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Full Length Research Paper

Inositol-P_6 induced structural changes in duck major haemoglobin

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The reactivities of 5,5'-Dithiobis (2-nitrobenzoate) with the sulphydryl groups of major haemoglobin of duck (*Anas platyrhinchos*) have been carried out in the presence of inositol hexakisphosphate, inositol-P_6. The time course of the reaction is biphasic. The addition of inositol-P_6 changed the pH dependence profile of the apparent second-order rate constant of both oxy- and carbonmonoxyhaemoglobin for the reaction with CysF9(93)β. Inositol-P_6 reduced the rate of reaction with CysF9(93)β of the carbonmonoxy derivative by about 2 to 9-fold, depending on the pH. In contrast, the addition of inositol-P_6 increased the rates of reaction of the oxy- and aquomet derivatives. This difference might have risen from the fact that the salt bridge that is normally formed in human haemoglobins between HisHC3(146)β and AspFG(94)β was not formed in the presence of inositol-P_6 in liganded derivatives whose pH profiles were changed. Consequently, HisHC3(146)β comes sufficiently close to the CysF9(93)β sulphydryl group to interact with it electrostatically and so influences its reactivity directly. However, inositol-P_6 changed only that of carbonmonoxyhaemoglobin for CysB5(23)β. For the reaction with CysB5(23)β, inositol-P_6 reduced the rate in the carbonmonoxy derivative with increasing pH up to about 15-fold at pH 9.0, and increased the rates of reaction of the oxy- and aquomet derivatives by about 2 to 3-fold.

Key words: 5,5'-Dithiobis (2-nitrobenzoate), duck, haemoglobin, inositol-P_6, sulphydryl group.

INTRODUCTION

Although, atomic details of haemoglobin have been determined by x-ray crystallographic analysis (Perutz, 1970, 1989), there are still various features, especially structure-function relationship of the molecule that remains unexplained or controversial. This is because the crystal structure only shows the structure at a moment in time, whereas the structure is dynamic in solution. Structural changes in proteins have been said to play important role in biological functions, such as allosteric regulation (Perutz, 1989), energy transformations (Abrahams at al., 1994) and molecular recognition (Kato et al., 1991). Inositol hexakisphosphate (IHP), inositol-P_6, is a strong allosteric effector that can give insight into structural changes in haemoglobin when bound to the molecule in solution, hence this study. Structural changes in solution are not only affected by allosteric effectors (including H^+) but also by ionic strength. So far, we only have insights into what the structure might look like in solution by studying reaction kinetics and thermodynamics, since it is often very difficult to interpret NMR data of macromolecule in solution. A compromise for haemoglobin structural determination by X-ray crystallography and implied structures from solution is the encapsulation of haemoglobin in transparent sol-gel (Wo et al., 1994; Shibayama and Saigo, 1995; Bruno et al., 2001). Under this condition, the quaternary transition is dramatically reduced (Das et al., 1999; Shibayama and Saigo, 1999), thereby making it possible to detect the tertiary conformational properties. The rate of tertiary transitions is also decreased by several orders of magnitute (Shibayama and Saigo, 2001). Under this condition, haemoglobin is said to retain most of its functional and structural properties (Shibayama and Saigo, 1995,

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2001; Bettati and Mozzarelli, 1997), though not all. A structural study in native environment of the molecule is therefore preferred.

Several animal haemoglobins have properties that cannot be readily explained by their amino acids sequence and known model because they vary widely in their interactions with allosteric effector molecules, such as Inositol-P_6, and H^+. The lower oxygen affinity of haemoglobin within red blood than that of haemoglobin in free solution has been attributed to the presence of organic phosphates in the red blood cell (Benesch and Benesch, 1967, 1969; Gibson and Gray, 1970). This difference is of great importance to the physiological functions of haemoglobin. Human red blood cells contain 2, 3 bisphosphoglycerate (BPG) at about the same molar concentration as haemoglobin. However, in avian and turtle erythrocytes, inositol pentakisphosphate is present instead of 2, 3-bisphophoglycerate (Vandecasserie et al., 1971). These organic phosphates regulate oxygen binding properties of haemoglobin by binding at the entrance of the molecular dyad axis in deoxyhaemoglobin (Arnone, 1972; Arnone and Perutz, 1974; Brygier et al., 1975; Okonjo, 1980). There is evidence that the same binding site is involved in the R as in the T structure. The resultant effect is a decrease in the reactivity of the sulphydryl groups at position F9(93)β (Okonjo et al., 1995). Other conformational changes have also been attributed to the binding of organic phosphates to haemoglobins (Babalola and Nwozo, 2002).

Some of the organic phosphates which act as allosteric effectors in haemoglobins include inositol hexakis-phosphate (inositol-P6), inositol pentakisphosphate (inositol-P5), 2,3-bisphosphoglycerate (BPG), adenosine monophosphate (AMP), cyclic adenosine mono-phosphate (CAMP), adenosine bisphosphate (ABP) and adenosine trisphosphate (ATP). All the organic phos-phates are molecules with strong negative charges binding at the allosteric site (Arnone and Perutz, 1974). The stoichiometry of binding of organic phosphate to both oxy and deoxy haemoglobin has been shown to be one organic phosphate per haemoglobin molecule (Rollema and Bauer, 1974). Avian haemoglobins are of interest with respect to pentakisphosphate because they posses a relatively high number of positively charged residues at the phosphate binding site as compared to human haemoglobin (Rollema and Bauer, 1974). This gives rise to a very high affinity for organic phosphate even in the R state. It also makes it possible to study the interaction of inositol hexakisphosphate with the protein over a wide pH range. Such measurements provide information on the nature of the groups interacting with the organic phos-phate. Different interesting structural information has been obtained from the reactivities of each of human (Okonjo et al., 1995, 1996), guinea pig (Babalola et al., 2002) and avian (Brygier et al., 1975; Okonjo and Nwozo, 1997; Babalola and Nwozo, 2002) haemoglobins bound to organic phosphate. Avian haemoglobins have parti-cularly been of great interest because of the multiple reactive sulphydryl groups they possess.

