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Full Length Research Paper

Ligand-dependent reactivity of cysF9(93)β of minor duck hemoglobin induced by inositol hexakisphosphate

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The reactivities of the sulphydryl groups of minor component of duck hemoglobin with 5,5[1]-dithiobis-(2-nitrobenzoic acid), DTNB, in the presence of inositol hexakisphosphate (inositol-P_6) have been studied. The reaction has been monitored as a function of pH (pH 7.0 – 9.0) and at ionic strength of 50 mmolL^{-1}. The carbonmonoxy and aquomet derivatives were used for the study. Biphasic time courses were obtained under all experimental conditions and the two kinetic phases were well separated along the time axis. From the observed pseudo-first order rate constants, k_{obs}, the apparent second-order rate constants, k_{app}, were calculated. Inositol-P_6 reduced the rates of all reactions for the two derivatives for both CysF9(93)β and CysB5(23)β. The reaction of CysF9(93)β gave an increase of 0.88 unit of pK_a for the aquomet derivative but a decrease of 0.40 unit of pK_a for the carbonmonoxy derivative. The salt-bridge formed between HisHC3 and AspFG1 is more strengthened by inositol-P_6 in aquomet than in carbonmonoxy derivative.

Key words: Duck, hemoglobin, sulphydryl, inositol-P_6.

INTRODUCTION

In hemoglobin, the reactivity of a sulphydryl group is an indicator of the structure in its immediate neighbourhood. The oxygen affinity of hemoglobin within red blood cells is lower than that of hemoglobin in free solution (Benesch and Benesch, 1967; Benesch and Benesch, 1969; Gibson and Gray, 1970). Human red blood cells contain 2,3-bisphosphoglycerate (BPG) at about the same molar concentration as hemoglobin (Arnone, 1972). However, in avian and turtle erythrocytes, inositol pentakisphosphate is present instead of BPG (Vandecasserie et al., 1971). The organic phosphates regulate oxygen binding properties of hemoglobin by binding at the entrance of the molecular dyad axis in deoxyhemoglobin (Arnone and Perutz, 1974; Brygier et al., 1975; Okonjo, 1980). All the phosphates 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 deoxyhemoglobin has been shown to be one organic phosphate per hemoglobin molecule (Rollema and Bauer, 1979).

Avian haemoglobins are interesting with respect to their interaction with inositol pentakisphosphate. This is because they posses a relatively high number of positively charged residues at the phosphate-binding site as compared to human hemoglobin. This gives rise to a very high affinity for organic phosphates even in the relaxed (R) state. This makes it possible to study the interaction of inositol hexakisphosphate with the protein over a wide range of pH. Such measurements provide information on the stoichiometry of effector binding to oxyhemoglobin and on the nature of the groups interacting with the organic phosphate (Okonjo and Nwozo, 1997; Babalola and Nwozo, 2002)

MATERIALS AND METHODS

Live ducks were bought from Oje market in Ibadan, Nigeria. Blood was obtained from the duck (*Anas platyrhnchos*) by venous punc-

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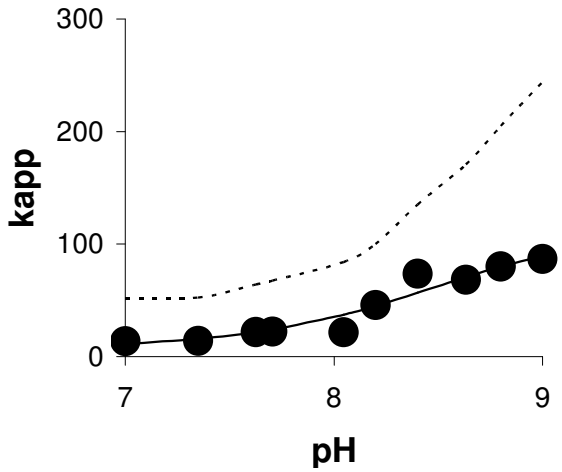

Figure 1. Dependence of the apparent second-order rate constant, kapp, on pH for the reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with the fast phase of minor duck carbonmonoxyhemoglobin the presence (•) of inositol-P_6 at 10 µM HbCO, 300 µM DTNB, 10 µM inositol-P_6, I= 0.05 M, phosphate buffer pH 7.0-8.0, borate buffer pH 8.2-9.0, λ= 412 nm and temperature of 20°C, (Broken line shows the absence of inositol-P_6)

