The Interaction of Inositol Hexaphosphate with Methaemoglobin

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1. Inositol hexaphosphate causes the shape of the oxidation–reduction equilibrium curve to become hyperbolic at acid pH values. 2. Inositol hexaphosphate also causes a decrease in the alkaline oxidation Bohr effect at these same pH values. 3. These results support the idea that inositol hexaphosphate causes methaemoglobin to take up the deoxyhaemoglobin quaternary structure at pH 6.5.

The shape of the oxygen equilibrium curve of haemoglobin is practically independent of pH (Antonini *et al.*, 1962). In contrast, the shape of the oxidation–reduction (redox) equilibrium curve is pH dependent (Antonini *et al.*, 1964). At pH 6.5 the value of the Hill's constant n is 1.3, and at pH 8.5 the value of n is 2.5, where n is defined as:

$$n = 2.303 \frac{RT}{F} \cdot \frac{d[\log(y/1-y)]}{dE}$$

where y is the percentage oxidation, E the redox potential and F is Faraday's constant.

Two explanations have been offered for this pH dependence of the redox equilibrium. The first suggests that there is a pH-dependent difference in the intrinsic redox potentials of the α and β chains in the intact tetramer (Brunori *et al.*, 1968). Thus at pH 6.5 there would be a large difference in redox potential between the α and β chains, which would give a low value of n due to pseudo non-co-operativity, whereas at pH 8.5 there would be little difference, allowing the true value of n to be expressed. This explanation found support when the pH dependence of the redox potentials of the isolated chains was examined (Banerjee & Cassoly, 1969).

The second explanation was first advanced by Perutz (1972) and forms the basis for the studies by M. F. Perutz, A. R. Fersht, S. R. Simon, J. G. Beestone & E. F. Slade (unpublished results). These are summarized by Perutz (1973) and are quoted in the rest of the paper as the latter reference. This explanation suggests that the change in shape of the redox equilibrium with pH follows the form predicted by the allosteric theory of Monod *et al.* (1965). This theory has been found useful in describing many functional aspects of haemoglobin (Perutz, 1970; Edelstein, 1971; Ogata & McConnell, 1972b; Shulman *et al.*, 1972; Szabo & Karplus, 1972) and will be used to describe the results presented in the present paper. The theory defines two forms of haemoglobin, one having low ligand affinity (the T structure) with

the quaternary structure of deoxyhaemoglobin, and another having high ligand affinity (the R structure) with the quaternary structure of oxyhaemoglobin. The theory proposes that the transition between these two structures is responsible for the co-operativity observed in the reaction of haemoglobin with ligands.

Perutz (1970) proposed a mechanism for the reaction of haemoglobin with ligands, which, though derived from the allosteric theory of Monod *et al.* (1965), differed from it in one important aspect: this was that the tertiary structure of each subunit changes when ligand is bound in either the R or T structures. Thus in the oxidation reaction the α chain could take up four different structures depending on its oxidation state and on the quaternary structure of the tetramer: $[\alpha_{\text{deoxy}}]^T$; $[\alpha_{\text{deoxy}}]^R$; $[\alpha_{\text{met}}]^T$; $[\alpha_{\text{met}}]^R$; and similarly for the β -chain. However, this modification of the allosteric theory states that the change in tertiary structure caused by the oxidation of any one chain is completely non-co-operative unless accompanied by a transition of the quaternary structure from T to R (Perutz, 1972; Ogata & McConnell, 1972b). If this transition is inhibited by stabilizing the molecule in either the deoxy (T) or the oxy (R) quaternary structure then the allosteric theory predicts that no co-operativity will be observed.

The equilibrium between the two alternative quaternary structures depends on the tertiary structure of the subunits, and this in turn, according to Perutz (1970), is regulated mainly by the displacement of the iron atoms from the plane of the porphyrin ring. In oxyhaemoglobin, which is low-spin ferrous, the iron atoms lie in the plane of the ring; in deoxyhaemoglobin, which is high-spin ferrous, they are displaced from the ring by 0.075 nm (0.75 Å). In aquomethaemoglobin, which is high-spin ferric, the displacement is 0.03 nm (0.3 Å) or about halfway between oxy- and deoxy-haemoglobin. Perutz (1973) then argued that the tertiary structure of the methaemoglobin chains might be equally stable in an oxy-like or a deoxy-like conformation, and that a