There is high degree of homology between the amino acid sequence of the β chain of duck and those of other avians (Niessing, 1981). This homology is as high as 97% with chicken. In the present study, we extended the previous studies on pigeon (Babalola and Nwozo, 2002) and chicken (Okonjo and Nwozo, 1997) to the major haemoglobin of duck. Amazingly, the effect of inositol hexakisphosphate on the reaction of 5,5'-Dithiobis (2-nitrobenzoate) (DTNB) with duck major haemoglobin was more pronounced in terms of structural changes.

MATERIALS AND METHODS

The collection of blood samples and haemoglobin preparation are as previously described (Babalola and Nwozo, 2002; Babarinde et al., 2005; Babarinde and Babalola, 2005). The experimental procedure was also similar to the previous ones except that 10 µM inostol-P_6 (sodium salt) was added to the 'stripped haemoglobin' solution (Tamburrini et al., 1994; Okonjo et al., 1995, 1996).

Kinetics

The reaction of DTNB with haemoglobin derivatives was monitored on a Zeiss PMQ II ultraviolet-visible spectrometer under pseudo-first-order conditions. The reaction of DTNB with the haemoglobins was monitored as previously described (Babalola et al., 2002; Babalola and Nwozo, 2002; Babarinde et al., 2005; Babarinde and Babalola, 2005). This was achieved by reacting each haemoglobin sample with at least a ten-fold excess of DTNB over the concentration of reacting sulphydryl groups. The reactions were monitored at an ionic strength of 50 mM so that electrostatic interaction will not be screened out. 10 µM (heam) haemoglobin solutions were prepared using different buffers (phosphate buffers pH 7.0 to 8.0 and borate buffers pH 8.2 to 9.0), each of ionic strength 50 mM. Inositol-P_6 was added to the haemoglobin solution before making up to the mark to give an inositol-P_6 concentration of 10 µM.

The solutions were allowed to equilibrate at 20°C in a Lauda 30D Table Cryostat. A 10 ml aliquot of the haemoglobin solution was then transferred to a 2 × 2 cm cuvette, which was placed in the cell compartment of the Zeiss UV-VIS spectrophotometer thermostated at 20°C. A calculated volume of the 20 mM DTNB stock solution that would give a final concentration of at least 100 µM in the 10 ml haemoglobin solution was placed on a glass rod, one end of which had been shaped into the form of a shallow spoon. The data were recorded on a Philips PM8261 Xt chart recorder. All reactions were allowed to proceed to completion. Each experiment was repeated three times. The procedure for data analysis has been described in detail (Okonjo et al., 1995). The analyses gave two kinetic phases. The apparent second-order-rate constant (k_{app}) was calculated from the pseudo-first-order rate constants (k_{obs}). The reactions were carried out at 20°C in phosphate buffers (pH 7.0 to 8.0) and borate buffers (pH 8.2 to 9.0) of ionic strength 50 mM and haemoglobin concentration of 10 µM (haem).

Curve fitting

In order to be able to determine the nature of the ionizable groups that influence the DTNB reaction, it was necessary to determine the reactivity of the sulphydryl groups as a function of the pH. The pH-

dependence profiles obtained were fitted to model equation with user-friendly computer software as was previously done (Babalola et al., 2002; Babarinde et al., 2005). The direct approach to the curve-fitting problem (the full-variable approach) minimized the objective function χ^2 with respect to the rate constants, k_j, and the ionization constants, K_j. Another approach (the restricted-variable approach) was to express the rate constants (which occur linearly in the objective function) in terms of the ionization parameters by solving a least-squares problem, and then to minimize the objective function with respect to the ionization parameters alone. Both approaches are supported by the software. An automatic non-negativity constraint was also imposed by working directly with the pK values of the ionization parameters.

RESULTS

Sulphydryl titrations

The amino acid sequence of duck major haemoglobin revealed that there are ten sulphydryl groups in its tetramer molecule and located at positions G11(104)α, H13(130)α, B5(23)β, F9(93)β and H4(126)β. An earlier static titration (Boyer titration) done on duck major haemoglobin stripped of organic phosphates (Babarinde et al., 2005) showed that only two pairs located at positions B5(23)β and F9(93)β are reactive towards DTNB.

Reactions of DTNB with sulphydryl groups in the absence of allosteric effectors

The reaction of DTNB with the CysF9(93)β and CysB5(23)β sulphydryl groups of oxy, carbonmonoxy and aquomet derivatives of duck major haemoglobin stripped of organic phosphates gave simple profiles resembling the titration curves of diprotic acid (Figures 1 to 6, dotted lines) for their pH dependence of k_{app} (Babarinde et al., 2005). These profiles have been previously reported and analysed (Babarinde et al., 2005) using Equation (1):

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

This equation is based on the finding that there is a Bohr effect in R-state haemoglobin involving HisHC3(146)β (Kwiatkowski and Noble, 1982). This proves that the histidine forms a salt bridge with AspFG1(94)β, resulting in CysF9(93)β being sterically hindered by TyrHC2(145)β (Perutz, 1970). When the histidine ionizes, the salt bridge is broken and the steric hindrance to the approach of DTNB to the sulphydryl group is removed. Then, the reactivity of the sulphydryl should increase as the histidine ionizes with increasing pH. In this equation, k_1 is the limiting apparent second order rate constant at high pH for the DTNB reaction when the reactivity of the sulphydryl group is linked to the ionization of HisHC3 (146)β, with ionization constant K_1; k_2 is the limiting apparent second order rate constant at high pH when the sulphydryl reactivity is linked to the ionization of itself, with ionization constant K_2. The standard error in the determination of k_{app} was not more than 20% for the slow phase and 10% for the fast phases.

