

HIGH IONIC STRENGTH OR PRESENCE OF INOSITOL HEXAKISPHOSPHATE REVERSES THE UNUSUALLY LOW pK_a OF CYSH3(125)B OF GUINEA PIG HAEMOGLOBIN

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ABSTRACT. The kinetics of the reaction of 5,5'-dithiobis(2-nitrobenzoate) with guinea pig oxy- and carbonmonoxyhaemoglobin are biphasic. The two phases differ in rate by two orders of magnitude. For the fast phase, quantitative analysis of the pH dependence of the apparent second order rate constant, k_{app} , shows that it has all the characteristics associated with the reaction of CysF9(93) β , a sulphhydryl group that is invariant in all mammalian haemoglobins. The slow kinetic phase is assigned to CysH3(125) β . Quantitative analysis of the pH dependence of k_{app} for this phase at 50 mM ionic strength gave an unusually low pK_a of 6.0 for this sulphhydryl group. Published data on guinea pig haemoglobin show that it has a much-enhanced acid Bohr effect compared to human haemoglobin. This indicates that CysH3(125) β functions as an acid Bohr group in guinea pig haemoglobin. Comparison with human haemoglobin indicates that this cysteine reduces the alkaline Bohr effect of guinea pig haemoglobin between pH 7 and 6, and enhances its acid Bohr effect below pH 6. From kinetic data collected at high salt concentration, and in the presence of inositol hexakisphosphate, it is shown that the pK_a of the sulphhydryl increases to ca 9 and 7.7, respectively. Under these conditions, CysH3(125) β ceases to be an acid Bohr group.

KEY WORDS: Haemoglobin, CysH3(125) β , pK_a , Bohr effect, Guinea pig

INTRODUCTION

In the past four decades, the CysF9(93) β sulphhydryl group of haemoglobin has been employed as an indicator of tertiary and quaternary structures [1-7]. It has been suggested, following the mutation F9(93) $\beta^{Cys \rightarrow Ala}$, that CysF9(93) β might regulate the interplay between the HisHC3(146) β /AspFG1(94) β salt bridge and chloride ion, thereby modifying the alkaline Bohr effect of human haemoglobin. Furthermore, Ho and co-workers have concluded that mutations or chemical modifications at the F9(93) β site affect the salt bridge and the organic phosphate-binding site and also destabilize the T quaternary state [8]. By studying the reactivity of CysF9(93) β as a function of pH it is demonstrated that inositol hexakisphosphate (inositol- P_6), an organic phosphate, raises the pK_a of HisHC3(146) β of human haemoglobins A and S by one pK_a unit, thereby strengthening the HisHC3/AspFG1 salt bridge and reducing the reactivity of the sulphhydryl group [9]. These interesting findings raise the possibility that sulphhydryl groups located at other positions in the haemoglobin molecule might provide equally interesting information. Since CysF9(93) β is highly conserved in the vast majority of haemoglobins, for most cases such information can only be obtained by studying haemoglobins with multiple reactive sulphhydryl groups [10-11].

Guinea pig haemoglobin is an example of haemoglobin with multiple reactive sulphhydryl groups. According to its amino acid sequence [12] guinea pig haemoglobin has three kinds of sulphhydryl groups. These are located at positions G11(104) α , F9(93) β and H3(125) β . CysG11(104) α is at the $\alpha_1\beta_1$ subunit interface in both the T and R quaternary structures. It is therefore 'masked' and unreactive towards any sulphhydryl reagent [13]. CysF9(93) β , on the other hand, is reactive in both the T and R quaternary structures, although its rate of reaction is

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lower in deoxy- than in oxyhaemoglobin and carbonmonoxyhaemoglobin. Position H3(125) β is at the $\alpha_1\beta_1$ subunit interface in the T quaternary structure (and is also masked) but is partially exposed to the solvent in the R quaternary structure [14]. Consequently, CysH3(125) β is not expected to be reactive but was found to be reactive in oxy-, carbonmonoxy and methaemoglobin [15].

