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REGULAR ARTICLE

Equilibrium Studies of the Reaction of Turkey (*Meleagris gallopavo*) Haemoglobin Sulphydryl Groups with 5,5'-dithiobis(2-nitrobenzoate): Tertiary Conformational Change in Turkey Haemoglobin Induced by Inositol hexakisphosphate

Ehi-Eromosele C.O.*, Edobor-Osoh A., Ajanaku C.O., Anake W.U., Aladesuyi O., Oduselu G.O.

Department of Chemistry, Covenant University, P.M.B. 1023, Ota, Ogun State, Nigeria

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Corresponding Author:
Email: cyril_720@yahoo.com
Phone: +234-8039576084

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ABSTRACT

The red blood cell of turkey contains two haemoglobin types, major and minor components. In the present study, the equilibrium constant, K_{equ}, for the reaction of 5,5'-dithiobis(2-nitrobenzoate), DTNB, with the sulphydryl group of the major turkey aquomethaemoglobin was determined at 25°C as a function of pH. K_{equ} varies by about 2 to 3 orders of magnitude between pH 5.6 and 9.0 for both haemoglobin [stripped and in the presence of inositol hexakisphosphate (inositol-P_6)]. Calculations from the pH dependence of K_{equ} showed that in the r ⇌ t tertiary conformational transition of aquomethaemoglobin, the t isomer population was 0.26 %. In the presence of inositol-P_6, the t isomer population increased to 9.08 %. The results showed that while inositol-P_6 increased the relative population of the **t** tertiary conformation by changing the relative distribution of two protein conformations, it had no effect on K_{equ}. The effect of Inositol-P_6 on the nature and number of groups linked to the DTNB reaction was also determined.

1. Introduction

In biology, sulphur frequently occurs in the form of cysteine, an amino acid that fulfils a wide range of functions in proteins (Giles et al., 2003). Of the twenty naturally occurring amino acids that proteins are composed of, the cysteine groups are by far the most reactive. The thiolate anion, RS^-, is over 500 times more nucleophilic than the corresponding alkoxy analogue, RO^- (Streitweisser, 1956). This high reactivity of the thiolate anion has placed sulphydryl groups among the most important functional groups to be considered when investigating the reactivities, functions, mechanisms and conformations of biological macromolecules. Sulphydryl groups have been shown to facilitate or inhibit many reactions in living systems (Braunitzer et al., 1964). The thiol groups do this by direct catalysis, by binding to substrates or by being involved in so-called allosteric interactions (Colman and Chu, 1969).

Haemoglobin is the paradigm of allosteric proteins (Eaton et al., 2007). Allostery in proteins, though extensively studied, has recently experienced resurgence partly due to the extension of the concept to systems of different complexity (Whitty, 2008; Garcia et al., 2011; Ronda et al., 2013). Different allosteric models have been proposed to explain the structure function dynamics relation-

ships of haemoglobin, but none has been able to give quantitative accounts for all of the structural, equilibrium and kinetic properties of haemoglobin (Eaton et al., 2007). However, the tertiary two state allosteric model has been the most promising.

The CysF9[93]β sulphydryl group has been used to monitor tertiary and quaternary structure change in haemoglobin (Baldwin, 1980; Shaanan, 1983; Okonjo *et al.* 1989, 2008, 2009, 2010). The CysF9 [93]β sulphydryl group of haemoglobin in the R (and also in the T) quaternary state of haemoglobin exists in two conformations relative to the main chain: cis-to-amino and cis-to-carbonyl (Shaanan, 1983; Okonjo *et al.* 1989). These two conformations are coupled to two tertiary isomeric forms of haemoglobin (r and t, respectively) in dynamic equilibrium (Okonjo *et al.* 1989).