solution of methaemoglobin might contain both quaternary forms in dynamic equilibrium. Thus Perutz (1973) proposes that methaemoglobin at pH 6.5, consisting almost wholly of the high-spin ferric aquomet form, contains a small but significant amount of T structure (about 10%). This is the structure normally associated with unliganded haemoglobin, hence in the oxidation of deoxyhaemoglobin at pH 6.5 there would be an incomplete transition from the T to R forms, leading the allosteric theory to predict the low value of n observed (Antonini *et al.*, 1964). This equilibrium between the R and T forms of methaemoglobin would be pH dependent for two reasons. At high pH the concentration of the low-spin hydroxymethaemoglobin rises relative to that of the high-spin aquo form; in the low-spin form the iron lies in the plane of the porphyrin ring, which favours the R state. Also at high pH the salt bridges between the subunits that stabilize the quaternary deoxy structure become weaker. Consequently the fraction of methaemoglobin molecules in the T state should decrease with increasing pH. Thus methaemoglobin at pH 8.5 would contain extremely small amounts of T structure (less than 0.01%), like oxyhaemoglobin at both pH 6.5 and 8.5; thus a complete transition from the R to T structures is possible, allowing full expression of co-operativity according to the allosteric theory, in agreement with the observed rise in n with pH for the redox equilibrium (Antonini *et al.*, 1964).

A quantitative analysis of these predictions in terms of the allosteric theory has not yet been attempted. However, similar analyses have been described for modified or abnormal haemoglobins which might have alterations in the relative amounts of the R or T structures in either the liganded or unliganded forms (Edelstein, 1971; Ogata & McConnell, 1972a; Shulman *et al.*, 1972; Szabo & Karplus, 1972), and a reasonable fit with the observed values of n was found.

Most of the evidence presented by Perutz (1973) for methaemoglobin at pH 6.5 having a small amount of T structure was based on the use of IHP.* This substance considerably lowers the oxygen affinity of normal haemoglobin (Benesch *et al.*, 1968a), probably due to its binding much more strongly to the T form than to the R form (Gray & Gibson, 1971). In methaemoglobin at pH 6.5, allosteric theory predicts that IHP would bind so strongly to the very small amount of T structure present that it pushes the equilibrium over in favour of the T structure. Hence methaemoglobin at pH 6.5 in the presence of IHP could be almost wholly in the deoxy quaternary structure. The various states of methaemoglobin in the presence and absence of IHP at pH 6.5 could be described by Scheme 1.

*Abbreviation: IHP, inositol hexaphosphate.

Scheme 1. Various states of methaemoglobin in the presence and absence of inositol hexaphosphate at pH 6.5

Thus at pH 6.5, in the absence of IHP, methaemoglobin would be predominantly in the R_{met} form, while in the presence of IHP it would be mainly in the $IHP-T_{met}$ form. At pH 8.5, the affinity for IHP is diminished and also the amount of T_{met} in the absence of IHP is so small that addition of IHP increases the amount of the $IHP-T_{met}$ form by only a negligibly small amount; thus only two forms of methaemoglobin would be present, the R_{met} form and the $IHP-R_{met}$ form. Similarly for oxyhaemoglobin at both pH 6.5 and 8.5 only two forms would be present, the R_{oxy} form and the $IHP-R_{oxy}$ form. In deoxyhaemoglobin under all these conditions only two forms would be present: T_{deoxy} and $IHP-T_{deoxy}$. At present none of the equilibrium constants for any of the reactions described above has been measured, except for an approximate value for the reaction of IHP with R_{oxy} (Gray & Gibson, 1971), and an overall equilibrium constant for the reaction of methaemoglobin with IHP at pH 6.5 (Perutz, 1973).

Perutz (1973) presented evidence showing that addition of IHP to methaemoglobin at pH 6.5 caused changes in the absorption spectra, the u.v. circular dichroism, the paramagnetic susceptibility and a lowering of the reactivity of the thiol groups all consistent with a change in quaternary structure to the deoxy form, since an identical series of changes occur in the transition from oxy- to deoxy-haemoglobin. At pH 8.5 addition of IHP to methaemoglobin was without effect on any of these parameters.