A comprehensive pH dependence kinetic study at 50 mM ionic strength of the reaction of DTNB with the sulphhydryl groups of guinea pig haemoglobin in the R quaternary structure has shown that there are two reacting sulphhydryl groups. The fast phase resembled the titration curve of a diprotic acid and has been assigned to the reaction of CysF9(93) β . Its reactivity is linked to two ionisable groups with pK_a s of 6.4 ± 0.1 and 7.8 ± 0.2 which were assigned to HisHC3(146) β and CysF9(93) β , respectively [15]. The pH dependence profile of the slow phase on the other hand resembled the titration curve of a monoprotic acid. Its reactivity is linked to a single ionisable group with a pK_a of 6.1 ± 0.2 which has been assigned to CysH3(125) β because of the presence of LysA5(8) β which is only 0.4 nm away from it [15].

The monoprotic nature of the pH dependence profile of k_{app} for the slow phase, the unusually low pK_a of CysH3(125) β and the extremely fast kinetics of CysF9(93) β in the reaction of DTNB, 5,5'-dithiobis(2-nitrobenzoate), with guinea pig haemoglobin at 0.05 M ionic strength has generated interest in the understanding of the reaction in relation to the structure and functions of guinea pig haemoglobin. This study was designed to study the effect of increased ionic strength and the presence of allosteric effector (inositol hexakisphosphate) on the reactivity of guinea pig haemoglobin with DTNB.

EXPERIMENTAL

Male guinea pigs were purchased from a local market at Ibadan, Nigeria. Haemoglobin was prepared from blood by well-known laboratory procedures except that, prior to the lysis step, the red blood cells were washed thrice with cold isotonic saline containing $11.5 \text{ g NaCl L}^{-1}$ instead of the usual 9.5 g L^{-1} . The red cells lysed prematurely at the latter concentration. Prior to use for the experiments, each haemoglobin sample was 'deionized' by passage through a Dintzis ion-exchange column [16].

Kinetic experiments were carried out at 20°C in phosphate ($\text{pH} \leq 8$) and borate ($\text{pH} > 8$) buffers. The kinetics of the reaction between haemoglobin and DTNB were studied under pseudo-first order conditions, with DTNB in 20-fold excess over the concentration of reacting sulphhydryl groups. The kinetics was monitored at 412 nm on a Zeiss PMQ II UV/visible spectrophotometer equipped with thermostated cell compartment. Haemoglobin solutions were equilibrated at 20°C in a Lauda TUK 30 Table Kryostat. The results obtained from this instrument are comparable to those monitored at 450 nm on a Dionex stopped-flow apparatus coupled to an on-line data acquisition system [15]. The kinetic traces (transmittance as a function of time) were recorded on a Philips PM 8261 X-t chart recorder. Each kinetic run was repeated three times under the same experimental conditions and was allowed enough time to proceed to completion. After converting transmittance to absorbance, the data were analysed with a 1990 update of DISCRETE, a computer program for analysis of multiple exponential signals [17, 18]. Apparent second order rate constants, k_{app} , were obtained from k_{obs} by dividing the latter by the DTNB concentration because plots of k_{obs} against the DTNB concentration were found to be linear, with zero intercepts as shown in Figures 2a and 2b of reference [15]. Unless otherwise indicated, the final concentration of haemoglobin after mixing was $10 \mu\text{M}$ heme (5 μM in reacting sulphhydryl groups).

RESULTS AND DISCUSSION

It has been demonstrated that DTNB is more sensitive to sulphhydryl groups environments than other sulphhydryl reagents, because it carries two negative charges [19]. For this reason this reagent has been chosen for the study of the reactivity of the sulphhydryl groups of guinea pig haemoglobin. However, DTNB has one disadvantage: it contains a disulphide bond, and the rate of hydrolysis of disulphide bonds increases markedly above pH 9 [20]. Hence the DTNB reactions were not monitored above pH 9 because of uncertainty about its integrity under these conditions.

The static titration of guinea pig haemoglobin with DTNB has shown that the haemoglobin has four reacting sulphhydryl groups, a pair of CysF9(93)β and CysH3(125)β [15]. This supports why the time course of the DTNB reaction is biphasic. The fast phase is about two orders of magnitude faster than the slow phase, just like what was obtained for the reaction at 50 mM ionic strength. The reaction of DTNB with guinea pig haemoglobin (oxy- and carbonmonoxy-derivatives) was carried out both at increased ionic strength (200 mM) and in the presence of inositol hexakisphosphate.