Heterotropic effector molecules such as 2, 3-bisphosphoglycerate (2,3-BPG), inositol hexakisphosphate (inositol-P6) and bezafibrate — lower the oxygen affinity of haemoglobin (Vandecasserie, 1971; Lalezari, 1990; Marden, 1990; Yokoyama, 2006) and the reactivity of the CysF9[93]β sulphydryl group. Contrary to the established text-book view, these compounds are able to reduce oxygen affinity without switching the protein to the T (tense) state (Yokoyama, 2006). Recent studies demonstrate binding of allosteric effectors to liganded Hb, contributing a new understanding of the allosteric transition and structural determinants regulating Hb function (Qiuying, 2005). These findings indicate that structural changes at the quaternary level are not the only determinants of oxygen affinity or of CysF9[93]β reactivity but that structural changes at the tertiary level must also be considered as important determinants of both parameters (Okonjo et al., 2009).

In this study, we present the equilibrium study of the reaction of the sulphydryl groups of the aquomethaemoglobin of turkey with 5,5'- dithiobis (2-nitrobenzoate) – DTNB. From the equilibrium studies, K_{rt}, the equilibrium constant for the r ⇌ t tertiary structure transition in turkey aquomethaemoglobins and the influence of allosteric effectors on this transition is calculated. Also, the effect of Inositol-P_6 on the nature and number of groups influencing the reaction of turkey sulphydryl groups with DTNB is presented.

2. Materials and Methods

Inositol hexakisphosphate and 5,5'-dithiobis(2-nitrobenzoate), DTNB, were obtained from Sigma while resin Zeolite D.M.F was obtained from Permutit Company Limited.

Turkey blood was obtained from a local bird farm. The blood was collected in bottles containing heparin anticoagulant.

2.1. Preparation of Haemoglobin

Haemoglobin was prepared using the procedures outlined in Okonjo et al., 2007. The blood sample was centrifuged for 20 min at 5^{o}C at high speed of about 18,000 rpm using refrigerated centrifuge (model GL- 18B, Gallenkamp, England). The red blood cells were washed three times with isotonic saline solution (9.5 g NaCl/dm^3). After each washing, the resulting mixture was centrifuged at 10,000 rpm for 15 min. The sediment (erythrocytes) was lysed with an equal volume of ice-distilled water to yield a mixture of haemolysate solution and red cell debris. The mixture was centrifuged at 10,000 rpm for 20 min to remove all impurities. The haemolysate was decanted from the cake of cell debris, after which sodium chloride (5 % w/v) was added. This mixture was left for 20 minutes at 5^{o}C in a refrigerator. The haemolysate was futher centrifuged at a speed of 18,000 rpm for 20 min. Lower molecular weight impurities contained in the haemolysate were then removed by dialyzing it in a 5 dm^3 flask against distilled water (pH 7.0) at 5^{o}C using polyvinyl chloride dialysis tubing for three hours. This procedure was repeated two more times but in 10 mmol dm^{-3} phosphate buffer (pH 6.5). The haemoglobin solution was stored in a deep freezer until it was needed. The oxyhaemoglobin so prepared was converted to carbonmonoxyhaemoglobin and stored frozen. Oxyhaemoglobin was made from carbonmonoxyhaemoglobin by photolysis. Aquomethaemoglobin was made from oxyhaemoglobin by oxidation with a 2-fold molar excess of $K_3Fe(CN)_6$.

2.2. Separation of Haemoglobin

Separation of haemoglobin was done using the procedures outlined in Okonjo et al., 2009. Turkey haemolysate contains two haemoglobins, major and minor. The separation of the haemoglobins was achieved as follows in a cold room at 5 °C. A 3 cm (diameter) by 30 cm column of Whatman CMC-52 carboxymethylcellulose, a microgranular, preswollen cation exchanger, was used. The resin was pre-equilibrated with 10 mmol dm^{-3} phosphate buffer, pH 6.5. The minor haemoglobin was completely

eluted with the pH 6.5 buffer, whereas the major component remained bound to the resin. The major haemoglobin was eluted with phosphate buffer, pH 8.0, ionic strength 0.2 mol dm^{-3}. The haemoglobins were stored in the freezer and thawed when required. Prior to use for experiments, each haemoglobin was passed through a Dintzis ion exchange column (Dintzis, 1952) to remove endogenous organic phosphates and undesired ions.