Reactions of DTNB with sulphydryl groups in the presence of allosteric effectors

Time course for the reaction and phase assignments

The time course of the reaction of DTNB with duck major haemoglobin in the presence of inositol-P_6 is biphasic (data not shown). The two phases are well resolved by the computer program we used and these are similar to what was obtained for duck major haemoglobin in the absence of organic phosphates. The fast kinetic phase has been attributed to the reaction of CysF9(93)β, while the slow phase is assigned to the reaction of CysB5(23)β. The assignments of these kinetic phases are in agreement with previous assignments (Okonjo et al., 1995, 1996).

Quantitative analyses of pH-dependence profiles

CysF9(93)β of oxyhaemoglobin

Figure 1 shows the dependence of the apparent second order rate constant, k_{app}, on pH for the reaction of DTNB with CysF9(93)β of duck major oxyhaemoglobin in the presence of inositol-P_6. The value of k_{app} decreased with increasing pH up to pH 8.2, after which the value became constant. This pH dependence profile is completely different from the one obtained in the absence of organic phosphate (dotted line). Our initial expectation was that, the curve reported in Figure 1 would need a simple one-term equation to be fitted since it is not bowl-shaped. All efforts to fit that curve (Figure 1) with Equation 3 did not yield a good theoretical fit. These trials are represented by dashed line in Figure 1. The theoretical fits were particularly bad at higher pH values.

$$k_{app} = \frac{k_1 [H^+]}{K_1 + [H^+]} \quad (2)$$

The definition of the terms in Equation (2) is similar to the definitions of the first term in equation (3). The clear implication of this is a change in conformation of the molecule on binding inositol-P_6. To the best of our knowledge, this type of profile has not been obtained for the reaction of DTNB with haemoglobin sulphydryl group before. The closest in nature are the bowl-shaped profiles obtained for pigeon (Babalola and Nwoso, 2002) and chicken (Okonjo and Nwoso, 1997). To better understand the nature of the new profile, we fitted the data points in Figure 1 with the equation (3) that was also used to fit the

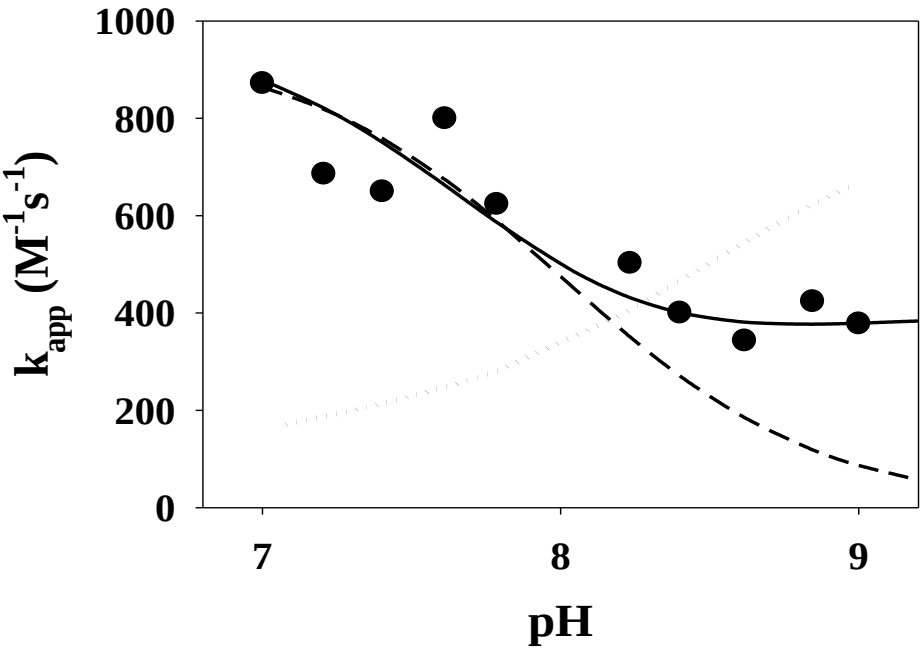

Figure 1. Dependence of k_{app} on pH for the reaction of DTNB with CysF9(93)β of duck major oxyhaemoglobin in the presence of IHP. Full-line is the theoretical best fit for the plot using Equation (3), dashed-line is the best obtainable fit using Equation (2) and the dotted-line shows the reaction profile in the absence of inositol-P_6 (Babarinde et al., 2005). Conditions: HbO_2 = 10 µM haem; inositol-P_6 = 10 µM; I = 50 mM; Phosphate buffer pH = 7.0 to 8.0; borate buffer pH = 8.2 to 9.0; DTNB = 300 µM ; T = 20°C; λ = 450 nm.

bowl-shaped profiles.

$$k_{app} = \frac{k_1 [H^+]}{K_1 + [H^+]} + \frac{k_2 K_2}{K_2 + [H^+]} \quad (3)$$

Theoretically, bowl-shaped profiles can only be accounted for by assuming that there is an ionizable cationic group close to the reacting sulphydryl group. At low pH the cationic group is positively charged and the reaction of the negatively charged DTNB is fast. As the cationic group ionizes to its neutral form with increasing pH, the DTNB reaction slows down. In Equation (3), k_1 is the limiting apparent second-order rate constant at low pH for the DTNB reaction when the sulphydryl reactivity is linked to the ionization of a neighbouring cationic group, with ionization constant K_1. The second fractional term in Equation (2) is similar to those of Equation (1) and the parameters are similarly defined. To our amazement, Equation (3) gave a perfect theoretical fit to the pH dependence profile in Figure 1, though it is not bowl-shaped.