These proposals of Perutz (1973) represent an opportunity to test the general applicability of the allosteric theory of Monod *et al.* (1965) to the reaction of haemoglobin with ligands. Two main predictions follow from these proposals. First, IHP should cause an overall lowering in the value of n for the redox equilibrium at acid pH values, since it increases the amount of T form present in methaemoglobin solutions. Secondly, the lowering of the reactivity of the thiol groups on deoxygenation of haemoglobin is associated with the formation of the salt bridge between histidine 146 β and aspartic acid 94 β in deoxyhaemoglobin where this bridge crosses in front of the thiol group; in oxyhaemoglobin the reactivity of the thiol group is high because histidine 146 β

floats free and does not form its salt bridge (Perutz *et al.*, 1969; Geraci & Sada, 1972). This salt bridge is responsible for about half the alkaline Bohr effect (Kilmartin *et al.*, 1973). The alkaline Bohr effect is due to groups in haemoglobin changing their average pK values from 7.8 to 6.8 on ligation (Rossi-Bernardi & Roughton, 1967). The lowering of the reactivity of the thiol groups of methaemoglobin on combination with IHP at pH 6.5 suggests that if the transition to the T structure does occur then it is accompanied by closure of the salt bridge involving histidine 146 β . Thus in the oxidation of haemoglobin at pH 6.5 in the presence of IHP this salt bridge would remain intact, and the Bohr effect would be decreased. A measurement of this decrease will tell us whether the other salt bridges involved in the Bohr effect also remain intact; thus if methaemoglobin is in the T form under these conditions then this provides information on the configuration of the salt bridges in this form of methaemoglobin.

Experimental

Materials

Reagents. Phytic acid (inositol hexaphosphate) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Toluyene Blue was obtained from G. T. Gurr, Searle Scientific Services, High Wycombe, Bucks., U.K. Dichlorophenol-indophenol was obtained from BDH (Chemicals) Ltd., Poole, Dorset, U.K.

Gases. Nitrogen (Oxygen-Free Nitrogen) was obtained from British Oxygen Co., Ipswich, U.K. and Oxygen (Medical Grade) was obtained from British Oxygen Co., London S.W.19, U.K.

Human blood. Human blood was obtained as partially filled bottles less than 1 week old from the National Blood Transfusion Centre, Cambridge, U.K.

Methods

Preparation of human deoxyhaemoglobin and methaemoglobin. Human carbonmonoxyhaemoglobin was prepared as described by Kilmartin & Rossi-Bernardi (1971). Carbon monoxide was removed as described by these authors and 18 ml of oxyhaemoglobin solution (150 mg/ml) was passed through a Sephadex G-25 (fine) column (2.5 cm $\times$ 20 cm) equilibrated with 0.001 M- Na_2HPO_4 to remove most of the salt. The solution was then passed through a 2.5 cm $\times$ 20 cm Dintzis column (Nozaki & Tanford, 1967) (this is a mixed-bed ion-exchange column used to de-salt the haemoglobin solution), KCl was added to a final concentration of 0.1 M and the solution deoxygenated as described by Kilmartin & Rossi-Bernardi (1971). Total phosphate analysis (Chen *et al.*, 1956) of the haemoglobin solution

showed less than 0.02 mol of inorganic phosphate/mol of haemoglobin tetramer.

Methaemoglobin was prepared by addition of 5 mol of NaNO_2 /mol of haem iron to a solution of oxyhaemoglobin at pH 7.0 at 20°C for 5 min. The solution was then immediately passed through a Sephadex G-25 (fine) column equilibrated with 0.001 M- Na_2HPO_4 to remove excess of nitrite and then through a Dintzis column as described above. If necessary, the solution was also deoxygenated.

Preparation of free β chains in the met form. The β chains were prepared as described by Geraci *et al.* (1969) and the carbon monoxide was removed as described above. The oxy β chains (20 mg/ml) were made 0.2 M in sodium phosphate, pH 6.6 (2.025 g of Na_2HPO_4 and 3.342 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ /l) and 1 mM in EDTA. After addition of 1.2 mol of $\text{K}_3\text{Fe}(\text{CN})_6$ /mol of haem iron at 20°C, the mixture was left for 10 min and then passed through a column of Sephadex G-25 (fine) equilibrated with 0.2 M-sodium phosphate-1 mM-EDTA, pH 6.6.