Stripped haemoglobin at ionic strength 200 mM

(i) *Fast kinetic phase.* Figure 1 shows the variation of k_{app} with pH for the fast phase of the reaction of DTNB with stripped guinea pig haemoglobin, that is, freed from endogenous organic phosphate and other undesired ions by passage through a Dintzis ion-exchange column [16] at an ionic strength of 200 mM. It is seen that each profile resembles the titration curve of a diprotic acid. Best-fit curve for similar results earlier obtained [15] at 50 mM ionic strength are indicated by the dashed line. The fast kinetic phase of the DTNB reaction is tentatively assigned to CysF9(93)β. To test the correctness of this assignment, kinetic experiments were carried out at 200 mM ionic strength. It was previously reported that an increase in ionic strength from 50 to 200 mM practically had no effect on the pK_as of HisHC3(146)β and CysF9(93)β of stripped human haemoglobin [9]. Profiles similar to those reported in Figure 1 were earlier accounted for in terms of the fractional population of the thiol anion form of the sulphhydryl [9-11, 19, 21, 22]. Moreover, a salt bridge is formed in R state haemoglobin between HisHC3(146)β and AspFG1(94)β [23], and this salt bridge sterically hinders access to CysF9(93)β [24]. These considerations give rise to the two-term equation, Eq. 1 [19].

$$k_{app} = k_1 \frac{K_1}{K_1 + [H^+]} + k_2 \frac{K_2}{K_2 + [H^+]} \quad (1)$$

In Eq. 1, k_1 is the limiting apparent second order rate constant at high pH for the DTNB reaction when the reactivity of CysF9(93)β is linked to the ionization of HisHC3(146)β, with ionization constant K_1 ; k_2 is the limiting second order rate constant at high pH when the reactivity of the same sulphhydryl is linked to its own ionization, with ionization constant K_2 . The first fractional term in the above equation is the fraction of the neutral form of the histidine; the second fractional term is the fraction of the thiol anion form of the sulphhydryl. Eq. 1 is employed to analyze the profiles shown in Figure 1.

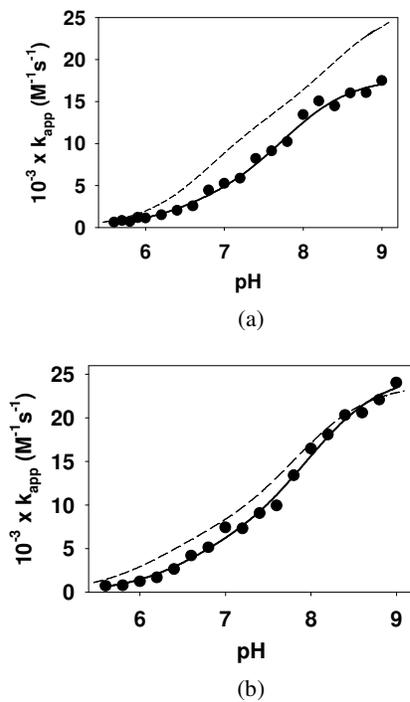


Figure 1. Reaction of 5,5'-dithiobis(2-nitrobenzoate) with CysF9(93) β of stripped guinea pig haemoglobin at ionic strength 200 mM. Variation of k_{app} with pH. (a) oxyhaemoglobin; (b) carbonmonoxyhaemoglobin. The lines through the experimental points are theoretical best-fit calculated with the parameters reported in Table 1, using Eq. 1 of the text. Each experimental point is the mean of 5 determinations and is subject to a standard error of 5%. Conditions: concentrations after mixing – haemoglobin, 10 μ M heme; DTNB, 200 μ M; phosphate buffers, pH \leq 8; borate buffers, pH \geq 8; Temperature, 20 $^{\circ}$ C.