CysF9(93)β of carbonmonoxyhaemoglobin

The pH dependence of k_{app} for the reaction of DTNB with CysF9(93)β of duck major carbonmonoxyhaemoglobin in the presence of inositol-P_6 is shown in Figure 2. This profile is similar to the one obtained for oxyhaemoglobin. However, it is clearly shown in Figure 2 (inset) that inositol-P_6 did not only change the profile of the pH dependence, it also drastically reduced the second apparent rate constant up to 9 fold at pH 9. Figure 2 was therefore fitted with Equation (3). The fitting parameters are reported in Table 1. The long dashed-line is the fit done using Equation (2).

CysF9(93)β of aquomethaemoglobin

Figure 3 shows the pH dependence profile of the reaction of DTNB with CysF9(93)β of duck major aquo-methaemoglobin in the presence of inositol-P_6. Inositol-P_6 had no effect on the pH dependence profile, but increased the second order apparent rate constant contrary to expectation (Okonjo et al., 1995, 1996). The profile resembles that of the ionization of a simple diprotic acid. Figure 3 was therefore fitted with Equation (1) and the fitting parameters are reported in Table 1.

CysB5(23)β of oxyhaemoglobin

The slow kinetic phase in the reaction of duck major

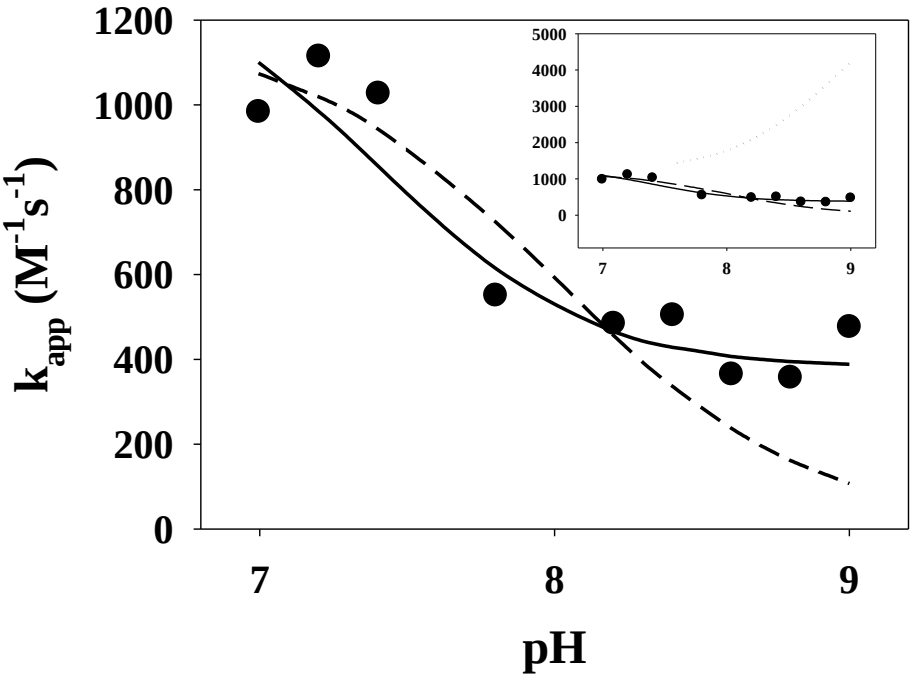

Figure 2. Dependence of k_{app} on pH for the reaction of DTNB with CysF9(93)β of duck major carbonmoxyhaemoglobin in the presence of IHP. Full-line is the theoretical best fit for the plot using Equation (3), dashed-line is the best obtainable fit using Equation (2) and the dotted-line (in the inset) shows the reaction profile in the absence of inositol-P_6 (Babarinde et al., 2005) for comparison. Conditions: HbCO = 10 µM haem; inositol-P_6 = 10 µM; I = 50 mM; phosphate buffer pH = 7.0 to 8.0; borate buffer pH = 8.2 to 9.0; DTNB = 300 µM; T = 20°C; λ = 450 nm.