2.3. Equilibrium Constant Determination for the Reaction of DTNB with Sulphydryl Groups

The method as suggested by Okonjo et al., 2009 for the determination of equilibrium constant, K_{equ}, was used with some modifications. For measurements involving stripped haemoglobin, 3 cm^3 aliquots of a 50 µmol (haem) dm^{-3} solution in a buffer of known pH, ionic strength 0.05 mol dm^{-3}, prepared as detailed in (Okonjo et al., 2007) were accurately measured into eighteen clean, dry test tubes. Varying small volumes (2 to 35 mm^3) of the stock 29.07 mmol dm^{-3} DTNB were added to each of the test tubes. The contents of each test tube were stirred and allowed to equilibrate at 25°C for about 6 hours.

The absorbance of the mixture in each tube was read at 412, 450 and 470 nm with Helios Zeta uv–visible spectrophotometer using a 1 cm light path cuvette. The reference solution was a 50 µmol (haem) dm^{-3} haemoglobin solution to which no DTNB had been added. Measurements in the presence of inositol-P_6 were similarly carried out, except that 3 cm^3 aliquots of haemoglobin (25 µmol (haem) dm−3) were used. The inositol-P_6 concentration was 25 µmol dm^{-3}, that is, a 4:1 molar ratio with respect to haemoglobin tetramers. The volume of stock DTNB added to the tubes containing haemoglobin was between 2 and 35 mm^3. The absorbance of the 25 µmol (haem) dm^{-3} reference solution was adjusted to zero by pressing the 'auto zero' button. The absorbance change of each haemoglobin/DTNB mixture was then determined at 412, 450 and 470 nm relative to this zero absorbance. A molar absorption coefficient of 14,000 mol^{-1} dm3 cm^{-1} was assumed for 5-thio-2-nitrobenzoate (TNB), the chromophoric product of the reaction.

3. Results

The reaction between CysF9[93]β sulphydryl group and DTNB is known to be a reversible reaction (Okonjo et al., 2008, 2009, 2010) which can be depicted as:

$$PSH + DTNB \overset{Q_{SH}}{\rightleftharpoons} H^+ + PS^- + DTNB \overset{K_{equ}}{\rightleftharpoons} H^+ + PS.ST + TNB^- \overset{Q_{TNB}}{\rightleftharpoons} PS.ST + TNBH \ldots\ldots(1)$$

PSH is haemoglobin with the sulphydryl in its protonated, unreacting (with DTNB) form; PS^- is the corresponding (reacting) anion form in the *cis*-to-amino conformation; PS.ST is the mixed disulfide formed after reaction with DTNB, and it is in the *cis* -to-carbonyl conformation (Shaanan, 1983; Okonjo et al., 1989); TNB^- is 5-thio-2-nitrobenzoate, the anionic, chromophoric product of the reaction; TNBH is the protonated form of TNB^-; Q_{SH} and Q_{TNB} are the ionization constants of a sulphydryl group and TNBH, respectively; K_{equ} is the equilibrium constant for the formation of the mixed disulfide, that is, the DTNB reaction step. The equation used to calculate K_{equ} from Equation 1 is:

$$K_{equ} = \frac{[TNB^-]^2\left\{1+\frac{[H^+]}{Q_{TNB}}\right\}\left\{1+\frac{[H^+]}{Q_{SH}}\right\}}{\left\{[P]_{total}-[TNB^-]\left(1+\frac{[H^+]}{Q_{TNB}}\right)\right\}\left\{[DTNB]_{total}-[TNB^-]\left(1+\frac{[H^+]}{Q_{TNB}}\right)\right\}} \ldots\ldots\ldots(2)$$

It is important to state that turkey haemoglobin, like those of other avians, contain another sulphydryl reactive group (CysB5[23]β). So this was considered in calculating the K_{equ}, since the concentration of the DTNB-reactive sulfhydryl groups in these haemoglobins is twice that of the mammalian hemoglobins at the same haemoglobin concentration (Okonjo et al., 2008).

