Response of the Bohr Group Salt Bridges to Ligation of the T State of Haemoglobin Kansas

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In his stereochemical mechanism for haemoglobin, Perutz (1970) proposed that ligation of the T state of haemoglobin caused tertiary structure changes culminating in the breakage of Bohr group salt bridges. The bonds changed or broken in this pathway are partially responsible for the free energy of co-operativity. The Perutz scheme was supported by kinetic and equilibrium data on haemoglobin A but not by Anderson’s (1975) crystallographic work on haemoglobin Kansas, (Asn102(G4)β→ Thr) showing the Bohr group salt bridges to be intact in the liganded T state. We have confirmed Anderson’s findings by showing that the pH of a Bohr group, His 146β, remained unchanged on ligation of the T state of haemoglobin Kansas with NO, and that there was little T state Bohr effect with either NO or oxygen. To reconcile these seemingly contradictory findings in haemoglobins A and Kansas we propose that the unusual properties of the liganded T state of haemoglobin Kansas are part of a general occurrence and will be expressed in any haemoglobin with low ligand affinity whether it be caused by inositol hexaphosphate, crystallization into the deoxy form, or ligation with NO or high spin ligand. This would mean that there would be no unique stereochemical mechanism for haemoglobin.

1. Introduction

Co-operativity in haemoglobin is caused by a reversible transition between the low affinity deoxy or T state and the high affinity oxy or R state (Monod et al., 1965). The R state has the same high ligand affinity as the free α and β subunits (Noble, 1969; Tyuma et al., 1971), so the subunits in the T state must have different bonds which lower their affinity for ligand. A first step in any molecular theory of co-operativity must be the identification of these extra bonds. When the structure of deoxy-haemoglobin was solved (Muirhead & Greer, 1970; Perutz & Bolton, 1970) several salt bridges were found, both between and within the subunits, which were absent in the R structure. These included the Bohr group salt bridges, Val18 (Kilmartin &
Rossi-Bernardi, 1969) whose α amino group forms a salt bridge with an inorganic anion positioned between it and the guanidinium of Arg141α (Arnone et al., 1976), and His146β (Kilmartin & Wootton, 1970) whose imidazole forms a salt bridge with the carboxyl of Asp94β (Perutz et al., 1969). Perutz (1970) suggested that all the salt bridges including the Bohr groups were partially responsible for the low affinity of the T state. At that time the only chemical evidence supporting this proposal concerned the Bohr group salt bridges. Kinetic experiments of Antonini et al. (1965) had shown CO uptake and Bohr proton release to be simultaneous, suggesting that ligation within the T state broke the Bohr group salt bridges. Linkage theory (Wyman, 1964) then predicts a reciprocal relationship between the ligand affinity of the T state and the affinity of the Bohr groups for protons. So formation of a salt bridge lowers the ligand affinity of the T state as proposed by Perutz (1970). This can be tested in two alternative ways: either by X-ray analysis of crystals of unliganded and liganded haemoglobin in the T state to see if the salt bridges break, or by measuring the effect of removing the salt bridge on the ligand affinity of the T state, $K_T$ (mm Hg$^{-1}$).

The results of crystallographic experiments argued against the Perutz scheme since oxidation of neither the T state of normal haemoglobin (Anderson, 1973) nor haemoglobin Iwate (Greer, 1971) resulted in breakage of Bohr group salt bridges. A possible reason for this failure might have been the use of the high-spin aquomet ligand which would cause only small tertiary structural changes around the haem group. However, this explanation was invalidated when Anderson (1975) used the low-spin ligand carbon monoxide on the T state of haemoglobin Kansas (Asn102(G4), &+Thr) and again found the Bohr group salt bridges unbroken.

To test Perutz’ mechanism by the effect of salt bridge removal on $K_T$, oxygen equilibrium curves have to be measured very accurately, because the value of $K_T$ has to be determined from that part of the equilibrium curve which lies between 1 and 2% saturation (Imai et al., 1970). This part gives the first Adair constant $K_1$ (mm Hg$^{-1}$) which is equal to $K_T$ provided Hill’s constant $n > 2.0$ (Baldwin, 1975). While the crystallographic work failed to confirm Perutz’ mechanism, the oxygen equilibrium measurements of Imai & Yonetani (1975) supported it by showing that breakage of the Bohr group salt bridges on increasing the pH increased the $K_T$. Ackers et al. (1975) criticized this work because the haemoglobin concentration (1 mg/ml) was not high enough to prevent dissociation into dimers whose presence would change the position of the bottom asymptote and raise the apparent value of $K_1$. However, when these measurements were repeated at a much higher haemoglobin concentration (50 mg/ml), where the effect of dimerization is negligible, then identical results were obtained (Kilmartin et al., 1978). Similar changes in $K_T$ were found from oxygen equilibrium curves measured at 40 mg/ml of deo-(His146β) haemoglobin and haemoglobin specifically carbamylated at the α amino group of Val1α (Kilmartin et al., 1978). So these experiments showing breakage of Bohr group salt bridges on ligation of the T state are in conflict with the crystallographic results.