The best-fit parameters are reported in Table 1. The mean value of pK_1 is 6.53 ± 0.05 ; that of pK_2 is 7.9 ± 0.1 . Comparison with the corresponding parameters in Table 1 (of [14]) for ionic strength 50 mM (6.4 and 7.9, respectively) shows that the increase in ionic strength has produced no remarkable effect on the pK_a values of HisHC3(146) β and CysF9(93) β of guinea pig haemoglobin. This finding is identical to the finding for human haemoglobin and provides evidence supporting the assignment of the fast kinetic phase to the reaction of CysF9(93) β .

(ii) *Slow kinetic phase.* The effect of high salt concentration (ionic strength, 200 mM) on the slow kinetic phase of the DTNB reaction is reported in Figure 2. It is seen that k_{app} remains almost constant between pH 5.6 and 6.6. Above pH 7, however, k_{app} increases rapidly and monotonically with increase in pH. These profiles are fitted with the one-term equation, Eq. 2:

$$k_{app} = k \frac{K}{K + [H^+]} \quad (2)$$

In Eq. 2, k is the limiting apparent second order rate constant at high pH for the reaction of DTNB with the sulphhydryl group whose ionization constant is K . The fractional term is the fraction of the thiol anion form of the sulphhydryl group, the form that reacts with DTNB [21, 22]. The best-fit parameters are reported in the legend to Figure 2. The mean value of pK_a for the reacting sulphhydryl group is *ca* 9. This value is only approximate, because it is equal to the upper limit of the pH range of this study. The effect of high salt concentration on the pK_a of the sulphhydryl group to which the slow kinetic phase may be assigned is quite remarkable: a change of 3 pK_a units (compare with the dashed curve that shows similar result at 50 mM ionic strength). This is in sharp contrast to the lack of an effect on the fast phase. This remarkable change in pK_a strongly indicates that the slowly reacting sulphhydryl is not CysF9(93) β . Therefore the slow phase is assigned to CysH3(125) β . This marked change in pK_a has implications for the ability of CysH3(125) β to act as an acid Bohr group.

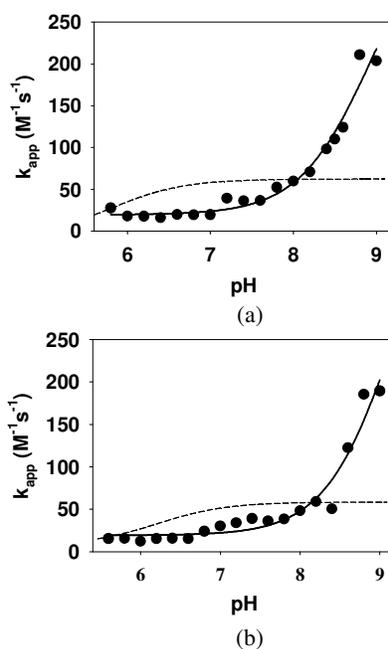


Figure 2. Reaction of 5,5'-dithiobis(2-nitrobenzoate) with CysH3(125) β of stripped guinea pig haemoglobin at ionic strength 200 mM. Variation of k_{app} with pH. (a) oxyhaemoglobin; (b) carbonmonoxyhaemoglobin. The lines through the experimental points are theoretical best-fit calculated with Eq. 2 of the text. The fitting parameters are (a) oxyhaemoglobin: $pK = 8.94$; $k = 371.5 \text{ M}^{-1}\text{s}^{-1}$; (b) carbonmonoxyhaemoglobin: $pK = 9.17$; $k = 460.4 \text{ M}^{-1}\text{s}^{-1}$. Baselines of 19.4 and $19.2 \text{ M}^{-1}\text{s}^{-1}$, respectively. All conditions are as in Figure 1.