A full derivation of Eq. (2) has been reported in Okonjo et al., 2006. A computer programme written on a *MicroMaths Scientist software* aided the calculation of K_{equ} from the experimental data.

The results obtained for the equilibrium constants for the stripped haemoglobin and the haemoglobin in the presence of inositol-P_6 are presented in Table 1.0. Figure 1 shows the graph of $-\log_{10}K_{equ}$ with pH for the reaction of stripped aquomethaemoglobin with DTNB. Table 2 shows the results of the analyses for the major aquomethaemoglobin (stripped) (Fig. 1) and those for the major aquomethaemoglobin in the presence of inositol-P_6 (Fig. 2). Figure 2 shows the graph of $-\log_{10}K_{equ}$ with pH for the reaction of aquomethaemoglobin with DTNB in the presence of inositol-P_6. Figure 3 shows the graph that compares the variation of $-\log_{10}K_{equ}$ with pH for the reaction of stripped aquomethaemoglobin with DTNB (open symbols) and the variation of $-\log_{10}K_{equ}$ with pH for the reaction of aquomethaemoglobin with DTNB in the presence of inositol-P_6 (filled symbols).

pH (stripped)	$-\log_{10}K_{equ}$	pH (+ inositol-P_6)	$-\log_{10}K_{equ}$
4.97	-3.040	5.20	-3.040
5.28	-2.960	5.79	-2.800
5.36	-2.950	5.80	-3.080
5.85	-2.790	5.81	-2.860
6.12	-2.810	6.16	-2.540
6.37	-2.430	6.37	-2.410
6.53	-2.160	6.45	-2.350
6.71	-2.340	6.62	-2.220
6.90	-2.210	6.79	-2.060
7.00	-1.960	7.07	-1.820
7.05	-1.720	7.12	-1.790
7.14	-1.730	7.23	-1.580
7.13	-1.700	7.19	-1.780
7.84	-0.950	7.86	-1.200
7.97	-1.040	8.02	-1.040
8.32	-0.900	8.33	-0.780
8.41	-0.780	8.61	-0.900
8.64	-0.780	8.77	-0.700

Table 1: Dependence of $-\log_{10}K_{equ}$ on pH for the reaction of stripped (and in the presence of inositol-P_6) turkey aquomethaemoglobin with DTNB at 25°C.
Conditions: phosphate buffers pH 5.6- 7.8 and borate buffers pH 8.0- 9.0 (ionic strength 0.05 mmol dm^{-3}). The pH values reported above are those of experimental solutions.

Parameters	Stripped	In the presence of inositol-P_6
pQ_{1r}	5.124	5.884
pQ_{1t}	5.210	4.856
pQ_{2r}	8.216	5.837
pQ_{2t}	8.218	9.159
K_{E3}/K_{E2}	0.007343	0.7844
K_{E3}/K_{E1}	0.0001	0.0600
pK_{E3}	-0.6904	-0.6892
K_{rt3}	0.002604	0.09988

Table 2: Best fit parameters used to fit the equilibrium data for the reaction of DTNB with the sulphydryl groups of turkey aquomethaemoglobin (stripped) and the haemoglobin in the presence of inositol-P_6.