Here we attempt to resolve the paradoxical inconsistency between the chemical results on haemoglobin A and the crystallographic ones on haemoglobin Kansas by further chemical studies on haemoglobin Kansas. We have measured the Bohr effect of its T structure by three methods. First, we have measured the pK of His146β by proton nuclear magnetic resonance in NO-haemoglobin Kansas with inositol hexaphosphate which remains in the liganded T state ($T_4$) up to pH 8.0 (Salhany...
Earlier n.m.r. measurements on haemoglobin A had shown that the pK of His146β changes from 8.0 in deoxyhaemoglobin to 7.1 in CO-haemoglobin (Kil- 

martin et al., 1975). Secondly, we have measured the NO Bohr effect in the T state; and thirdly, we have measured the maximum value of the T state Bohr effect for oxygen from the pH dependence of the apparent first Adair constant $K_1$ (mm Hg$^{-1}$).

These three types of measurements confirm Anderson’s (1975) finding that the Bohr group salt bridges are not broken on ligation of the T state of haemoglobin Kansas. We attempt to resolve the conflict between the broken salt bridges in the stripped haemoglobin A T$_3$ state and the unbroken ones in the Kansas T$_4$ state by proposing that the stereochemical mechanism of Perutz (1970) is not unique. It is applicable to the T state of stripped haemoglobin A but not to the T states of haemoglobins with low oxygen affinity caused by mutations or IHP. Here the salt bridges remain unbroken on ligation suggesting that in these T states they are not involved in the free energy of co-operativity.

2. Materials and Methods

(a) Haemoglobins

Des-(His146β) haemoglobin Kansas was prepared from purified haemoglobin Kansas (Ogawa et al., 1972) by the same procedure as for des-(His146β) haemoglobin A (Kilmartin et al., 1975). For n.m.r. measurements the haemoglobin solutions were passed through a Sephadex G25 column equilibrated with $^2$H$_2$O and for oxygen equilibrium curves the haemoglobins were further purified by preparative isoelectric focusing (Righetti & Drysdale, 1971).

(b) n.m.r. Measurements

Anaerobic pH adjustment, filling of n.m.r. tubes and subsequent pH measurement were carried out as before (Kilmartin et al., 1973a). The haemoglobin concentration was between 3 and 7 mM in haem and contained 20 to 40 mM Cl$^-$ and a twofold excess of IHP per tetramer.

n.m.r. spectra were measured on a Brucker HX270 spectrometer at 30°C. Data acquisition was made in both Fourier transform mode with water presaturation and continuous wave mode for between 15 and 25 min. Convolution difference spectra (Campbell et al., 1973) were obtained by processing signals in time domain. The free induction decay signal was multiplied by $(1 - \exp(-AT))$ with $A$ equivalent to 20 Hz line broadening ($T$ is accepted symbol for absolute temperature). Spectra in continuous wave mode were Fourier transformed into time domain for processing. Peak positions are expressed in parts per million from 2,2-dimethyl-2-silapentane sulphonate.

(c) Oxygen Equilibrium and Bohr Effect Measurements

Oxygen equilibrium curves were measured by the Imai method (Imai et al., 1970) and the data processed as described by Kilmartin et al. (1977). Data from deoxy and reoxy curves were in excellent agreement.

The Bohr effect and the change in charge on binding IHP were measured as described previously (Kilmartin, 1973) except that CO was used as ligand. The NO Bohr effect was measured by a modification of Chien's (1973) method; the pH of 0.5 ml deoxyhaemoglobin (50 mg/ml) was measured with a Beckman 39000 combination electrode; it was then drawn into a 0.5-ml gas-tight Hamilton syringe containing 40 μl NO (Melville & Gowenlock, 1964). The syringe was shaken by hand for 2 min to mix the contents which were then injected back into the cell containing the pH electrode. The pH change was noted and neutralised by appropriate addition of deoxygenated HCl or KOH.

† Abbreviations used: n.m.r., nuclear magnetic resonance; T$_0$ or R$_0$, unliganded T or R state; T$_4$ or R$_4$, T state or R state with all 4 haems liganded; IHP, inositol hexaphosphate; p.p.m., parts per million.
3. Results

(a) Measurement of the pK of His\textsubscript{146β} in the liganded $T$ state of haemoglobin Kansas

Before measuring the pK of His\textsubscript{146β} in the $T_4$ state of haemoglobin Kansas it was necessary to perform several control experiments. Firstly these were to show that IHP, necessary for stabilising the Kansas $T_4$ state (Ogawa et al., 1972) and known to change the alkaline Bohr effect (Kilmartin, 1973), does not change it by affecting the pK of His\textsubscript{146β} in either $T_0$ or $R_4$. Such an effect would complicate our measurements considerably. Secondly, it must be shown that His\textsubscript{146β} has normal pK values in the haemoglobin Kansas $T_0$ and $R_4$ states.