Measurement of redox equilibrium curves. Redox equilibrium curves were measured by the method of mixtures as described by Antonini *et al.* (1964) between 20 and 70% oxidation. Haemoglobin concentration was 6.4 mg/ml (0.1 mM in haemoglobin tetramer) in 0.1 M-KCl and 0.02 mM-Toluyene Blue and 0.02 mM-dichlorophenol-indophenol. When necessary IHP at a final concentration of 1 mM was added. The two redox dyes were always added anaerobically to the deoxyhaemoglobin solution, since in the presence of oxygen they tended to oxidize the deoxyhaemoglobin. The methaemoglobin concentration in the deoxyhaemoglobin solutions after the addition of the redox dyes was measured by addition of deoxygenated ferricyanide and following the oxidation from the change in pH. The methaemoglobin concentration averaged 10%. The pH and redox potential were measured with a radiometer pH meter 26 by using a glass electrode G202C, calomel electrode K4112, and platinum electrode P101, all manufactured by Radiometer. Equilibrium (defined as a change in redox potential of less than 0.2 mV) was usually attained within 5 min, and remained constant over at least 20 min. The pH was kept constant throughout the equilibrium curve by appropriate additions of deoxygenated 0.03 M-HCl-0.07 M-KCl or 0.03 M-KOH-0.07 M-KCl.

Measurement of the effect of IHP on the redox potential of β chains. An equal mixture of met and oxy β chains (10 mg/ml) in 0.2 M-sodium phosphate-1 mM-EDTA, pH 6.6, with or without 1 mM-IHP, was deoxygenated, and deoxygenated Toluyene Blue solution added to a final concentration of 0.02 mM. The spectrum of this mixture was measured by withdrawing a sample with a syringe, previously washed out with the mixture to remove oxygen, and injecting the sample into a deoxygenated cuvette. The recorded

spectrum corresponded exactly to that calculated from the addition of the two spectra of β chains in the met and deoxy forms. The recorded redox potentials of β chains were constant to within 1 mV over a period of 30 min.

Measurement of the Bohr effect. A solution of deoxyhaemoglobin (10 mg/ml) in 0.1 M-KCl was oxidized by stoichiometric addition of deoxygenated potassium ferricyanide (Brunori *et al.*, 1965), or oxygenated by blowing oxygen over it, and the amount of acid or alkali necessary to restore the pH to its original value was recorded.

Measurement of the change in charge on addition of IHP or ferrocyanide to haemoglobin. A stock solution of IHP was prepared and adjusted to a particular pH. A portion, corresponding to 1.5-fold excess per haemoglobin tetramer, was added to 0.1 M-KCl and the pH recorded. The appropriate form of haemoglobin was adjusted to the latter pH value and the portion of IHP added. The amount of acid necessary to restore the pH to its original value was recorded.

Since potassium ferrocyanide does not buffer in the neutral pH range, pH adjustment was not necessary before addition to the haemoglobin solution.

Results

Effects of IHP

Effect on the redox equilibrium curves. The effect of IHP on the redox potential at half oxidation and the value of the Hill's constant n at various pH values is shown in Fig. 1. A large change in redox potential is caused by IHP. The value of n for the redox equilibrium is lowered by IHP at all pH values studied except 8.5.

Effect on the redox potential of β chains. The spectra of the met β chains prepared here were slightly different from those prepared by Banerjee & Cassoly (1969); in particular the peak at 530 nm was smaller in the met β chains prepared here. The met chains were quite stable in 0.2 M-sodium phosphate-1 mM-EDTA, pH 6.6; however, in lower phosphate concentrations or at more alkaline pH values the met chains were unstable. The recorded redox potential of β chains (10 mg/ml) at 50% oxidation in 0.2 M-sodium phosphate-1 mM-EDTA, pH 6.6, was 89 mV at 25°C; addition of a tenfold excess of IHP per β chain tetramer caused only a very slight change to 90 mV.

It was not established whether the met β chains were present as β_4 tetramers similar to oxy β chains (Bucci *et al.*, 1965) nor was the effect of IHP on this aggregation investigated.

