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′-dithiobis-(2-nitrobenzoic acid), DTNB, in the presence of inositol hexakisphosphate (inositol-P6) 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, kobs, the apparent second-order rate constants, kapp, were calculated. Inositol-P6 reduced the rates of all reactions for the two derivatives for both CysF9(93) and CysB5(23). The reaction of CysF9(93) gave an increase of 0.88 unit of pKa for the aquomet derivative but a decrease of 0.40 unit of pKa for the carbonmonoxy derivative. The salt-bridge formed between HisHC3 and AspFG1 is more strengthened by inositol-P6 in aquomet than in carbonmonoxy derivative.

Key words: Duck, hemoglobin, sulphydryl, inositol-P6.

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 possess 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 platyrhynchos) by venous punc-
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 7.0-8.0, 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 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$_6$)](image)

RESULTS AND DISCUSSION

Analysis of simple profiles in the presence of inositol-P$_6$

The apparent second-order rate constant, kapp, 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, $k_{app}$, 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, $\lambda$= 412 nm and temperature of 20°C, (Broken line shows the absence of inositol-P$_6$).

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) sulphhydryl group is linked to the ionization of HisHC3(146), with ionization constant $K_1$, $k_2$ is the limiting apparent second order rate constant at high pH when the sulphhydryl reactivity is linked to the ionization of CysF9(93), 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, $k_{app}$, 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-P$_6$, I= 0.05 M, phosphate buffer pH 7.0-8.0, borate buffer pH 8.2-9.0, $\lambda$= 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, $k_{app}$, 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, $\lambda$= 412 nm and temperature of 20°C, (Broken line shows the absence of inositol-P$_6$).
The bowl shape was also observed for the fast phase of pigeon hemoglobin in the presence of inositol-P₆ 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-P₆ 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]βVal) mutation resulted in much difference in their reactivities both in the presence and absence of inositol-P₆ (Okonjo et al., 1996).

Moreover, it is observed that pKᵥ value for aquomet (Table 1) increases by 0.88 unit of pKᵥ when the hemoglobin is bound to inositol-P₆. The increase of 0.88 unit in pKᵥ 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 Fe³⁺ 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 pKᵥ 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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REFERENCES


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* Best-fit parameters for the stripped hemoglobin

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* Best-fit parameters for the stripped hemoglobin

Table 1. Reaction of DTNB with minor duck hemoglobin in the presence of inositol-P₆ 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).

Table 2. Reaction of DTNB with hemoglobin in the presence of Inositol-P₆ 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).