(i) Effect of inositol hexaphosphate on the pK of His\textsubscript{146β} in the $T_0$ and $R_4$ states of haemoglobin $A$

It was not possible to measure the effect of IHP on the pK of His\textsubscript{146β} in the $T_0$ and $R_4$ states of haemoglobin Kansas because at low pH the CO form is switched to $T_4$ (Ogawa et al., 1972) and because it was impossible to prepare the large quantities of des-(His\textsubscript{146β}) Kansas necessary for this experiment. Instead, we used haemoglobin $A$ and identified the $C_β$ proton of His\textsubscript{146β} in the aromatic n.m.r. spectrum of deoxy- and CO-haemoglobin $A$ with IHP in the usual way, by comparison with des-(His\textsubscript{146β}) haemoglobin $A$ under the same conditions (Kilmartin et al., 1973a).

Figure 1 shows a typical continuous wave spectrum of the aromatic proton resonances of the deoxy forms of haemoglobin $A$ (Fig. 1(a)) and des-(His\textsubscript{146β}) haemoglobin (Fig. 1(b)) at pH 8.76 where most of the titratable histidines are unprotonated and should lie between $-7$ and $-8$ p.p.m. from 2,2-dimethyl-2-silapentane sulphonate. The other two spectra (Fig. 1(c) and (d)) are the convoluted difference spectra (Campbell et al., 1973) where the broad components seen in the continuous wave spectra are greatly reduced in intensity. The correspondence of the sharp peaks (5 to 10 Hz in full-width at half-height) was quite easily seen between the continuous wave and convoluted spectra. All the sharp peaks seen in Figure 1(c) below $-7.4$ p.p.m. were titratable and the peak indicated by an arrow was assigned to the $C_β$ proton of His\textsubscript{146β}. The single proton peak of haemoglobin $A$ at 7.29 p.p.m. in Figure 1(a) and (c) was also missing in des-(His\textsubscript{146β}) haemoglobin $A$ (Fig. 1(b) and (d)). This peak is probably the $C_β$ proton of His\textsubscript{146β}.

The $C_β$ proton peak of His\textsubscript{146β} was assigned at various pH values by the procedure described above. A few of the titratable sharp peaks between $-7$ and $-9$ p.p.m. in des-(His\textsubscript{146β}) haemoglobin were found at slightly different positions from those in haemoglobin $A$, indicating some disturbance generated by the removal of His\textsubscript{146β}. Otherwise most of the titratable peaks in the $C_β$ proton resonance region in des-(His\textsubscript{146β}) haemoglobin were at identical positions to those of haemoglobin $A$. The plot of the pH titration curve of the $C_β$ proton of His\textsubscript{146β} gave a pK of 8.2 (Fig. 2). This is only slightly higher than the pK of 8.0 found for deoxyhaemoglobin in the absence of IHP (broken line in Fig. 2).

The pH titration curves of the other $C_β$ proton peaks were difficult to resolve. It was possible to distinguish between 11 and 13 titratable $C_β$ protons per $αβ$ dimer out of a total of 15 protons, excluding the proximal and distal histidines of the $α$ and $β$ chains. There were between three and four protons with pK values above 7.8, in contrast to deoxyhaemoglobin in the absence of IHP which had only two (Kilmartin, unpublished work). The two extra high pK histidines are probably His\textsubscript{28} and His\textsubscript{143β}. 
since the close proximity of the negatively charged phosphate groups in IHP (Armone & Perutz, 1974) would be expected to raise their pK values.

In the R₄ state, the C₂ proton of His₁₄₆β was identified by comparing the aromatic n.m.r. spectra of CO-haemoglobin A and des-(His₁₄₆β) CO-haemoglobin taken between pH 6.5 and 8.3 (Fig. 3). But above pH 8.3 the identification of the peak became uncertain because several peaks changed their position in different ways in the two haemoglobins. This uncertainty would not significantly affect the pK value because the His₁₄₆β C₂ proton has already changed its position by the expected amount of 1 p.p.m. Figure 2 shows that the pK value of His₁₄₆β in CO-haemoglobin with IHP is 7.2 compared with 7.1 in the absence of IHP (Kilmartin et al., 1973a). There were no peaks with pK values higher than 7.8.

We can conclude that IHP has only a slight effect on the pK of His₁₄₆β in T₀ and R₄ states of haemoglobin A.