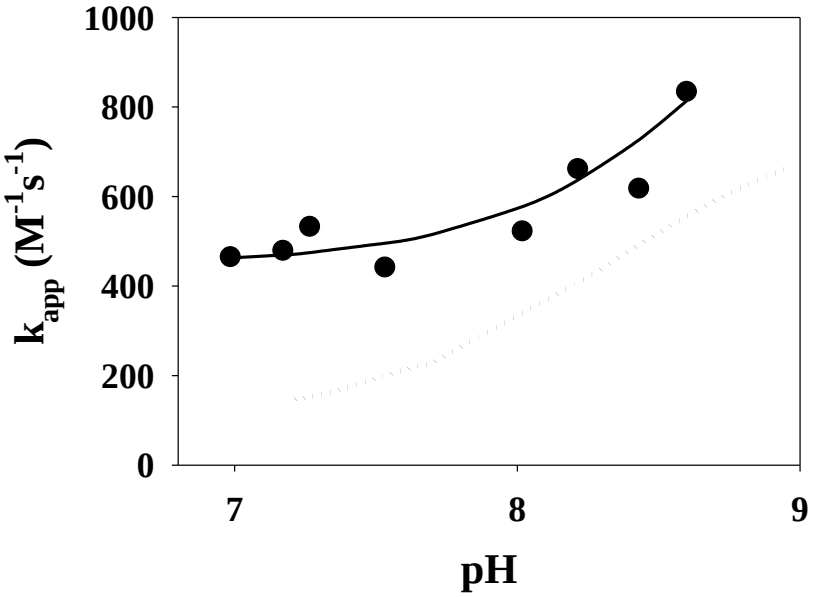

Figure 3. Dependence of k_{app} on pH for the reaction of DTNB with CysF9(93)β of duck major aquomethaemoglobin in the presence of IHP. Full-line is the theoretical best fit for the plot using Equation 1 and the dotted-line shows the reaction profile in the absence of inositol-P_6 (Babarinde et al., 2005). Conditions: aquometHb = 10 µM haem; inositol-P_6 = 10 µM; I = 50 mM; phosphate buffer pH = 7.0 to 8.0; borate buffer pH = 8.2 to 9.0; DTNB = 300 µM; T = 20°C; λ =450 nm.

Table 1. Best-fit parameters using the different equations (in superscripts) employed to fit data in Figures 1to 3 for the reaction of DTNB with CysF9(93)β of major duck inositol-bound haemoglobin at an ionic strength of 50 mM in the presence of inositol-P_6: fast kinetic phase.

Derivative	pK_1	pK_2	k_1 ($M^{-1}s^{-1}$)	k_2 ($M^{-1}s^{-1}$)
Oxy[3]	7.8	8.4	1000	400
Carbonmonoxy[3]	7.5	8.0	1400	380
Aquomet[1]	5.0	8.9	453	1100

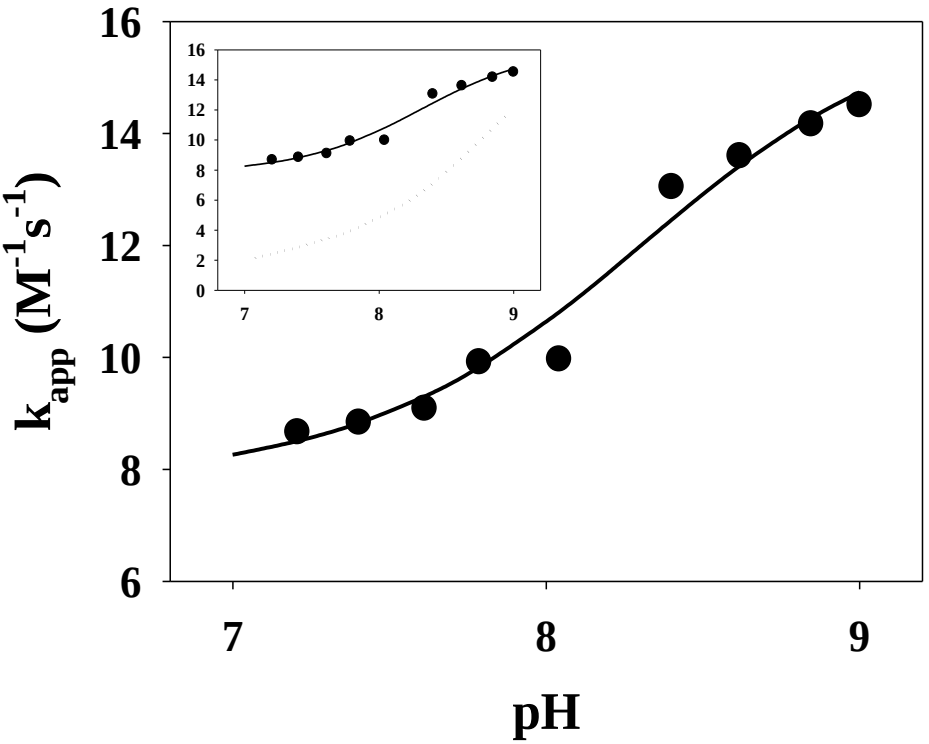

Figure 4. Dependence of k_{app} on pH for the reaction of DTNB with CysB5(23)β of duck major oxyhaemoglobin in the presence of IHP. Full-line is the theoretical best fit for the plot using Equation 1 and the dotted-line (in the inset) shows the reaction profile in the absence of inositol-P_6 (Babarinde et al., 2005) for comparison. Conditions: HbO_2 = 10 µM haem; inositol-P_6 = 10 µM; I = 50 mM; phosphate buffer pH = 7.0 – 8.0; borate buffer pH = 8.2 – 9.0; DTNB = 300 µM ; T = 20 ˚C; λ = 450 nm.

haemoglobin with DTNB is the reaction of CysB5(23)β with DTNB. Figure 4 shows the pH dependence profile of k_{app} for the reaction of DTNB with CysB5(23)β of duck major oxyhaemoglobin in the presence of inositol-P_6. The profile resembles that of the ionization of a simple diprotic acid and was therefore fitted with Equation (1). The fitting parameters are shown in Table 2. The inset of Figure 2 shows a comparison of the reaction in the presence and absence of inositol-P_6. Inositol-P_6 increased the rate of reaction contrary to expectations. Apparently, the profile of the reaction of CysF9(93)β and CysB5(23)β of duck major oxyhaemoglobin has similar profiles in the absence of inositol-P_6 but different profiles in the presence of inositol-P_6. This indicated that either the B5 or F9 position on β-chain is affected by the binding of inositol-P_6 to duck major haemoglobin.