Effect of inositol hexakisphosphate at ionic strength 50 mM

(i) *Fast kinetic phase.* The endogenous organic phosphate in red blood cells is 2,3-bisphosphoglycerate (2,3-BPG) but inositol hexakisphosphate (inositol- P_6) is used more often in haemoglobin studies. This organic phosphate reduces the reactivity of CysF9(93) β and increases the pK_a of HisHC3(146) β in human haemoglobin by 1 pK_a unit [9]. Figure 3 shows the pH

dependence profiles of k_{app} for the fast kinetic phase of the reaction of DTNB with guinea pig haemoglobin in the presence of inositol- P_6 . For each data set the theoretical best-fit line to the data for the corresponding stripped haemoglobin derivative is included as a dotted line for comparison. It is seen that inositol- P_6 reduces the reactivity of CysF9(93) β throughout the experimental pH range. At pH 7.4 the reduction in reactivity is about 2-fold. The data points in Figure 3 give a profile that appears to resemble the titration curve of a diprotic acid. However, on quantitative analysis with Eq. 1, bad fits were obtained for the data. In particular, either pK_1 values were about 1 or the k_1 values were negative. However, the data were fitted successfully with Eq. 2. The lines through the data points in Figure 3 are the best-fit lines calculated with the parameters in the legend to Figure 3. Only a single pK_a of 7.8 was obtained. Since inositol- P_6 increases the pK_a of HisHC3(146) β of human haemoglobin by 1 pK_a unit [9], the single pK_a obtained from the data in Figure 3 suggests that the pK_a of this histidine in guinea pig haemoglobin has been increased from 6.5 in stripped haemoglobin (Figure 1; Table 1 of reference 15) to 7.8 in the presence of inositol- P_6 , an increase of 1.3 pK_a units. In other words, in the presence of inositol- P_6 the pK_a s of HisHC3(146) β and CysF9(93) β of guinea pig haemoglobin coincide. Although a pK_a of 7.8 may seem low for this sulphhydryl, it is within the error range of the value 8.1 ± 0.3 obtained for stripped haemoglobin (Table 1).

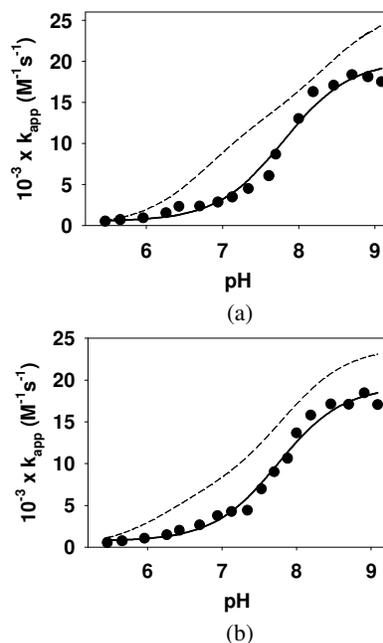


Figure 3. Reaction of 5,5'-dithiobis(2-nitrobenzoate) with CysF9(93) β of guinea pig haemoglobin in the presence of inositol- P_6 at ionic strength 50 mM. Variation of k_{app} with pH. (a) oxyhaemoglobin; (b) carbonmonoxyhaemoglobin. The lines through the experimental points are theoretical best-fit calculated with Eq. 2 of the text. The fitting parameters are (a) oxyhaemoglobin: $pK = 7.8$; $k = 19573 \text{ M}^{-1}\text{s}^{-1}$; (b) carbonmonoxyhaemoglobin: $pK = 7.75$; $k = 18555 \text{ M}^{-1}\text{s}^{-1}$. Baselines of 523 and 716 $\text{M}^{-1}\text{s}^{-1}$, respectively, were required for the fits. Apart from the addition of inositol- P_6 to the buffers, all other conditions are as in Figure 1. The dashed-lines are theoretical best-fit lines to the data for stripped haemoglobin [15].

Table 1. Reaction of 5,5'-dithiobis(2-nitrobenzoate) with derivatives of stripped guinea pig haemoglobin (fast kinetic phase). Best-fit parameters employed to fit the data in Figure 5 according to Eq. 1 of the text. (Ionic strength, 200 mM).

Derivative	pK ₁	pK ₂	k ₁ (M ⁻¹ s ⁻¹)	k ₂ (M ⁻¹ s ⁻¹)
Oxy	6.49	7.76	3710	14161
Carbonmonoxy	6.58	7.98	6133	19026

(ii) *Slow kinetic phase.* Figure 4 shows the effect of inositol-P₆ on the reactivity of CysH3(125)β. For comparison the theoretical best-fit lines to the data for stripped haemoglobin are shown as full lines. It is clear that the organic phosphate drastically reduces the reactivity of this sulphhydryl. At pH 7.4 the reduction in reactivity is about 6-fold for oxy- and carbonmonoxyhaemoglobin. Also, the pK_a of the sulphhydryl is increased from 6 to about 7.7, a change of 1.7 pK_a units. As it is shown below, this result has implications for the ability of CysH3(125)β to act as an acid Bohr group.