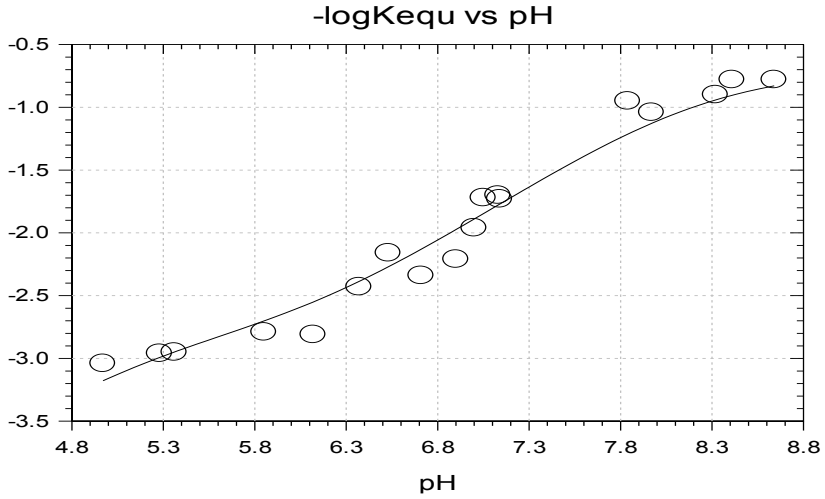


Figure 1: Variation of $-\log_{10}K_{equ}$ with pH for the reaction of stripped turkey aquomethaemoglobin with DTNB at 25°C. Conditions: phosphate buffers, pH 5.6 - 7.8 and borate buffers, pH 8.0 - 9.0; ionic strength, 50 mmol dm^{-3} (added salt, NaCl); [Hb], 50 µmol (haem) dm^{-3}. The lines through the data points are the theoretical best-fit lines drawn with the parameter reported in Table 2.

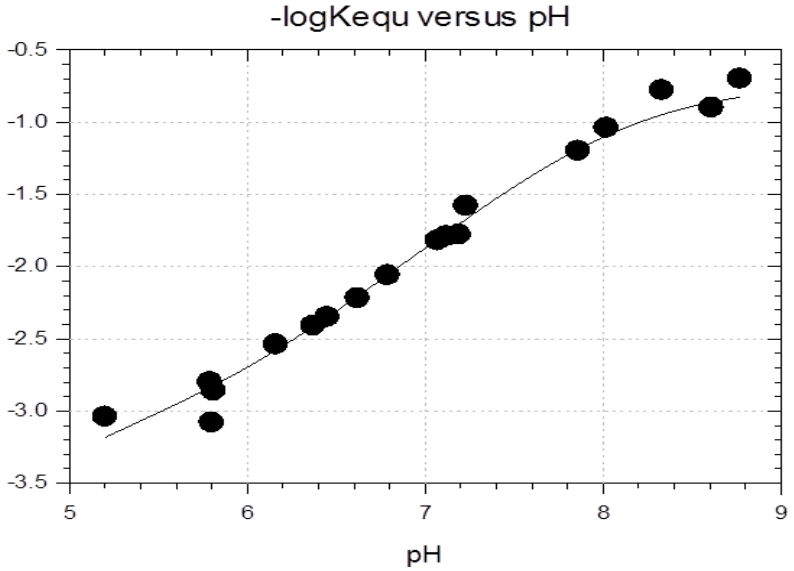


Figure 2: Variation of $-\log_{10}K_{equ}$ with pH for the reaction of turkey aquomethaemoglobin with DTNB in the presence of inositol-P_6 at 25°C. Conditions: phosphate buffers, pH 5.6 - 7.8 and borate buffers, pH 8.0 - 9.0; ionic strength, 50 mmol dm^{-3} (added salt, NaCl); [Hb], 50 µmol (haem) dm^{-3}; [inositol-P_6], 50 µmol (haem) dm^{-3}. The lines through the data points are the theoretical best- fit lines drawn with the parameter reported in Table 2.