(ii) pK values of His₁₄₆β in the T₀ and R₄ structures of haemoglobin Kansas with and without inositol hexaphosphate

It was not possible to measure the pK of His₁₄₆β by proton n.m.r. methods in haemoglobin Kansas for the reasons mentioned in the previous section. Instead we have compared the Bohr effects of the two haemoglobins. This is a very sensitive...
Fig. 2. pH dependence of the chemical shift of the C$_2$ proton of His$_{146\beta}$ in: —○—○—, deoxy-haemoglobin A with IHP (pK = 8.2); —▲—▲—, NO-haemoglobin Kansas with IHP (pK = 8.0±0.1); and —●—●—, CO-haemoglobin A with IHP (pK = 7.1±0.1). The broken (pK = 8.0) and dotted lines (pK = 7.1) are for deoxy and CO-haemoglobin A, taken from Kilmartin et al. (1973a). DSS, see Fig. 1 legend.

Fig. 3. Convoluted difference spectra of CO-haemoglobin A (a) and des-(His$_{146\beta}$) CO-haemoglobin A (b) with IHP at pH 7.38 and 30°C. The arrow indicates the C$_2$ proton of His$_{146\beta}$. DSS, see Fig. 1 legend.
Figure 4 shows the CO Bohr effect of haemoglobins A and Kansas with and without IHP. These measurements were done at a fairly low haemoglobin concentration (10 mg/ml) so that even with IHP at pH below 7 haemoglobin Kansas still partially changes its quaternary structure on ligation (Ogawa et al., 1972; Edelstein, 1975). The Bohr effect of stripped haemoglobin Kansas does not seem to be concentration-dependent because its value at pH 7.4 was unaltered between 10 mg/ml and 50 mg/ml. The results show that the CO Bohr effects of haemoglobins A and Kansas are similar at alkaline pH, but different at acid pH, probably on account of a change in the acid Bohr groups of haemoglobin Kansas; this occurs frequently in different haemoglobins (Kilmartin & Rossi-Bernardi, 1973; Kilmartin et al., 1973b). Figure 4(a) shows that the altered Bohr effect of haemoglobin Kansas can be explained by its having normal alkaline Bohr groups but acid Bohr groups with a pH change larger than normal. Even if an alkaline Bohr group of haemoglobin Kansas is altered, this would not include His146β because its main contribution occurs between pH 7.6 and 8.0 (Kilmartin et al., 1973a) where the Bohr effects of haemoglobins A and Kansas are almost the same. Similar results were also found on addition of IHP (Fig. 4(b)).

We can conclude from this section that the pK values of His146β in the Tₐ and Rₐ structures of haemoglobin Kansas are the same as in haemoglobin A.

(iii) Measurement of the pK value of His146β in the Tₐ state of haemoglobin Kansas

It is not possible to estimate the pK of His146β in CO-haemoglobin Kansas with IHP which is Tₐ up to pH 7.0 (Ogawa et al., 1972) because under these conditions
des-(His146β) haemoglobin Kansas was not T₄ but R₄ (the -14 p.p.m. line characteristic of the T state (Patel et al., 1970) was absent). This was presumably due to the removal of the stabilizing His146β-Asp94β salt bridge. Instead we measured the pK in NO-haemoglobin Kansas with IHP which is T₄ up to pH 8.0 and is converted to R₄ between pH 8.0 and 9.0 (Salhany et al., 1975). At pH 7.0 des-(His146β) NO-haemoglobin Kansas with IHP showed the -14 p.p.m. line diagnostic of the T₄ state and could therefore be used to identify the C₂ proton of His146β.

Figure 5(a) and (b) shows continuous wave spectra of the aromatic proton resonance of NO-haemoglobin Kansas with IHP and des-(His146β) NO-haemoglobin Kansas with IHP at pH 7.25. Figure 5(c) and (d) shows the convolution difference spectra obtained from Figure 5(a) and (b), respectively. The peak indicated by the arrow at -8.3 p.p.m. was identified as the C₂ proton of His146β. This peak was also identified by spectral comparison at three other pH values, pH 6.57, 7.08 and 7.85. At other pH values the peak was followed only in unmodified NO-haemoglobin Kansas. At high pH values it was not possible to assign the His146β peak unequivocally among several peaks, probably because of a transition from T₄ to R₄ known to occur between pH 8.0 and 9.0. The T₄ state of des-(His146β) NO haemoglobin Kansas with IHP would be even more unstable; a comparison of the aromatic spectra showed that it was T₄ up to pH 7.85, above which it probably began to change to R₄. A pK value of 8.0±0.1 for His146β in NO haemoglobin Kansas with IHP was measured from the plot shown.