Effect on the oxidation Bohr effect. The oxidation Bohr effect in the absence of IHP is shown in Fig. 2. This experiment is carried out by oxidizing deoxy-

Fig. 1. Effect of IHP on the redox potential and Hill's constant (n) of haemoglobin

(a) Redox potential at half oxidation of human haemoglobin (6.4 mg/ml) in 0.1 M-KCl (○), or with a tenfold excess of IHP per haemoglobin tetramer in 0.1 M-KCl (●). (b) Values of n under the same conditions.

haemoglobin with potassium ferricyanide; hence at the end of it there is left a mixture of methaemoglobin and 1 mol of ferrocyanide/mol of haem. If this ferrocyanide binds to methaemoglobin then protons might be taken up or released, seriously affecting the observed value of the oxidation Bohr effect. This proton release or uptake can be measured from the pH change on addition of ferrocyanide to methaemoglobin and the amount of acid or alkali necessary to restore the pH to its original value. This change in charge, shown in Fig. 2, is quite considerable at acid pH values and decreases the observed value of the oxidation Bohr effect. This change in charge must be added to the observed oxidation Bohr effect to obtain the true oxidation Bohr effect as shown in Fig. 3.

The effect of IHP on the oxidation Bohr effect is also shown in Fig. 3. Here no correction for the proton uptake on binding of ferrocyanide to methaemoglobin is necessary because this is negligible in the presence of IHP (Fig. 2). Presumably methaemoglobin binds both ferrocyanide and IHP at the same site, but its affinity for IHP is much higher than for ferrocyanide. At pH 6.5, where IHP is thought to

Fig. 2. Oxidation Bohr effect and change in charge on binding of ferrocyanide to methaemoglobin with and without IHP

Oxidation Bohr effect of human haemoglobin (10mg/ml) in 0.1M-KCl (○). Uptake of protons/haem on binding ferrocyanide to methaemoglobin (10mg/ml) in 0.1M-KCl (□) or in 0.1M-KCl with a 1.5-fold excess of IHP per haemoglobin tetramer (■).

Fig. 3. Corrected oxidation Bohr effect with and without IHP

Oxidation Bohr effect of human haemoglobin (10mg/ml) in 0.1M-KCl after correction for proton uptake due to ferrocyanide binding to methaemoglobin (○); oxidation Bohr effect in 0.1M-KCl with a 1.5-fold (■) or 10-fold (●) excess of IHP per haemoglobin tetramer. The calculated values of the oxidation Bohr effect (Δ) found by addition of the values of the oxidation Bohr effect in the absence of IHP to the difference in proton uptake on binding IHP between met- and deoxy-haemoglobin are shown for comparison, with a line drawn through the measurements of the oxidation Bohr effect in the presence of the same amount of IHP (■). The values of $[(\Delta E_{\frac{1}{2}} F)/(2.303 RT)]/\Delta pH$ (Δ) calculated from the redox equilibrium data in Fig. 1 are shown for comparison with the oxidation Bohr effect measurements with the same amount of IHP (●).

change methaemoglobin from mainly the R form to mainly the T form, the oxidation Bohr effect is diminished as expected. It would be interesting to know how much of the oxidation Bohr effect remains at pH 6.5. A quantitative estimate of this is complicated by: (i) the residual oxidation Bohr effect (Brunori *et al.*, 1964) (this is an extra Bohr effect over and above the oxygenation Bohr effect and observed even in single-chain haemproteins); (ii) the acid Bohr effect due to groups in haemoglobin changing their average pK values from 5.0 to 5.6 on ligation (Rossi-Bernardi & Roughton, 1967); (iii) the proton uptake associated with the binding of IHP to the R and T forms. This problem will be dealt with in the Discussion section below.

Change in charge on binding of IHP to haemoglobin

An alternative way of measuring how much of the oxidation Bohr effect is suppressed when IHP interacts with methaemoglobin is to measure the change in charge associated with this binding and compare it with similarly induced changes in charge in oxy- and deoxy-haemoglobin, as shown in Fig. 4. Both cyanomethaemoglobin and oxyhaemoglobin take up very similar amounts of protons in the presence of IHP since they are both low-spin with the iron atoms lying in the plane of the porphyrin ring; thus they probably exist wholly in the R structure even in the presence of IHP since IHP causes only small changes

Fig. 4. Change in charge on binding of IHP to various forms of haemoglobin

Uptake of protons/haem observed on mixing solutions of methaemoglobin ($\square$), cyanomethaemoglobin (Δ), oxyhaemoglobin ($\bullet$), deoxyhaemoglobin ($\circ$) and IHP of the same initial pH. The haemoglobin (10 mg/ml) was in 0.1 M-KCl, and the IHP was added in a 1.5-fold excess over haemoglobin tetramers.

in the u.v. circular dichroism, or the reactivity of the thiol groups of these haemoglobins (Perutz, 1973; M. F. Perutz, A. R. Fersht, S. R. Simon, J. G. Beestone & E. F. Slade, unpublished results). Deoxyhaemoglobin releases different amounts of protons at all pH values because it has the T structure and IHP binds to this in a way that is different from that to the R structure.