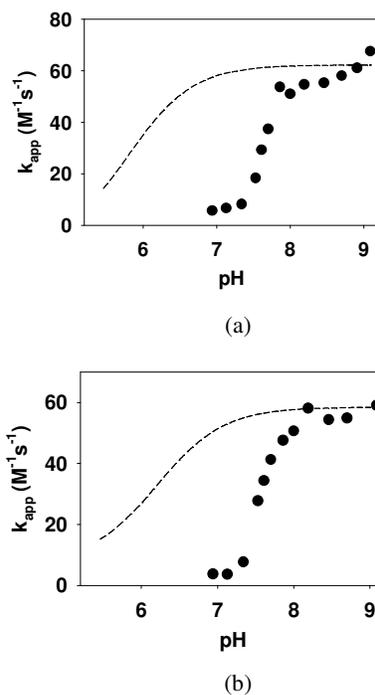


Figure 4. Reaction of 5,5'-dithiobis(2-nitrobenzoate) with CysH3(125)β of guinea pig haemoglobin in the presence of inositol-P₆ at ionic strength 50 mM. Variation of k_{app} with pH. (a) oxyhaemoglobin; (b) carbonmonoxyhaemoglobin. Apart from the addition of inositol-P₆ to the buffers, all other conditions are as in Figure 3. The dashed-lines are theoretical best-fit to the data for stripped haemoglobin [15].

Comparison with recent reports and implications of results

The kinetics of the reaction of guinea pig haemoglobin with DTNB have been reported [25]. In that report considerable attention was paid to the fast kinetic phase because its rate was unusual for haemoglobin; the slow kinetic phase was completely ignored. As it shall be shown below, by ignoring the slow phase the author lost very interesting information on guinea pig haemoglobin. Moreover, some of the conclusions arrived at on the fast phase are erroneous because of the insufficient amount of data used for the quantitative analysis of the pH dependence profile of the apparent second order rate constant: five experimental points over the pH range 5 to 9. In particular, the author failed to observe the biphasic nature of the pH dependence profile (Figure 1; Table 1) and, consequently, erroneously assigned the fast kinetic phase to CysH3(125) β instead of CysF9(93) β .

The very high rates obtained for the reaction of DTNB with CysF9(93) β of guinea pig haemoglobin are about 2 orders of magnitude faster than what was previously reported for other haemoglobins [9-11, 22]. At present there is no explanation for these very high rates. Nevertheless, this highly reactive sulphhydryl shows all the characteristics that are typical of the behavior of CysF9(93) β : biphasic pH dependence profile of k_{app} (Figure 1, Table 1); coupling of its reaction to the ionization of HisHC3(146) β ; lack of effect of high salt concentration on the pK_a s of the two coupled residues; increase in the pK_a of the histidine by *ca* 1 pK_a unit in the presence of inositol- P_6 , without any corresponding effect on the pK_a of the sulphhydryl. These findings are in sharp contrast to the results obtained for the slowly reacting sulphhydryl: *monophasic* pH dependence profile of k_{app} (Figure 2); lack of coupling of its reaction to the ionization of any other ionizable group; remarkable effect of high salt concentration on the pK_a of the reacting sulphhydryl; increase in the pK_a of the sulphhydryl itself by inositol- P_6 . These latter findings are not characteristic of CysF9(93) β . Therefore the slow kinetic phase is assigned to CysH3(125) β .