CysB5(23)β of carbonmonoxyhaemoglobin

The pH dependence profile for the reaction of CysB5(23)β of duck major carbonmonoyhaemoglobin with DTNB in the presence of inositol-P_6 is reported in Figure 5. The k_{app} decreased with increase in pH. This is similar to what was obtained for the reaction of CysF9(93)β, the profile was therefore fitted with equation (3). The fitting parameters are reported in Table 2. The profile is completely different from what was obtained in the absence of inositol-P_6 and there is a drastic reduction in the rate of the reaction.

Table 2. Best-fit parameters using the different equations (in superscripts) employed to fit data in Figures 4 to 6 for the reaction of DTNB with CysB5(23)β major duck inositol-bound haemoglobin at ionic strength of 50 mM in the presence of inositol-P_6: slow kinetic phase.

Derivative	pK_1	pK_2	k_1 ($M^{-1}s^{-1}$)	k_2 ($M^{-1}s^{-1}$)
Oxy[1]	5.0	8.3	7.96	8.16
Carbonmonoxy [3]	7.5	6.0	10.50	1.50
Aquomet[1]	5.0	7.9	3.58	12.05

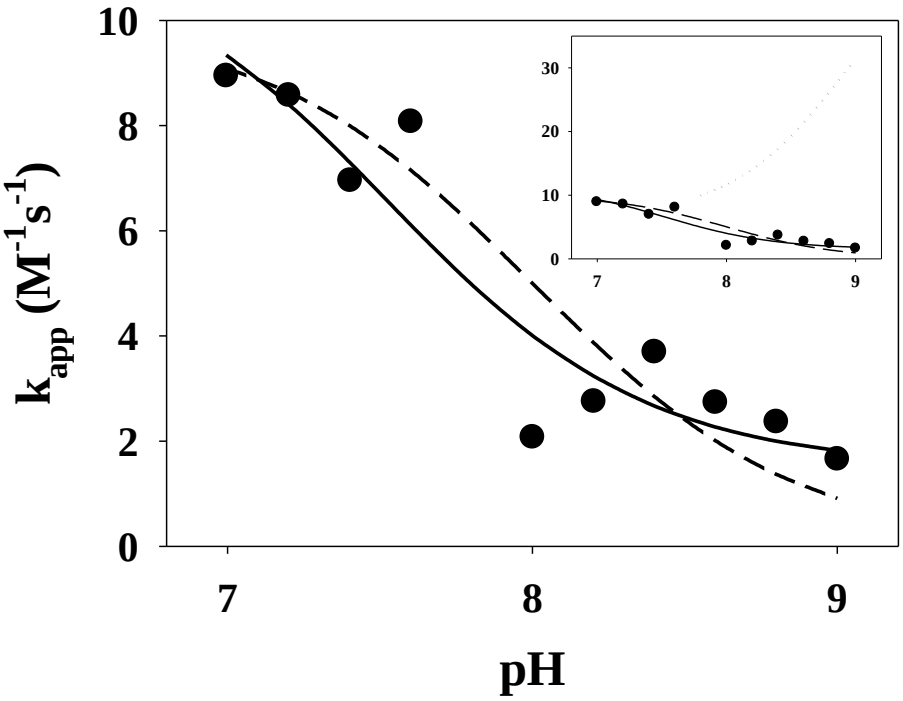

Figure 5. Dependence of k_{app} on pH for the reaction of DTNB with CysB5(23)β of duck major carbonmoxyhaemoglobin in the presence of IHP. Full-line is the theoretical best fit for the plot using Equation (3), dashed-line is the best obtainable fit using Equation (2) and the dotted-line (in the inset) shows the reaction profile in the absence of inositol-P_6 (Babarinde et al., 2005) for comparison. Conditions: HbCO = 10 µM haem; inositol-P_6 = 10 µM; I = 50 mM; phosphate buffer pH = 7.0 – 8.0; borate buffer pH = 8.2 to 9.0; DTNB = 300 µM; T = 20°C; λ = 450 nm.

CysB5(23)β of aquomethaemoglobin

The reaction of DTNB with CysB5(23)β of duck major aquomethaemoglobin is reported in Figure 6. The pH dependence profile resembles that of the titration of simple diprotic acid. The profile was fitted with Equation (1) and the fitting parameters are reported in Table 2. An attempt to fit the experimental data with Equation (2) did not yield a good fit as shown by dashed lines. Inositol-P_6 increased that rate of the reaction (compare experimental data with theoretical fit of data in the absence of inositol-P_6). Inositol-P_6 is known to reduce the rate of reaction of DTNB with haemoglobin sulphydryl groups. The implication here is that the binding of inositol-P_6 exposes CysB5(23)β more to the reaction of DTNB, though the profile did not change.

pKa values obtained from quantitative analyses

In Table 1, the mean pK value of 6.77 ± 1.27 observed for oxy, carbonmonoxy and aquomet haemoglobin derivatives are close to that of histidine. On the basis of our previous study (Babarinde et al., 2005; Babarinde and Babalola, 2005) this pK value was assigned to HisHC3(146)β. The mean pK_2 value is 8.43 ± 0.30. This value is within the range of pK values observed for cysteine in proteins. It was therefore assigned to CysF9(93)β. The CysF9(93)β is responsible for the fast kinetic phase. Also in Table 2, the mean pK_1 value of 5.83±0.83 observed for the three haemoglobin derivatives is close to that of histidine. It was therefore assigned to HisG19(117)β as previously reported. The mean pK_2 value of 7.4 ± 0.77 is within the normal range of pK