![Continuous wave and convolution difference spectra of NO-haemoglobin Kansas and des-(His146β) haemoglobin Kansas with IHP at pH 7.35 and 30°C.](image)

**Fig. 5.** Continuous wave (a) and (b)) and convoluted difference spectra ((c) and (d)) of NO-haemoglobin Kansas ((a) and (c)) and NO-des-(His146β) haemoglobin Kansas ((b) and (d)) with IHP at pH 7.35 and 30°C. The arrows indicate the C₂ proton of His146β.
in Figure 2. The uncertainty in the peak position at high pH did not significantly affect the pK value.

The number of titratable peaks between $-7.5$ and $-8.5$ p.p.m. in Figure 5 was $12 \pm 1$ protons per $\alpha \beta$ dimer. There were four protons with pK values higher than 7.8.

We conclude that there is only a slight change in pK value of His146$\beta$ on ligation of the T state of haemoglobin Kansas with NO, indicating that the His146$\beta$ salt bridge remains unbroken. This result agrees with Anderson's (1975) crystallographic work using CO to ligate the T state of haemoglobin Kansas.

(b) NO Bohr effect measurements of the T state of haemoglobin Kansas

The NO Bohr effect of the T state of haemoglobin Kansas with IHP should be decreased if the His146$\beta$-Asp94$\beta$ salt bridge remains unbroken on ligation. If the other Bohr group salt bridges are also unbroken the NO Bohr effect should be abolished. NO haemoglobin Kansas is actually T$_4$ both with and without IHP up to pH 8.0 (Salhany et al., 1975) so it is possible to measure breakage of Bohr group salt bridges in the stripped T state of haemoglobin Kansas. These results are shown in Figure 6(a) and (b). The NO Bohr effect of haemoglobin Kansas in the T state is practically abolished. The residual value is due to a small excess of free NO reacting with water to produce acid (Chien, 1973) giving a larger than normal blank. This confirms the n.m.r. work showing that the His146$\beta$-Asp94$\beta$ salt bridge remains un-

![Graphs showing NO Bohr effect measurements of haemoglobins A and Kansas.](image)

FIG. 6. (a) and (b) NO Bohr effect of haemoglobins A ($\bigtriangleup$, $\bigtriangleup$) and Kansas ($\bigcirc$, $\bigcirc$). (a) Stripped, (b) 1.5-fold excess of IHP per tetramer. Solid symbols indicate absence of a quaternary structure change, half-solid symbols a partial change, and open symbols no quaternary structure change as determined by Salhany et al. (1974,1975).

(c) and (d) Proton uptake found on mixing IHP (1.5-fold excess per tetramer) with haemoglobin solutions of the same pH. (c) Normal haemoglobin: $\bigtriangleup\bigtriangleup$, NO form; $\bigtriangleup\bigtriangleup$, deoxy form, and (d) haemoglobin Kansas: $\bigcirc\bigcirc$, NO form; $\bigcirc\bigcirc$, deoxy form. Haemoglobin concentration 50 mg/ml in 0.1 M-KCl at 25°C.
broken in the NO $T_4$ state of haemoglobin Kansas with IHP. It proves, in fact, that all the alkaline Bohr group salt bridges remain intact in NO $T_4$ even in the absence of IHP.

Stripped haemoglobin A changes its quaternary structure on reaction with NO and has a normal Bohr effect (Chien, 1973 and Fig. 6(a)). Addition of IHP to NO-haemoglobin A below pH 7.0 changes it from $R_4$ to $T_4$ (Salhany et al., 1974; Perutz et al., 1976), so that no quaternary structure change occurs on ligation. The NO Bohr effect is considerably decreased under these conditions (Fig. 6(b)). Unfortunately we cannot conclude from this that the alkaline Bohr group salt bridges remain unbroken, for reasons explained in the Discussion.

(c) Estimation of the Bohr effect of the $T$ state of haemoglobin Kansas

At high haemoglobin concentration where the oxy form remains tetrameric the first Adair constant $K_1$ can be measured from the bottom asymptote of the oxygen equilibrium curve (Akers et al., 1975). When co-operativity is high, $K_1$ is equal to $K_T$ (Baldwin, 1975) and measurement of the pH dependence of $K_1$ gives the oxygen Bohr effect of the $T$ state $\Delta \log K_T/\Delta \text{pH}$. Measurements of $K_1$ in haemoglobin A at high protein concentrations (Kilmartin et al., 1978) show that the $T$ state of stripped haemoglobin A has a normal Bohr effect since $\Delta \log K_T/\Delta \text{pH}$ is close to $\Delta \log p_{50}/\Delta \text{pH}$.