If IHP causes methaemoglobin to change from the R to T structure then the proton uptake on addition of IHP should be at least as great as that observed on binding to the T structure. In addition, if any of the Bohr group salt bridges were formed in this R to T transition then this proton uptake should be greater than the T structure proton uptake. If all the Bohr group salt bridges were formed, including the acid Bohr groups, then the predicted curve for proton uptake at pH values where IHP caused the R to T transition in methaemoglobin would be given by the addition of the curve for proton uptake on binding to the T structure to the curve for the Bohr effect of haemoglobin in 0.1 M-KCl (Fig. 5).

The measured proton uptake on addition of IHP to methaemoglobin is seen to be steadily greater than to deoxyhaemoglobin between pH 6.5 and 7.0 in agreement with the proposals of Perutz (1973) that IHP causes the R to T transition in methaemoglobin with formation of some of the Bohr group salt bridges at these pH values. At pH 8.5, where Perutz

Fig. 5. Oxygenation Bohr effect with and without IHP

Oxygenation Bohr effect of human haemoglobin (10 mg/ml) in 0.1 M-KCl ($\circ$) or in 0.1 M-KCl with a 1.5-fold excess of IHP per haemoglobin tetramer ($\bullet$). Calculated values (Δ) of the Bohr effect in the presence of IHP are shown for comparison. These are obtained by addition of the value of the oxygenation Bohr effect in the absence of IHP to the difference in proton uptake caused by IHP on binding to oxy- and deoxy-haemoglobin seen in Fig. 4.

(1973) proposes that methaemoglobin behaves similarly to oxyhaemoglobin, the proton uptakes on binding IHP to the two forms of haemoglobin are identical and quite different from deoxyhaemoglobin.

As an internal check of these results the oxygenation or oxidation Bohr effects were measured in the presence of IHP. If there are no complications, then the oxygenation Bohr effect should correspond to the sum of the protons released in the absence of IHP plus the difference in proton binding between deoxy- and oxy-haemoglobin caused by the binding of IHP. Similarly the oxidation Bohr effect should correspond to the sum of the protons released in the absence of IHP plus the difference in proton binding between deoxy- and met-haemoglobin caused by the binding of IHP. These differences caused by the binding of IHP are obtained by subtraction of the appropriate two lines in Fig. 4. The results of these calculations are in the form of open triangles in Figs. 3 and 5. The agreement between observed and calculated curves shows that the changes in the Bohr effect due to the presence of IHP are consistent with the uptake of protons observed on its binding to the different forms of haemoglobin.

Discussion

The redox equilibrium curves presented here support the proposal of Perutz (1973) that at pH 6.5 IHP causes the quaternary structure of methaemoglobin to change from the R to the T form. Thus in the presence of IHP the allosteric theory of Monod *et al.* (1965) would predict a value of the Hill's constant of 1.0, in excellent agreement with the values of n observed in Fig. 1. These values are accurate to only about ± 0.1 , owing to the difficulties in measuring the amount of methaemoglobin in the deoxyhaemoglobin solutions at the start of the experiment.

An alternative explanation for the lowering of the value of n for the redox equilibrium by IHP is that IHP has altered the redox potential of either the α or β chains in haemoglobin. The results described here cannot reject such an explanation; however, the evidence of Perutz (1973), coupled with the lack of any effect of IHP on the redox potential of isolated β chains, does not support it, although the latter experiment does not exclude the possibility that IHP could affect the redox potential of β chains in the intact haemoglobin tetramer. The effect of IHP on the redox potential of isolated α chains was not measured here because it was found difficult to prepare stable α chains in the met form. If IHP affects the redox potential of one of the chains then it would most likely be the β chains since 2,3-diphosphoglycerate, an analogue of IHP, binds strongly to isolated β chains (Benesch *et al.*, 1968b).