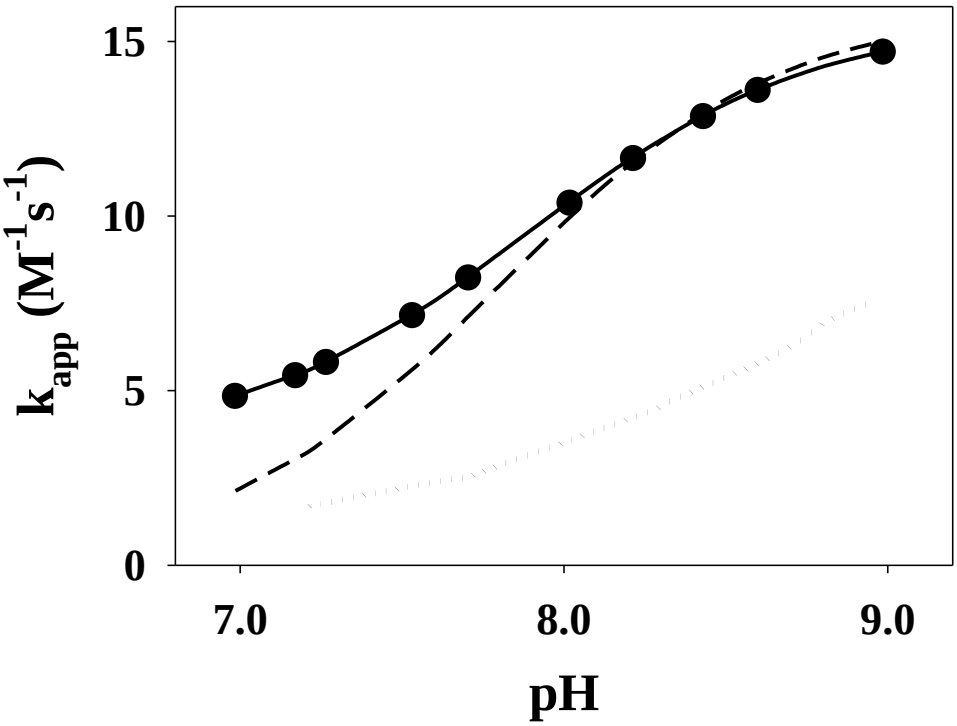

Figure 6. Dependence of k_{app} on pH for the reaction of DTNB with CysB5(23)β of duck major aquomethaemoglobin in the presence of IHP. Full-line is the theoretical best fit for the plot using Equation 1, dashed-line is the best obtainable fit using Equation (2) and the dotted-line shows the reaction profile in the absence of inositol-P_6 (Babarinde et al., 2005) for comparison. Conditions: AquometHb = 10 µM haem; inositol-P_6 = 10 µM; I = 50 mM; phosphate buffer pH = 7.0 to 8.0; borate buffer pH = 8.2 to 9.0; DTNB = 300 µM; T = 20°C; λ = 450 nm.

values for cysteine in proteins. This value was therefore assigned to CysB5(23) in line with previous assignment.

DISCUSSION

The reaction of DTNB with the stripped major duck haemoglobin gave biphasic kinetics as we reported earlier (Babarinde et al., 2005; Babarinde and Babalola, 2005). Such profiles for the fast phase have been previously assigned to the CysF9(93)β sulphydryl group (Okonjo, et al., 1995, 1996). This assignment has been based on the fact that the CysF9(93)β is exposed, while the CysB5(23)β position is in hydrophobic region in hemoglobin (Huang et al., 1990; Cirotto and Geraci, 1973). The profiles for the three derivatives differ, implying that the haem ligand affects the nature of the reaction. On binding to the haem, the haemoglobin molecule undergoes conformational changes, which cause changes in the reactivity of the sulphydryl group (Cheng et al., 2002).

The variation of apparent second order rate constant, k_{app}, with pH for the aquomet derivative resembles the titration curve of a monoprotic acid. This is similar to what has been previously observed for carbonmonoxy derivative of pigeon haemoglobin (Okonjo and Okia, 1993). It is expected that the profiles for the derivatives should resemble the titration curve of a diprotic acid knowing fully well that the reactivity of CysF9(93)β sulphydryl group is affected by the formation of salt bridge between HisHC3(143)β and AspFG1(94)β (Okonjo and Okia, 1993; Okonjo et al., 1995, 1996). Similar dramatic changes in profile have been reported for chicken (Okonjo and Nwozo, 1997) and pigeon (Babalola and Nwozo, 2002).

Also, inositol-P_6 is known to strengthen the salt bridge between His HC3(146)β and AspFG1(94)β, thereby reducing the reactivity of CysF9(93)β sulphydryl group (Okonjo et al., 1995, 1996). In human haemoglobin, for example, the pK_a of HisHC3(146)β is increased in the presence of inositol-P_6 by at least one pK_a unit. Human and duck haemoglobins differ in the number of groups involved in inositol-P_6 binding. In duck haemoglobin, compared to human haemoglobin, HisH21(143)β is replaced by arginine, and AsnH17(139)β is replaced by histidine. Moreover, an additional basic residue, namely arginine, is present in the H13(135)β position. The negatively charged inositol-P_6 is therefore expected to bind more tightly to duck haemoglobin than to human haemolglobin. Hence, in the presence of inositol-P_6, the

reactivity of CysF9(93)β of duck haemoglobin should be considerably reduced and the pK_a should be increased. Surprisingly, the result obtained is contrary to expectation. It is particularly noteworthy that k_{app} is generally higher for the inositol-P_6 bound haemoglobin than for the stripped one as shown in Figures 1 and 3.