Unfortunately, in haemoglobin Kansas the bottom asymptote of the oxygen equilibrium curve will measure only an apparent $K_1$ value because its oxy form dissociates into dimers more than oxyhaemoglobin A (Bonaventura & Riggs, 1968). The dissociation of oxyhaemoglobin Kansas is almost pH independent within the error of the measurement (Atha & Riggs, 1976), and causes the apparent or measured $K_1$ values ($K_1^*$) to deviate from the true $K_1$ values in proportion to the increase in oxygen affinity. This is because the fraction of high affinity $R_0$ state increases as the pH increases due to destabilization of the $T_0$ state. This means that $\Delta \log K_1^*/\Delta \text{pH}$ is always greater than $\Delta \log K_1/\Delta \text{pH}$ (which is equal to $\Delta \log K_T/\Delta \text{pH}$ in haemoglobin Kansas). So if $\Delta \log K_1/\Delta \text{pH}$ is less than $\Delta \log p_{50}/\Delta \text{pH}$, which happens in haemoglobin Kansas (Fig. 7(d)), then $\Delta \log K_T/\Delta \text{pH}$, the $T$ state Bohr effect, is less than $\Delta \log p_{50}/\Delta \text{pH}$, the $R$ to $T$ Bohr effect. This implies that some Bohr group salt bridges must remain intact on oxygenation of the haemoglobin Kansas $T$ state. This result agrees with the n.m.r. and Bohr effect measurements using NO as ligand for the $T$ state and with Anderson's (1975) crystallographic work using CO as ligand.

A factor which might complicate our analysis of the oxygen equilibrium curves of haemoglobin Kansas is the inherently low oxygen affinity of the isolated $\beta$ chain (Riggs & Gibson, 1973). Provided that this also occurs with CO then it seems unlikely that this is expressed in the tetramer because neither the $z\beta$ dimer nor the $T$ state of haemoglobin Kansas with IHP show any appreciable heterogeneity in CO binding (Ogawa, Mayer & Shulman, unpublished results).

(d) Comparison of the aromatic n.m.r. spectra of the $T_0$, $T_4$ and $R_4$ states of haemoglobin

Comparisons of the aromatic n.m.r. spectra of our samples at the same pH value showed three general types, these correspond to the $T_0$, $T_4$ and $R_4$ states.

Figure 8 shows the $T_0$ spectrum (deoxygenated haemoglobin A with IHP) compared with a

$\dagger p_{50}$ is the oxygen affinity at half-saturation.
Fig. 7. (a) and (b) Hill plots of oxygen equilibrium curves of stripped haemoglobins A (a) and Kansas (b) at pH 9-0, 7-9, 7-3 and 6-7 (left to right). Haemoglobin concentration 1 mg/ml at 10°C. 0·05 M-bis-Tris, 0·1 M-Cl\(^-\) was used for pH 6-7; and 7-3, 0·05 M-Tris, 0·1 M-Cl\(^-\) for pH 7·9 and 9·0. (c) and (d) Values of \(-\log K_1\) (\(\triangle\)) and \(\log p_{\text{H}}\) (\(\bigcirc\)) measured from the oxygen equilibrium curves above are plotted against pH. (c) Stripped haemoglobin A; (d) stripped haemoglobin Kansas. \(K_1\) is the apparent \(K_e\) value.

\(T_4\) spectrum (NO haemoglobin A or Kansas with IHP at pH 6-6). Distinct differences can be seen, but it is also clear that the mutation in haemoglobin Kansas has no effect on the spectrum in the \(T_4\) state. Comparison of NO- and CO-haemoglobin Kansas with IHP at pH 6-4 shows that the \(T_4\) spectrum is ligand-dependent (Fig. 9(a) and (b)). However, the ligand-dependent differences are much smaller than those between \(T_0\) and \(T_4\). The third type is the \(R_4\) spectrum (CO-haemoglobin A with IHP) and is shown in Figure 9(e).

Substantial differences would be expected in the aromatic spectra of \(T_4\) and \(R_4\) because in haemoglobin Kansas the Bohr effect occurs between these two states. Thus \(R_4\) with IHP has no C\(_2\) protons with pK values higher than 7·8 while \(T_4\) with IHP has four. These are probably His146β, another histidine contributing to the alkaline Bohr effect, and His2β and His143β whose pK values are raised by their close proximity to IHP (Arnone & Perutz, 1974).

The reason for the differences in aromatic proton spectra between \(T_0\) and \(T_4\) with IHP is less clear. The difference must be due to changes in the chemical shifts of the histidine protons and changes in the pK values of the histidines. Since there is no charge change between pH 7 and 8 (Fig. 6(b)) these changes in pK must be confined to the low pK histidines, and reflect some structural change linked to ligation.
4. Discussion

Our purpose in carrying out the work described in this paper was to test that part of the stereochemical mechanism of haemoglobin (Perutz, 1970) which involves the Bohr group salt bridges. Perutz predicted that ligation of the T state would break the Bohr group salt bridges. The change in $K_a$ between pH 7 and 9 (Imai & Yonetani, 1975; Kilmartin et al., 1978), equivalent to an energy change of about 1 kcal/mol, is in agreement with this proposal.