The large uptake of protons on binding of ferrocyanide to methaemoglobin introduces large errors in

the measurement of the oxidation Bohr effect at acid pH values. This is shown quantitatively when the effect of pH on the redox potential (Fig. 1) is compared with the direct measurement of the Bohr protons released on oxidation (Figs. 2 and 3). According to Wyman & Ingalls (1941) & Wyman (1964), these two quantities are directly related by the linkage equation:

$$\frac{\Delta E_{\frac{1}{2}} F}{2.303 RT} / \Delta \text{pH} = \Delta H^+$$

where ΔH^+ is the total change in charge on oxidation, $E_{\frac{1}{2}}$ is the redox potential at half oxidation and F is Faraday's constant. At pH 6.8, ΔH^+ for the uncorrected oxidation Bohr effect is 0.40 proton/haem (Fig. 2) and rises to 0.65 proton/haem (Fig. 3) after correction for ferrocyanide binding. The value calculated from the change in redox potential between pH 6.6 and 7.0 in Fig. 1 is 0.73 proton/haem, clearly in much better agreement with the value of 0.65 proton/haem for the corrected oxidation Bohr effect. Values of $[(\Delta E_{\frac{1}{2}} F)/(2.303 RT)]/\Delta \text{pH}$ calculated from the redox equilibrium data with IHP are in good agreement with the values measured directly under similar conditions (Fig. 3), except for a small deviation at pH 7.0.

This uptake of protons on binding of ferrocyanide to methaemoglobin might be relevant in considering the peculiar bow shape of the curves between pH 6.2 and 6.8, found by Brunori *et al.* (1965) for the release of Bohr protons as a function of oxidation. This bow disappears in the presence of a 1.5-fold excess of IHP per haemoglobin tetramer; however, the matter was not followed up in detail.

Perutz (1973) showed that addition of IHP lowers the reactivity of the thiol group of methaemoglobin at pH 6.5 to a value similar to that in deoxyhaemoglobin. Since this lowered reactivity is probably due to the formation of a salt bridge between histidine 146 β and aspartic acid 94 β (Perutz *et al.*, 1969; Geraci & Sada, 1972), this salt bridge should also be present after IHP is added to methaemoglobin at pH 6.5. Hence the oxidation Bohr effect with IHP at this pH should be reduced by at least the calculated contribution due to histidine 146 β . This has been estimated by Kilmartin *et al.* (1973) as 0.07 proton/haem at pH 6.5, a value calculated from the measured pK values of this group in carbonmonoxy- and deoxy-haemoglobin. If IHP caused the formation of the salt bridges associated with the whole of the alkaline Bohr effect in methaemoglobin, without affecting the acid Bohr groups, then it should decrease the oxidation Bohr effect by 0.33 proton/haem at pH 6.5 (Kilmartin *et al.*, 1973).

The actual value for the suppression of the oxidation Bohr effect can now be calculated from the two sets of data described in the Results section. First it

can be calculated from the determinations of the protons released by oxidation in the presence of IHP. This consists of four parts: (i) the residual oxidation Bohr effect (Brunori *et al.*, 1964), assumed to be unaffected by IHP; (ii) the difference in proton uptake, due to IHP binding, between the initial and final haemoglobin structures; (iii) the normal Bohr effect due to ligation, consisting of a contribution from both the acid and alkaline Bohr groups; (iv) the uptake of a OH^- by methaemoglobin. The last named reaction has a pK of 8.0 at 20°C (Brunori *et al.*, 1964); hence its contribution at pH 6.5 will be small and will not be considered here. IHP has no effect on this pK since it does not alter the spectrum of methaemoglobin at pH 8.0 (M. F. Perutz, unpublished results).

Part (i), the residual oxidation Bohr effect, is calculated by subtracting the value of the oxidation Bohr effect at pH 6.5 in the absence of IHP (0.48 proton/haem from Fig. 3) from the value of the oxygenation Bohr effect at pH 6.5 without IHP (0.31 proton/haem from Fig. 5), giving a value for the residual oxidation Bohr effect of 0.17 proton/haem at pH 6.5.

