–9 show that the intermediates with the β chains in the ferric form have a lower additional Bohr effect than the intermediates with the β chain in the ferrous form, which have an additional Bohr effect which is almost identical to the one of hemoglobin. This observation can be understood taking into account that in the T state IHP and DPG are bound at the entrance of the central cavity by a cluster of positively charged groups located on the β chains [26,27]. In the intermediates the geometry of this binding site is more likely to be similar to that in deoxyhemoglobin when the β chains are in the reduced form, than when they are in the ferric form. A similar difference in behaviour of the hybrids has been observed for the binding of DPG and some spin labels [28,29].

Secondly no difference is found between the low spin cyanomet hybrids and the high spin aquomet hybrids even at low pH. In other words the spin state of the heme iron of the two chains in the ferric form does not influence the Bohr effect of the intermediates studied.

Finally, since part of the Bohr effect of hemoglobin is due to difference in interaction of chloride ions with the T and R state [4,5], the question remains which part of the Bohr effect of the intermediates might be due to differences in interactions of these ions with the unligated and ligated hybrid. Studies on this subject and on the interaction of IHP separately with the oxy- and deoxy hybrids are in progress.

Acknowledgement

This work was supported by the Netherlands Organization for the Advancement of Pure Research (ZWO) under auspices of the Foundation for Chemical Research (SON).

The authors thank Mr. A.F.M. Simons, Mrs. P.M. Loontjens-Pieterse, Mr. B.J.H.C. Gröniger and Mr. P.A.A. van Puyenbroek for their assistance with the preparation of the hybrids, Dr. J.A.L.I. Walters for the ultracentrifuge experiments and Mr. J.W.M. van Kessel for the NMR experiments.

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