Since Anderson’s (1975) crystallographic work showed that the bridges remained intact in the CO T state of haemoglobin Kansas, in conflict with Perutz’ mechanism, we have now investigated the Bohr group salt bridges in the T state of haemoglobin...
Kansas. Our measurements of the pK of His146β in NO-haemoglobin Kansas with IHP, which is $T_4$, and our measurements of the Bohr effect of the T state with oxygen and NO have confirmed Anderson's finding, and contradict Perutz' (1970) proposal.

(a) Comparison of haemoglobin Kansas with other low affinity haemoglobin mutants

One possible explanation that might be advanced for the paradoxical difference between the behaviour of haemoglobins A and Kansas is that the T state of haemoglobin Kansas has been grossly altered by the mutation. This is unlikely for reasons summarized by Anderson (1975). Other low affinity mutants which have mutations at quite different parts of the molecule have functional properties strikingly similar to haemoglobin Kansas (Table 1). The mutants are non-co-operative so $p_{50} = K_1 = K_T$. The small Bohr effect observed in these haemoglobins means that their T state Bohr effect $(d\log K_T/dpH)$ is also much smaller than in haemoglobin A. It is interesting that at pH 7·0 the log $K_T$ values of around 1·7 for the mutants in Table 1 and probably also for haemoglobin Kansas are similar to the log $K_T$ value of 1·6 for haemoglobin A at about the same pH (Imai & Yonetani, 1975). However, at pH 9·0 the log $K_T$ values for the mutants in Table 1 and haemoglobin Kansas become much higher than log $K_T$ for haemoglobin A, showing that the pH-dependent salt bridges fail to break.

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<th>Haemoglobin</th>
<th>log $p_{50}$ pH 7·5</th>
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<th>$d\log p_{50}/dpH$ (max. value)</th>
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<td>1·0</td>
<td>0·21</td>
<td></td>
</tr>
<tr>
<td>M-Boston</td>
<td>1·7</td>
<td>1·0</td>
<td>$\leq 0·1$</td>
<td>Suzuki et al. (1965)</td>
</tr>
</tbody>
</table>

(b) Effect of inositol hexaphosphate on the T state of haemoglobin A

The similarity in functional properties of haemoglobin Kansas and other low affinity mutants suggests that this is part of a general occurrence in haemoglobin whereby mutations which cause low oxygen affinity alter the normal T state to inhibit breakage of Bohr group salt bridges. If this is correct, then IHP, a powerful allosteric effector which substantially lowers the oxygen affinity of haemoglobin A (Tyuma et al., 1973), ought to have the same effect on the T state of haemoglobin A. The n.m.r. spectra suggest that it does, since the aromatic n.m.r. spectrum of NO-haemoglobin A with IHP, which is $T_4$ at low pH, is very similar to that of NO-haemoglobin Kansas with IHP (Fig. 8(b) and (e)), and different from the $R_4$ spectrum of CO-haemoglobin A with IHP (Fig. 9(c)). This result suggests that the His146β–Asp94β salt bridge is unbroken in NO-haemoglobin A with IHP below pH 7·0. This is apparently confirmed by measurements of the NO Bohr effect of haemoglobin A under the same conditions. Figure 6(b) shows that this is very small and
Fig. 9. Comparison of the convoluted difference spectra of haemoglobin in T₄ and R₄ with IHP at pH 6.4+0.05 and 30°C. (a) NO-T₄ (NO-haemoglobin Kansas); (b) CO-T₄ (CO-haemoglobin Kansas); (c) CO-R₄ (CO-haemoglobin A).

similar to haemoglobin Kansas, suggesting that the Bohr group salt bridges remain unbroken in the NO T₄ state of haemoglobin with IHP.

However, there are problems in interpreting the state of Bohr group salt bridges from measurements of the Bohr effect in the presence of IHP. This is because of the large charge changes caused by the binding of IHP to haemoglobin, and is best explained by the following scheme:

In the above diagram the small letters by the equation arrows represent the proton release associated with the equilibrium reactions. Under conditions where a quaternary structure change occurs, the Bohr effect in stripped haemoglobin is a + b (open symbols in Fig. 6(a)) and the Bohr effect with IHP is f + g (open symbols in Fig. 6(b)). The proton uptake on binding IHP to the unliganded state is —c (open symbols
in Fig. 6(c) and to the ligand R state is \(-e\). These two are not equal for oxy- and deoxyhaemoglobin A (Kilmartin, 1973), therefore \(a + b \neq f + g\) and IHP changes the Bohr effect depending upon the relative values of \(c\) and \(e\). The magnitudes of these values are not proportional to the IHP binding constants at different pH values; consequently the Bohr effect in the presence of IHP can either be increased or decreased. In the absence of a quaternary structure change the Bohr effect \(f\) measured with IHP is due not only to \(pK\) changes of the Bohr groups but also to \(pK\) changes of groups in the IHP binding site (Arnone & Perutz, 1974) and of the bound IHP itself. Likewise the proton uptake upon IHP binding \((c, d)\) should include all these \(pK\) changes, although the value of \(c\) in the alkaline pH region seems to reflect mainly \(pK\) changes in the groups at the IHP binding site and IHP itself, since our measurements show the \(pK\) value of His146\(\beta\) in \(T_0\) is not affected much by IHP.