Low pK_a of CysH3(125) β

The most striking feature of the results of the reaction of DTNB with guinea pig haemoglobin is the low pK_a of 6 obtained for CysH3(125) β (dashed line Figure 2) [15]. To the best of our knowledge, such a low pK_a has not been reported before for a sulphhydryl group in haemoglobin. The normal pK_a of sulphhydryl groups in haemoglobin is between 8 and 9 [9-11, 22]. From the 3D structure of guinea pig haemoglobin, it can be seen that a cationic group, LysA5(8) β (pK_a *ca* 10.5), is located 0.4 nm away from CysH3(125) β . The positive electrostatic field produced by the lysine contributes to lowering the pK_a of the sulphhydryl. At a separation of 0.4 nm the attractive force between the lysine and the cysteine anion will give rise to the formation of a salt bridge.

In α -helix, a residue is separated from the next residue in the sequence by a translation of 1.5 Å (0.15 nm) along the helix axis [26]. Therefore, CysH3(125) β should be separated from the N terminus of the H helix by *ca* 3 Å (0.3 nm). At an ionic strength of 50 mM the radius of the ionic atmosphere is 20 Å (2 nm) [27]. The distance between LysA5(8) β and CysH3(125) β , *ca* 4 Å (0.4 nm), and that between CysH3(125) β and the N terminus of the H helix, *ca* 3 Å (0.3 nm), are less than the radius of the ionic atmosphere. Therefore, at ionic strength 50 mM, (i) LysA5(8) β should not be screened off electrostatically from CysH3(125) β , and the LysA5/CysH3 salt bridge should remain intact; and (ii) the effective positive charge at the N terminus of the H helix should not be screened off from CysH3(125) β and should be effective in reducing the pK_a of the sulphhydryl group. In contrast, at an ionic strength of 200 mM, the radius of the ionic atmosphere is less than 5 Å (0.5 nm) [27], that is, about the same as the distance, *ca*

4 Å (0.4 nm), separating LysA5(8)β from CysH3(125)β and the distance, *ca* 3 Å (0.3 nm), separating CysH3(125)β from the N terminus of the H helix.

Therefore, at ionic strength 200 mM, (i) the LysA5/CysH3 salt bridge should be broken and (ii) CysH3(125)β should be screened off electrostatically from the positive electrostatic field at the N terminus of the H helix. It is not surprising, therefore, that the pK_a of CysH3(125)β rises from 6 to 9 when the ionic strength changes from 50 to 200 mM (compare dashed line with the solid line in Figure 2).

At ionic strength 50 mM both the LysA5/CysH3 salt bridge and the effect on CysH3(125)β produced by the positive end of the α-helix dipole of helix H remain intact. However, it is found (Figure 4) that in the presence of inositol-P₆ at this ionic strength the pK_a of the sulphhydryl changed from 6 to *ca* 7.7. This implies that when inositol-P₆ binds to haemoglobin a structural transition occurs that breaks the LysA5/CysH3 salt bridge. The fact that the pK_a of the sulphhydryl does not change from 6 to about 9 expected at high salt concentration implies that inositol-P₆ binding does not alter the effect of the α-helix dipole on the sulphhydryl. If it did, the pK_a would have changed from 6 to at least *ca* 8.5.

Influence of CysH3(125)β on the Bohr effect

Above pH 6, when deoxyhaemoglobin is converted to liganded haemoglobin, protons are ejected into the solution. This is the alkaline Bohr effect. Below *ca* pH 6, protons are absorbed from solution on the binding of ligand to deoxyhaemoglobin. This is the acid or reverse of Bohr effect. The ionizable groups whose pK_a changes account for the acid and alkaline Bohr effects in human haemoglobin are known [28]. The same groups are present in guinea pig haemoglobin [12], and it is reasonable to expect that the Bohr effects of both haemoglobins should be about the same. This is far from being the case.