ture into a beaker containing 200 ml Acid Citrate Dextrose (ACD) anticoagulant. Hemoglobin was prepared from the blood according to normal well-known laboratory procedures (Babarinde et al., 2004; Babarinde and Babalola, 2004). In order to remove low molecular weight impurities present in the hemolysate, it was dialysed against dialyzing solution (5 mM phosphate buffer, pH 7 prepared with distilled water) at 5°C using polyvinyl chloride tube size 2 (made by Medicel International Limited, London). Three changes were made at intervals of three hours to reduce the salt level in the hemoglobin solution. The organic phosphates in the hemolysate, inositol pentakisphosphate, which is not easily removed except by gel filtration or ion exchange chromatography was removed by passage through a Dintzis ion-exchange column (Dintzis, 1952). The Dintzis column is a mixed bed of generated forms of Amberlite and Zerolit DM-F resins made by British Drug House. The hydrogen form of the resin was generated by washing a known volume of the Amberlite IR 120 resin packed in a column with about 10 times its volume of 3 M HCl. The resin was then washed with distilled water until the effluent was acid free, neutral to litmus. The acetate form was generated by washing the Amberlite IRA 400 resin packed in a column slowly with about 10 times its volume of 3 M HCl. The resin was then washed with distilled water until the effluent was acid free. Then, about 10 times its volume of 3 M sodium acetate was passed through the column. The resin was again washed with distilled water until the effluent gave no precipitate with $AgNO_3$ solution. The ammonium form was prepared by slowly passing 100 ml of 3 M NaCl through a column packed with Amberlite IR 120 resin. The resin was washed with distilled water until it gave no precipitate with $AgNO_3$ solution. Ten times its volume of 3 M NH_4Cl was slowly passed through the column and washed with water until the effluent gave no precipitate with $AgNO_3$ solution. Each of the mixed form and the mixed indicator form of Zerolit DM-F resins was simply washed with distilled water, without further treatment.

A 50 ml burette with a glass wool plugged end served as the column. The resins were packed into the column as described earlier (Babalola et al., 2002). The two components of duck hemolysate were separated as described by Babarinde et al. (2004) and Babarinde and Babalola (2004). The carbonmonoxy derivative of the hemoglobin was prepared by passing carbonmoxide gas through the prepared oxyhemoglobin derivative. The conversion from an oxy-derivative to a carbonmonoxy derivative is reversed by directing bright Tungsten lamp or sunlight on the latter while stirring in a glass container immersed in an ice bath to avoid being heated up.

The aquomet derivative was prepared by the addition of excess ferricyanide to oxyhemoglobin. The excess ferricyanide was removed by passage through the Dintzis column. Reagent grade inositol hexakisphophate (inositol-P_6) was made by Sigma Chemical Company Limited and used without further treatment. A 10 mM inositol-P_6 solution was prepared by dissolving 0.2370 g inositol-P_6 in distilled water in a 25 ml volumetric flask. The solution has a high pH of about 11. It was titrated to pH 6.5 using concentrated HCl, in order to stabilize the pH of the hemoglobin solution (Tamburrini et al., 1994). The DTNB used was made by Sigma Chemical Company Limited, London. It was used without further purification.

Kinetics

The kinetics of the DTNB reaction with the carbonmonoxy and aquomet derivatives of minor component of duck hemoglobin has been reported (Babarinde and Babalola, 2004). The present study was carried out for the same hemoglobin derivative in the presence of inositol-P_6. The Kinetic experiments were carried out under the same conditions; temperature of 20°C, pH (7.0 – 9.0), ionic strength of 50 $mmolL^{-1}$ and pseudo-first-order conditions. The experiments were performed as follow: A 10 µM inositol-P_6 in a given buffer, of ionic strength 50 $mmolL^{-1}$, was allowed to equilibrate at 20°C in a thermostat. 10 ml of this solution was then transferred to a 2 x 2 cm cuvette. The cuvette was placed in the cell compartment of a Zeiss PMQ II uv-vis spectrophotometer set at a wavelength of 412 nm and thermostated at 20°C with a Lauda 30 D Table Kryostat. A few mm^3 of a 50 mM solution of DTNB in 95% ethanol was placed on a glass rod, one end of which had been shaped into the form of a shallow spoon. The reaction was initiated by mixing the DTNB with the hemoglobin in the cuvette, with the glass rod serving as a stirrer.

The concentration of the DTNB in the reaction mixture was 100 µM. The kinetic traces (transmittance) as a function of time were recorded on a Philips PM 8261 Xt chart recorder. After the conversion of transmittance to absorbance, the apparent second order rate constant, k_{app}, for the reaction was calculated with the second order rate equation, using DISCRETE, computer software written for this purpose.