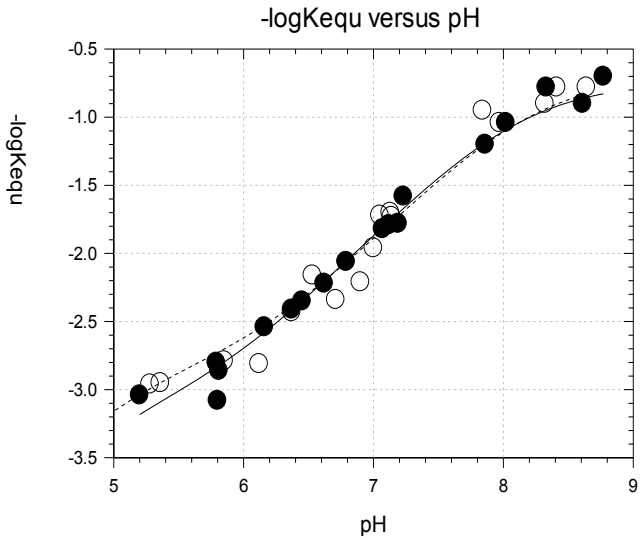


Figure 3: Variation of $-\log_{10}K_{equ}$ with pH for the reaction of turkey aquomethaemoglobin (stripped and with inositol-P_6) with DTNB at 25°C. Conditions: phosphate buffers, pH 5.6 - 7.8 and borate buffers, pH 8.0 - 9.0; ionic strength, 50 mmol dm^{-3} (added salt, NaCl); [Hb], 50 µmol (haem) dm^{-3}; [inositol-P_6], 50 µmol (haem) dm^{-3}. Open Symbols, stripped haemoglobin; filled symbols, haemoglobin + inositol-P_6.

4. Discussion

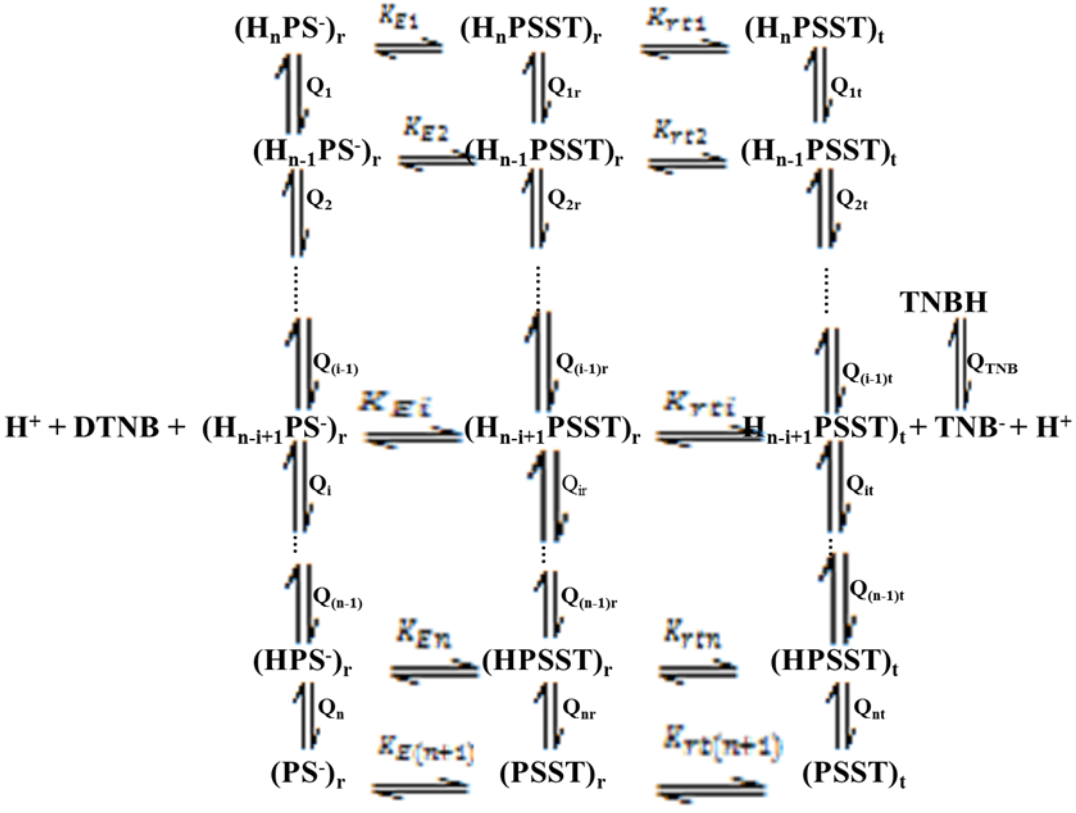
4.1. Analyses of pH dependence of K_{equ} data