Part (ii) is calculated from the difference in proton uptake on binding of IHP between the R and T structures, that is between oxy- and deoxy-haemoglobin. It should be pointed out that oxidation of deoxyhaemoglobin in the presence of IHP may not leave the T structure entirely unaltered, since the tertiary structure of the subunits changes (Anderson, 1973). Similarly, there are small differences in tertiary structure between carbonmonoxy- and met-haemoglobin (E. Heidner, personal communication), which suggest that there may be similar ones between oxy- and met-haemoglobin. However, for the purposes of this calculation, to measure the suppression of the oxidation Bohr effect by IHP, it will be assumed that these minor structural differences do not significantly affect the pK values of the Bohr groups or the pK values of the groups involved in the binding of IHP. Part (ii) is estimated from Fig. 4 by subtraction of the proton uptake on IHP binding to oxyhaemoglobin from that to deoxyhaemoglobin. At pH 6.5, this is -0.19 proton/haem; it is negative because oxyhaemoglobin takes up more protons on binding IHP than deoxyhaemoglobin, which would oppose the release of protons on oxidation.

Part (iii) may now be estimated by addition of parts (i) and (ii) and subtraction of this from the measured value of the oxidation Bohr effect at pH 6.5 in the presence of a 1.5-fold excess of IHP per haemoglobin tetramer (0.22 proton/haem from Fig. 3). This gives a value for part (iii), the normal Bohr effect in the presence of IHP, of 0.24 proton/haem at pH 6.5. This must be compared with the expected value of 0.33 proton/haem described earlier, assuming that the acid oxidation Bohr effect is not affected by IHP, which seems likely as IHP does not alter the oxidation

Bohr effect below pH 6.0. Subtraction of these two numbers then gives a value of 0.09 proton/haem for the suppression of the alkaline oxidation Bohr effect by IHP at pH 6.5.

An alternative and simpler way of measuring this value is to use the data presented in Fig. 4. If IHP causes methaemoglobin to change from the R to the T structure at pH 6.5, then the proton uptake on addition of IHP to a solution of methaemoglobin should be the sum of the proton uptake caused by adding IHP to the T structure (measured from the proton uptake on adding IHP to deoxyhaemoglobin) plus an unknown uptake due to the formation of the Bohr group salt bridges associated with the change in quaternary structure. This unknown uptake is equivalent to the suppression of the oxidation Bohr effect by IHP and can be estimated from Fig. 4 as the subtraction of the proton uptake on binding of IHP to methaemoglobin from that to deoxyhaemoglobin at pH 6.5 ($0.67 - 0.52 = 0.15$ proton/haem). This value is in reasonable agreement with the value of 0.09 proton/haem calculated earlier, considering the large number of manipulations involved in calculating the latter.

Two explanations can be invoked to account for this suppression of the oxidation Bohr effect by IHP, which cannot be distinguished on the basis of the data presented here. The first is that IHP combines with methaemoglobin differently from its combination with oxy-, deoxy- and cyano-methaemoglobin, so that the uptake of protons on binding is greater at around pH 7.0, leading to the suppression of the oxidation Bohr effect with IHP. The second explanation, favoured by the data of Perutz (1973), is that IHP causes the quaternary structure of methaemoglobin to change from mainly the R to mainly the T form, causing the formation of some of the salt bridges involved in the alkaline Bohr effect. Thus in the transition from deoxyhaemoglobin to methaemoglobin under these conditions some of the salt bridges remain formed, leading to a suppression of the oxidation Bohr effect. The calculated value of this suppression of 0.15 proton/haem at pH 6.5 is well in excess of the value of 0.07 proton/haem (Kilmartin *et al.*, 1973) to be expected if the salt bridge involving the alkaline Bohr group histidine 146 β is formed when IHP is added to methaemoglobin at pH 6.5 (Perutz, 1973). The difference between the measured suppression of 0.15 proton/haem and the calculated value for complete suppression of 0.33 proton/haem suggests that if IHP causes methaemoglobin to take up the T structure at pH 6.5 then this is not the same as the T structure observed in the crystal (Muirhead & Greer, 1970; Bolton & Perutz, 1970), since not all the salt bridges involved in the Bohr effect have been formed. A possible explanation for this might be that part of the oxidation Bohr effect is linked to the change in quaternary structure; this would include histidine

146 β and some other group or groups; and the rest is linked to changes in tertiary structure, that is the Bohr protons would be released on oxidation of the particular chain independently of whether the molecule was in the R or T structure. Thus if IHP causes the R to T transition in methaemoglobin at pH 6.5, the quaternary linked salt bridges, including histidine 146 β , would be formed, whereas the tertiary linked salt bridges would not since the chains are oxidized; this would lead to the observed partial suppression of the oxidation Bohr effect in the presence of IHP at pH 6.5.

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