RESULTS AND DISCUSSION

Analysis of simple profiles in the presence of inositol-P_6

The apparent second-order rate constant, k_{app}, for the reaction of DTNB with the sulphydryl groups for the stripped hemoglobin, with pH gives simple profiles (see broken lines in Figure 1 and 2). The presence of inositol-P_6 did not change the nature of the profiles. The simple profiles shown in Figures 1 and 2 resemble those previously reported for various hemoglobins (Okonjo et al., 1979; Okonjo and Aboluwoye, 1992; Okonjo and Adejoro, 1993; Okonjo and Okia, 1993; Babarinde and Babalola, 2004; Babarinde et al., 2004). Such profiles have been analysed with the equation:

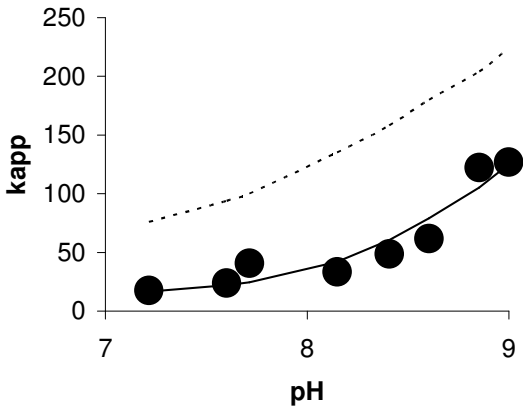

Figure 2. Dependence of the apparent second-order rate constant, kapp, on pH for the reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with the fast phase of minor duck aquomethemoglobin in the presence (•) of inositol-P_6 at 10 µM MetHb, 300 µM DTNB, 10 µM inositol-P^6, I= 0.05 M, phosphate buffer pH 7.0-8.0, borate buffer pH 8.2-9.0, λ= 412 nm and temperature of 20°C, (Broken line shows the absence of inositol-P_6).

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

The profiles in Figures 1 - 4 were analysed with the equation (1). 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 CysF9(93)β 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 CysF9(93)β, with ionization constant K_2. The analyses of the profiles in Figures 1 and 2 with equation (1) gave the best-fit parameters reported in Table 1. The analyses of the profiles in Figures 3 and 4 with equation (1) gave the best fit parameters reported in Table 2.

The pK_1 value of 5.00 is close to that earlier assigned to HisHC3 (146)β (Okonjo et al., 1996). It is therefore assigned to it. The mean pK_2 value of 8.70 ± 0.23 is normal for a free cysteine. It was therefore assigned to CysF9(93)β.

The mean pK_1 value of 5.16 ± 0.16 is close to that earlier assigned to histidine. It is therefore assigned to HisG19 (117)β. The mean pK_2 value of 8.72 ± 0.20 is normal for free Cysteine. It was therefore assigned to CysB5(23)β.

It is interesting to note that the pH dependence profiles of k_{app} for the fast and slow phases are similar and simple. Contrary to what was reported for each of Chicken (Okonjo and Nwozo, 1997) and pigeon (Babalola and Nwozo, 2002) where bowl shape was observed for the slow phase, simple profile was observed for duck minor hemoglobin.

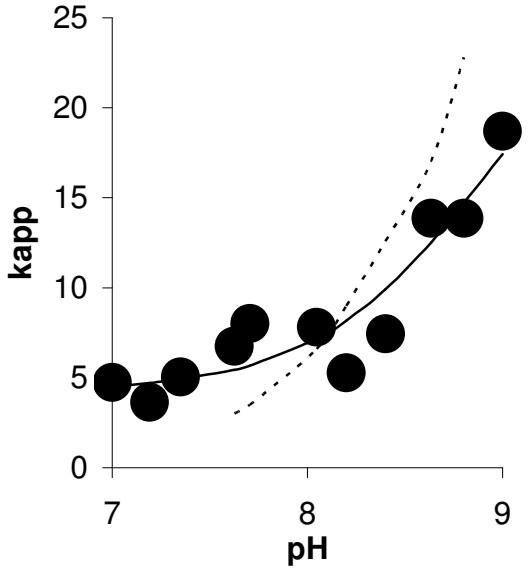

Figure 3. Dependence of the apparent second-order rate constant, kapp, on pH for the reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with the slow phase of minor duck carbonmonoxyhemoglobin in the presence (•) of inositol-P_6 at 10 µM HbCO, 300 µM DTNB, 10 µM inositol-P6, I= 0.05 M, phosphate buffer pH 7.0-8.0, borate buffer pH 8.2-9.0, λ= 412 nm and temperature of 20°C, (Broken line shows the absence of inositol-P_6)