The fairly strong pH dependences seen in Figures 1, 2 and 3 imply that the DTNB reaction (Eq. 1) is linked to the ionizations of groups on the haemoglobin molecule. To gain an understanding of the nature and number of these groups, and of the possible effect of inositol-P_6 on these groups and on the **r** ⇌ **t** tertiary conformational equilibrium, the data in Fig 3 was analysed quantitatively.

The following reaction scheme (Scheme 1) was used to account for the effects of pH on the reaction of DTNB with the sulphydryl groups of turkey (Okonjo et al., 2009). Scheme 1 and equation 3 were used to fit the equilibrium data.

A close examination of Scheme 1 reveals that DTNB first reacts with CysF9[93]β in the haemoglobin **r** isomer. After the mixed disulphide has been formed, the haemoglobin molecule undergoes an **r** ⇌ **t** transition.

In Scheme 1, $H_{n-i+1}PS^-$ (i = 1, 2,…, n) are species in which the sulphydryl is in its thiolate anion form, the form that reacts with DTNB; $H_{n-i+1}PS.ST$ (i = 1, 2, …, n) are the mixed disulphide species formed after the reaction of the sulphydryl with DTNB. Species marked with subscripts **r** and **t** are those in which the sulphydryl is in the **r** and **t** tertiary isomeric forms of haemoglobin, respectively. The various



Scheme 1.

(Source: Okonjo et al., 2009)

proton ionization constants are represented as Q_i, Q_{ir} and Q_{it} (i = 1, 2,…, n) to differentiate them from the equilibrium constants K_{Ei} (i = 1, 2,…, n+1) for the reaction of DTNB; and $K_{rt(n+1)}$ is the equilibrium constant at high pH for the **r** ⇌ **t** isomerization.

A relationship between K_{equ} and the parameters of Scheme 1 is represented with Eq. 3 (Okonjo et al., 2009).

$$K_{equ} = \frac{K_{E(n+1)}\{1 + \sum_{i=1}^{n}(H^+)^{n-i+1}(\prod_{j=i}^{n} Q_{jt})^{-1} + K_{rt(n+1)}(1 + \sum_{i=1}^{n}(H^+)^{n-i+1}(\prod_{j=i}^{n} Q_{jt})^{-1})\}}{1 + K_{E(n+1)}\{\sum_{i=1}^{n}(H^+)^{n-i+1}(\prod_{j=i}^{n} Q_{jr})^{-1} K_{Ei}^{-1}\}} \quad \ldots (3)$$

With the relationship in Eq. (3), the data in Figures 1, 2 and 3 were analysed and fitted successfully with n = 2.