In haemoglobin Kansas with NO as the haem ligand the values of \(a, f, c\) and \(d\) are all measurable over a reasonably wide pH range. The findings that \(c \approx d\) and \(a \approx f \approx 0\) are consistent with the absence of breakage of the Bohr group salt bridges in \(T_4\) and \(T_4\)-IHP. In stripped haemoglobin A the pH dependence of \(K_T\) indicates that \(a + b \approx a\) and all the Bohr group salt bridges are broken in \(T_4\). On addition of IHP NO-haemoglobin A could be switched to a \(T_4\)-IHP state with broken Bohr group salt bridges or a haemoglobin Kansas-type \(T_4\)-IHP state with unbroken salt bridges or a mixture of both. In the haemoglobin Kansas type \(T_4\) state \(d = c\) (Fig. 6(d)), suggesting that the IHP binding site has the same structure in \(T_0\) and NO-\(T_4\) and that the tertiary structure changes on ligation, which do not break the Bohr group salt bridges, also do not affect the IHP binding site. However, in the \(T_4\) state with broken Bohr group salt bridges there is evidence that the tertiary structure changes which break the Bohr group salt bridges also extend to the IHP binding site; this is that IHP changes the oxygen affinity of the \(T\) state (Tyuma et al., 1973). Linkage theory (Wyman, 1964) then predicts ligand-induced tertiary structure changes in the IHP binding site of the IHP-free or IHP-bound \(T\) state. If this finding is also applicable to NO, then the proton uptake on IHP binding to its site in \(T_0\) and \(T_4\) could be quite different, and if \(T_4\)-IHP were more protonated than \(T_0\)-IHP then any charge change due to \(pK\) change of the alkaline Bohr groups would be decreased. This could be the reason for the small NO Bohr effect in haemoglobin A with IHP. Conclusions about Bohr group salt-bridge behaviour cannot be made until independent measurements of the proton uptake on IHP binding to its site or sites in the various possible \(T\) states of haemoglobin are made. Consequently other measurements of the \(T\) state Bohr effect with IHP (Kilmartin, 1973; Olson & Gibson, 1973) must be treated with caution.

In this paper, there is some additional information, i.e. the similarity of the n.m.r. spectra of NO-haemoglobin A with IHP and NO-haemoglobin Kansas with IHP at low pH, suggesting that Bohr group salt bridges are unbroken in both haemoglobins. But to draw a clear conclusion about this more studies are required.

(c) Conclusions

We have shown that breakage of the Bohr group salt bridges which occurs on ligation of the \(T\) state of haemoglobin A is inhibited in haemoglobin Kansas. The effect of the mutation seems to be expressed in the liganded \(T\) state because the unliganded \(T\) states of haemoglobins \(A\) and Kansas are very similar; there are only slight changes in the crystal structure (Anderson, 1975), the change in charge on
adding IHP is the same (Fig. 6(c) and (d)) and the \( K_r \) values at pH 7 are similar (Fig. 7(c) and (d)).

Other mutants which cause low oxygen affinity (Table 1) and have mutations in different parts of the molecule have similar functional properties to haemoglobin Kansas, suggesting that this is a general occurrence in haemoglobin. This would take place in a variety of ways: by addition of IHP, crystallization into the deoxy form, or use of NO or high-spin ligands (Perutz et al., 1974,1976; Salhany et al., 1974,1975). We have evidence that suggests that IHP inhibits breakage of the His146–Asp94 salt bridge in the NO T₄ state of haemoglobin A since its aromatic n.m.r. spectrum (Fig. 8(c)) is very similar to the NO T₄ state of haemoglobin Kansas under the same conditions (Fig. 8(b)). Unfortunately Bohr effect measurements in these circumstances are inconclusive, so a definitive conclusion must await direct pK measurements of His146β.

If functional properties similar to haemoglobin Kansas are found in other mutants with low affinity then there would be no unique stereochemical mechanism for haemoglobin, and different bonds would be responsible for part of the energy of co-operativity depending on the conditions. Whether there are just two mechanisms, one for stripped haemoglobin A and another for haemoglobins with low oxygen affinity, or more likely a continuum of mechanisms, remains to be established.

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REFERENCES