This result implies that the salt bridge expected to be formed is only formed in the carbonmonxy derivative but not in the oxy and aquomet derivatives of major duck haemoglobin in the presence of inositol-P_6. This assumption explains the lack of an organic phosphate induced additional Bohr effect in duck haemoglobin and the relatively weak effect of inositol-P_6 on the oxygen affinity of major duck haemoglobin in relation to that of major chicken haemoglobin (Vandecasserie et al., 1973).The results in Figures 1 and 3 are not typical of the CysF9(93)β sulphydryl group reacting under the influence of the aforementioned salt bridge. It is known that the sulphydryl groups of haemoglobin react only in the thiolate form with DTNB. Presence of cationic group enhances this, thereby increasing the reactivity of the sulphydryl with DTNB. In the case of oxy and aquomet derivatives of major duck haemoglobins, inositol-P_6 does not strengthen the salt bridge but rather causes the histidine residue to be shifted away from the aspartate residue. This makes the HisH21(146)β to come within a close range to the CysF9(93)β residue, thereby resulting in electrostatic interaction that enhances its reactivity.

The pK_1 values obtained in Table 1 for oxy and carbonmonxy derivatives are close to the value expected for histidine. A close examination of the 3D structure of duck haemoglobin shows that there is no other histidine residue that is close enough to CysF9(93)β that could have caused such an electrostatic effect. On the other hand, inositol-P_6 bound carbonmonoxy derivative experiences the formation of the salt bridge between HisHC3(146)β and AspFG1(94)β, this is noticed in the high reduction in the k_{app} values with increase in pH. The effect of the addition of inositol-P_6 is a reduction in the rate of the DTNB reaction by about 9-fold at pH 9. The profile is typical of CysF9(93)β sulphydryl in inositol-P_6 bound haemoglobin.

Previous researchers have reported similar unusual pK values for ionisable groups in proteins (Kortemme and Creighton, 1995). Such alterations in the ionization groups are often to varying degree in the folded structures of proteins. Unusual pK_a values that are different from those apparent in the unfolded protein or in corresponding short peptides are often observed for groups within the active sites of enzymes and are important for catalysis. Perturbed pK_a values in folded proteins have been reported to have implications for protein stability. It has been generally accepted that the major factors influencing pK_a values in proteins are the desolvation of ionisable groups in the folded protein and their interaction with other charges or dipoles. Consequently, removing titratable groups in folded proteins from contact with the aqueous solvent renders ionization energetically unfavourable, thereby altering the pK_a value of the ionisable group.

The differences observed for the various derivatives can only be accounted for in terms of structural differences caused by the various haem ligands (Cheng et al., 2002). This indicates that in major duck haemoglobin, changes in the haem ligand give rise to structural changes in the neighbourhood of the CysF9(93)β and CysB5(23)β sulphydryl groups. Such structural differences have been previously observed for major chicken and pigeon haemoglobins (Okonjo and Nwozo, 1997; Babalola and Nwozo, 2002).

Figures 4 and 6 show the profiles of the reaction of DTNB with the inositol-P_6 bound oxy and aquomet haemoglobin, respectively. Each derivative reacts faster than the stripped one by about 2- fold. That is, addition of inositol-P_6 causes an increase in the rate of the reaction by about 2-fold. The two profiles show monotonic increase of k_{app} with increase in pH. On the other hand, Figure 5 shows the reverse for the carbonmonoxy haemoglobin in which inositol-P_6 reduces the rate of the reaction to about 15-fold at pH 9.0. The different reactivities observed are probably due to structural changes caused by the binding of the haem ligands and / or inositol-P_6. It has been reported that the cysteine thiolate ion (S^-) may function as a potent nucleophile in active-site chemistry of thiol proteases as well as in reactions with exogenous modifying agents (Raso et al., 2001). The cysteine sulphydryl may function as either a hydrogen bond donor or acceptor. It may also release its proton to facilitate sulphur ligation at metal coordination sites in proteins. Sulphydryls are also known to be vulnerable to aberrant oxidation, which can compromise protein function or stability.

Conclusion

It is clearly seen from the figures that the addition of inositol-P_6 had a strong effect on the reactivity of DTNB with the sulphydryl groups of major haemoglobin of duck (*Anas platyrhinchos*). The shape of the pH dependence profile of the apparent rate constant either changed or the apparent rate constant was altered. The addition of inositol-P_6 is seen to have increased the apparent rate constant in most cases contrary to what have been reported (Okonjo et al., 1995). This apparently signified a change in structure. The pH dependence profile of the apparent rate constant could only be fitted by two-term equations, meaning that the immediate environment of the sulphydryl groups did not change significantly. The major plausible reason for this finding is that the salt bridge that is normally formed in haemoglobins between HisHC3(146)β and AspFG(94)β was not formed in the presence of inositol-P_6 in liganded derivatives whose pH profiles were changed. Consequently, HisHC3(146)β

comes sufficiently close to the CysF9(93)β sulphydryl group to interact with it electrostatically, and so influences its reactivity directly. Unfortunately, the dynamic structure of the molecule in solution cannot be monitored by x-ray crystallography and the nuclear magnetic resonance data will be difficult to interpret.

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