4.2. Effect of pH and Inositol-P_6 on K_{equ}

Figure 3 shows the variation of $-\log_{10}K_{equ}$ with pH for the reaction of DTNB with stripped turkey major aquomethaemoglobin (open symbols) and aquomethaemoglobin in the presence of inositol-P_6 (filled symbols). For stripped haemoglobin, it is seen that K_{equ} varies by about 2 to 3 orders of 2magnitude between pH 5.6 and 9.0. Similar results were obtained in the presence of inositol-P_6. It is also seen from Figure 3 that inositol-P_6 has no effect on the affinity of CysF9[93]β and CysB5[23]β for DTNB. This result is interesting because inositol-P_6 is a heterotropic allosteric effector, and such effectors are known to reduce the oxygen affinity of haemoglobin. This result is in sharp contrast to the result obtained for the sheep haemoglobin, for which inositol-P_6 increases the affinity for DTNB (Okonjo et al., 2009). From table 2, it is also seen that inositol-P_6 has no effect on the value of K_{E3}, the limiting value of K_{equ} at high pH: from a value of 4.90 for stripped haemoglobin to a value of 4.89.

4.3 Effect of Inositol-P_6 on the Ionization Constants of Groups Coupled to the DTNB Reaction

The results of the analyses for the major aquomethaemoglobin (Figure 3) are presented in Table 2. From the analyses, two sites are detectable (Table 2). As can be seen in Table 2 for the stripped haemoglobin, pQ_{1r}/pQ_{1t} = 5.124/5.210 while pQ_{2r}/pQ_{2t} = 8.216/8.218. Thus in the transition from the **r** to the **t** isomer, the first ionizable group has its pQ increased marginally from 5.124 to 5.210; and the second group has its pQ increased from 8.216 to 8.218. On the basis of the above assignments, we assign the pQ_{1r} and pQ_{1t} values to HisNA2[2]β of turkey haemoglobin in the r and t tertiary conformations, respectively. pQ_{2r} and pQ_{2t} values, we assign to the terminal group of the NH_3^+ of the turkey haemoglobin in the **r** and **t** tertiary conformations, respectively.

Examination of the pQ values of the ionizable groups linked to the DTNB reaction (Tables 2) shows that inositol-P_6 increases pQ_{1r}, pQ_{2t} by a mean value of 0.9 pKa but decreases pQ_{2r} and pQ_{1t} by a mean value of 1.4 pKa units.

4.4 Effect of Inositol-P_6 on K_{rt3}

Ligand binding, allosteric effectors and the quaternary state affect the relative populations of t and r, with ligation and R favoring r (Ronda et al., 2013). Table 2 shows that inositol-P_6 increases K_{rt3} (the equilibrium constant of the **r** ⇌ **t** isomerization) 38.4-fold in the major haemoglobin of turkey. While in sheep major haemoglobin, K_{rt3} was observed to increase by 4.5-fold (Okonjo et al., 2009). From the value of K_{rt3} for the stripped haemoglobin, 0.002604 (see Table 2), a value of 0.26 % is obtained as the relative population of the **t** isomer. The value of K_{rt3} in the presence of inositol-P_6 is 0.09988; this gives a value of 9.08 % for the relative population of the **t** isomer. Therefore, inositol-P_6 increases the transition constant of turkey aquomethaemoglobin for DTNB. This increase of the **t** isomer population in the presence of inositol-P_6 demonstrates a tertiary conformational transition in the sulphydryl groups of the turkey aquomethaemoglobin.

5. Conclusion

These results clearly show that inositol-P_6 has no significant effect on the reaction of DTNB with the sulphydryl groups of turkey major aquomethaemoglobin. This result becomes more interesting when compared with the mammalian haemoglobins, which have lesser inositol-P_6 binding sites, yet inositol-P_6 increases their affinity with DTNB.

The present result further confirms that the structural transition induced by inositol-P_6 is not simply quaternary. It also involves the tertiary **r** ⇌ **t** transition. Hence, these results are consistent with the plasticity of tertiary structure within the constraints of the R quaternary structure and support the global allostery model of haemoglobin (Yonetani et al., 2002), implying that the heterotropic effector-linked tertiary structural changes are responsible for the modulation of haemoglobin functions (Lepeshkevich et al., 2009). Further work is being done on the equilibrium and kinetic studies of turkey haemoglobin derivatives to clarify these findings especially the effect of inositol-P_6 on the DTNB

reaction.

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