Bailey *et al.* [29] have determined the Bohr effects of human and guinea pig haemoglobins at an ionic strength of 50 mM. The alkaline Bohr effects of both haemoglobins do not differ much above pH 7 (Figure 1 of Bailey *et al.* [29]). Between pH 7 and 6, however, the alkaline Bohr effect of guinea pig haemoglobin is noticeably lower than that of human haemoglobin. Moreover, Δh⁺, the acid Bohr effect measured as the amount of proton absorbed per mole of O₂ bound, is much larger for guinea pig haemoglobin than for human haemoglobin. At pH 5.3 the values of Δh⁺ are 0.66 and 0.29, respectively (Figure 1 and Table 2 of Bailey *et al.* [29]), giving Δ(Δh⁺) = 0.37. These observations suggest that there might be an extra ionizable group present in guinea pig haemoglobin, but absent in human haemoglobin, which makes an acid Bohr contribution between pH 7 and 6 in guinea pig compared to human haemoglobin. In this way the alkaline Bohr effect of guinea pig haemoglobin is lower than that of human haemoglobin in this pH range. The same ionizable group makes an extra acid Bohr contribution below pH 6, making the acid Bohr effect of guinea pig haemoglobin larger than that of human haemoglobin. The low pK_a of 6 observed for CysH3(125)β in R state haemoglobin (Figure 4) suggests that this sulphhydryl might be just the right group to affect *both* the acid and alkaline Bohr effects, enhancing the former and decreasing the latter.

Effects of organic phosphate and high salt on the Bohr effect

It is noted that between pH 7 and 6 CysH3(125)β seems to make an acid Bohr contribution to the alkaline Bohr effect of stripped guinea pig haemoglobin when compared to human haemoglobin. In the presence of 2,3-bisphosphoglycerate (2,3-BPG), the alkaline Bohr effect of guinea pig haemoglobin is the same as that of human haemoglobin above pH 6 (Figure 3 of Bailey *et al.* [29]). This means that the acid Bohr contribution of CysH3(125)β between pH 7 and 6, observed in stripped guinea pig haemoglobin, has been eliminated in the presence of 2,3-

BPG. It also means that at low pH the extra acid Bohr effect of stripped guinea pig haemoglobin compared to human haemoglobin [$\Delta(\Delta h^+) = 0.37$ at pH 5.3], which is attributed above to the contribution of CysH3(125) β , will be eliminated in the presence of 2,3-BPG.

As noted in the Experimental section, guinea pig red cells lyse when they are washed with normal saline (9.5 g NaCl L⁻¹). For guinea pig red cells the isotonic saline is 11.5 g NaCl L⁻¹, that is, 200 mM NaCl. It is noted above that at an ionic strength of 50 mM the pK_a of CysH3(125) β is 6 (dashed line Figure 2). At an ionic strength of 200 mM, the physiological ionic strength for guinea pig red cells, the pK_a increases to 9 (Figure 2). Under this condition CysH3(125) β can no longer act as an acid Bohr group and therefore can no longer affect the alkaline Bohr effect. Moreover, in red blood cells organic phosphates are present. As noted above, inositol-P₆ increases the pK_a of CysH3(125) β from 6 to *ca* 7.7. The combination of organic phosphates and high ionic strength in guinea pig red blood cells should ensure that CysH3(125) β will have a high pK_a and therefore cannot serve as a Bohr group *in vivo*.

It is rather fortunate that under physiological conditions CysH3(125) β does not retain its low pK_a, since this would have adversely affected the normal functional properties of guinea pig haemoglobin. It is remarkable that among 177 tetrameric haemoglobins whose non- α chain amino acid sequences are listed by Kleinschmidt and Sgouros [30], no other haemoglobin has a cysteine residue at position H3(125). This makes guinea pig haemoglobin unique among haemoglobins. Clearly, the placement of a cysteine residue at this position is not evolutionarily favoured. It is worthy of note that CysH3(125) β is the first thiol group that has been implicated in the Bohr effect. Perutz *et al.* [28] have estimated that HisH21(143) β contributes about half of the acid Bohr effect of human haemoglobin. At pH 5.3 this will be half of 0.29, that is, 0.145. Assuming that the $\Delta(\Delta h^+)$ of 0.37 at pH 5.3 [29] arises from the contribution of CysH3(125) β alone, this amounts to a *ca* 3-fold higher contribution of CysH3(125) β to the acid Bohr effect of guinea pig haemoglobin compared to the contribution of HisH21(143) β . The acid Bohr contribution of HisH21(143) β arises because of a change in pK_a from 4.9 in deoxy- to 5.5 in oxyhaemoglobin [28], whereas the contribution of CysH3(125) β arises from a pK_a change from 4.3 in deoxy- (estimated from Ref. [29]) to 6.0 in oxyhaemoglobin.

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