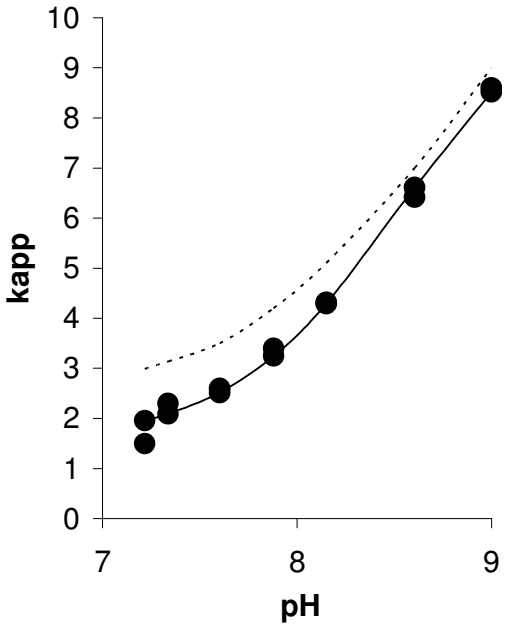

Figure 4. Dependence of the apparent second-order rate constant, kapp, on pH for the reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with the slow phase of minor duck aquomethemoglobin in the presence (•) of inositol-P_6 at 10 µM MetHb, 300 µM DTNB, 10 µM inositol-P_6, I= 0.05 M, phosphate buffer pH 7.0-8.0, borate buffer pH 8.2-9.0, λ= 412 nm and temperature of 20°C, (Broken line shows the absence of inositol-P_6).

Table 1. Reaction of DTNB with minor duck hemoglobin in the presence of inositol-P_6 at ionic strength of 50 mM: fast kinetic phase. Best-fit parameters used to fit the data in Figures 1 and 2 according to equation (1).

Derivative	pK_1	pK_2	k_1	K_2
Carbonmonoxy	5.00	8.47	169.70	142.84
	(5.00)*	(8.87)*	(43.94)*	(345.87)*
Aquomet	5.00	8.92	12.71	201.02
	(6.54)*	(8.04)*	(69.88)*	(155.88)*

* Best-fit parameters for the stripped hemoglobin

Table 2. Reaction of DTNB with hemoglobin in the presence of Inositol-P_6 at an ionic strength 50 mM: slow kinetic phase. Best-fit parameters used to fit the data in Figures 3 and 4 according to equation (1).

Derivative	pK_1	pK_2	k_1	k_2
Carbonmonoxy	5.00	8.91	4.30	23.75
	(5.00)*	(8.81)*	(0.44)*	(50.36)*
Aquomet	5.31	8.52	1.54	9.18
	(6.87)*	(8.59)*	(2.75)*	(2.75)*

* Best-fit parameters for the stripped hemoglobin

The bowl shape was also observed for the fast phase of pigeon hemoglobin in the presence of inositol-P_6 signifying that the expected salt-bridge between HisHC3(146)β and AspFG1(94)β was not formed. The structural implication of this is that inositol-P_6 rather than strengthening the HisHC3/AspFG1 salt bridge causes the histidine residue to move away from the aspartate residue thereby causing it to come closer to CysF9(93)β residue. This leads to increased reactivity of the CysF9(93)β. By contrast, such profiles reported for pigeon hemoglobin were not observed for minor duck hemoglobin. The overall implication of the differences observed in the reactivities of these hemoglobins is that the primary structure of each hemoglobin plays an important role in its reactivity. A similar observation has been reported for the difference in the reactivities of human hemoglobins A and S (HbA and HbS). In that case, a single point (A3[6]$\beta^{Glu \rightarrow Val}$) mutation resulted in much difference in their reactivities both in the presence and absence of inositol-P_6 (Okonjo et al., 1996).

Moreover, it is observed that pK_2 value for aquomet (Table 1) increases by 0.88 unit of pK_a when the hemoglobin was bound to inositol-P_6. The increase of 0.88 unit in pK_a value observed for aquomet-hemoglobin supports what was reported earlier for human hemoglobin A (HbA) that the water molecule attached to the sixth coordination position of the Fe^{+3} is also electrostatically linked to the sulphydryl (Okonjo et al., 1995). This implies that the salt bridge formed between HisHC3/AspFG1 was formed in the minor duck hemoglobin and it was strengthened in aquomet but was not as pronounced in the carbonmonoxy derivative. This suggests that the ligand bound to the heme contributes to the reactivity of the sulphydryl groups of inositol-bound minor duck hemoglobin. On the other hand, such high increase in pK_a value was not observed for carbonmonoxy derivative. The structural implication of this observation is that binding of CO to the heme causes CysF9(93)β to be more exposed for reaction hence an increase in reactivity. The binding of CO causes conformation changes that enhance the reactivity of the CysF9(93)β.

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