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Contrasting behavior of oxy- and carbonmonoxyhemoglobin: reaction of Ellman's reagent with CysF9[93]β of carbonmonoxyhemoglobin reveals tertiary level conformational differences between mammalian hemoglobins

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Abstract

The efficiency of oxygen transport function of hemoglobin is affected by their amino acid sequences amongst other things. To gauge how the amino acid sequences of mammals affect the affinity of their hemoglobins for oxygen, the pH dependent studies of the affinities of CysF9[93]β sulfhydryl group of oxy- and carbonmonoxyhemoglobin of four mammalian hemoglobins: rabbit, straw coloured fruit bat (SCFB), human A (HHbAA) and human S (HHbSS) for 5,5'-dithiobis(2-nitrobezoate) (DTNB) in dilute solution were reported. The affinities of DTNB for the hemoglobins in each of the derivatives decreases with increasing pH. Rabbit hemoglobin has an affinity that is at least twice that of HHbSS for DTNB. That of SCFB and HHbAA which were quite comparable were at least three times the affinity HHbSS at corresponding pH. The proportion of r-conformation in the oxy derivative of all the mammalian hemoglobins were between 73 and 80 % at high pH. The proportion of r-conformation in the carbonmonoxy derivative were 0.62% for SCFB; 8.0% for rabbit; 88.60 % for HHbAA and 91.87% for HHbSS. Carbonmonoxyhemoglobin is more sensitive to increasing differences in amino acid sequence and composition of hemoglobin compared to that in oxy-derivative. The restriction in the isomerization process of oxyhemoglobin was rationalized on the basis of ease of formation of hydrogen bond between O and N of α and β subunit by HisE7[63]β in oxyhemoglobin compared to COHb where such linkages are hindered. Single point mutation in human hemoglobin at position 6β result in significant difference in affinity of CysF9[93]β for DTNB.

Keywords: hemoglobin; sulfhydryl group; DTNB; tertiary isomer; amino acid.

INTRODUCTION

In the past two decades, studies of reversible reaction of 5,5'-dithiobis(2-nitrobenzoate) (DTNB) with CysF9[93]β of avian and mammalian hemoglobins have gained significant attention (Okonjo *et al*., 2008; Okonjo and Fodeke, 2006). This is because of the similarities between the binding properties of DTNB with hemoglobin and the binding properties of oxygen with hemoglobin. These similarities make DTNB a good candidate for the determination of hemoglobin tertiary and quaternary structures. Binding by DTNB to CysF9[93]β, just like the binding of oxygen to heme group is: (i) linked to the ionization of some ionizable groups on the amino acid side chains to which oxygen binding to hemoglobin is also linked; and (ii) DTNB reacts reversibly with CysF9[93]β in a manner similar to the reversible binding of oxygen to hemoglobin (Carrico *et al*., 1978); (iii) The affinity of DTNB for hemoglobin F9[93]β sulfhydryl group is pH dependent just the way oxygen binding affinity to hemoglobin heme group is (Thomas and Lumb 2012; Okonjo and Fodeke, 2006). The pH dependence of the equilibrium studies of the reaction of DTNB with CysF9[93]β of some avian and mammalian hemoglobins have been reported (Okonjo *et al*., 2008; Fodeke, 2017; Fodeke and Bolarinwa, 2024). The data from the experiment were analyzed on the assumption that in solution, hemoglobin exists in two tertiary conformations named r-

conformation and t-conformation. In the t-conformation, CysF9[93]β is cis to the main chain carbonyl group and is formed after DTNB binding to the CysF9[93]β of the hemoglobin. The r-conformation which is favoured in the unreacted thiolate anion form of the sulfhydryl group is cis to the main chain amino group. Since DTNB binds hemoglobin sulfhydryl group reversibly, the two conformations occur reversibly (Okonjo and Fodeke, 2006). Isomerization process should result in change in the immediate environment of at least some of the ionizable group that are linked to the equilibrium reaction and hence result in change in their pK_as. In an attempt to gain insight into how the difference in amino acid sequences and composition of hemoglobin affect their tertiary structure, the change in the pK_a of ionization of the ionizable groups and hence the hemoglobin function of oxygen transport, we embarked on comparing the affinities of DTNB for CysF9[93]β in dilute solutions of four mammalian hemoglobins; human hemoglobin A (HHbAA), straw coloured fruit bat hemoglobin (SCFBHb), rabbit hemoglobin (rabbit Hb) and human hemoglobin S (HHbSS) as a function of pH. Whereas, the difference between the amino acid sequences of HHbAA and HHbSS is the result of a single point mutation in which glutamic acid at position A6 of HHbAA β-chain is substituted with hydrophobic valine in HHbSS (Okonjo *et al*., 1996), more amino acid differences exist between human and other mammalian hemoglobins reported here. However, each of these hemoglobins has CysF9[93]β as the only hemoglobin sulfhydryl group that is reactive with DTNB (Fodeke *et al*., 2016a, 2016b; Fodeke, 2017). This should make the result of our experimental data easier to analyse and interpret. Report of the affinities of the CysF9[93]β of four mammalian hemoglobins with DTNB as a function pH were here presented and the experimental data were analysed based of previous finding that the tertiary structure exist in two isomeric forms "r" and "t" (Okonjo *et al*., 2009).

MATERIALS AND METHODS

Human hemoglobin S was obtained from the erythrocytes of a patient attending the sickle cell clinic of Obafemi Awolowo University. Straw coloured fruit bat hemoglobin was obtained from the blood of straw coloured fruit bat obtained from Obafemi Awolowo University. Male rabbits were purchased from local market and the blood from the animal was used for the preparation of hemoglobin. Hemoglobin A was obtained from a heathy donor at the Obafemi Awolowo University. 5,5-dithiobis(2-nitrobenzoic acid) 98% Lot # SHBG1688V is a product of Sigma Aldrich, USA. Grant thermostated water bath was used together with CC 60 Cryocool immersion cooler made by Thermoscientific Neslab. UV spectrophotometer UV-1800 is a product of Shimadzu Europa GmbH.

Methods

The Hemoglobins were prepared as previously described (Okonjo *et al*., 2006; Okonjo *et al*., 2008; Fodeke, 2022). The equilibrium constant of the reaction of CysF9[93]β sulfhydryl group with DTNB at 25°C was determined from absorbance measurement of the equilibrium solution of the reaction of DTNB with hemoglobin, at 412 nm. The reaction was carried out in dilute solution as was done earlier (Okonjo *et al*., 2006; Okonjo *et al*., 2009).

Data Analysis

The data were analyzed using the scheme which relates the different ionizable groups to the various equilibrium steps in the reaction of DTNB with hemoglobin sulfhydryl group. In the scheme which has previously been well described (Okonjo *et al*., 2007; Fodeke, 2017; Fodeke, 2022) the equilibrium constant of r↔t isomerization at high pH is $K_{rt(n+1)}$, where n is the total number of the ionizable groups linked to the equilibrium reaction of DTNB with the hemoglobin molecule at the specified pH. In analyzing the experimental data n = 2 was used at high pH. $pQ_{ir/t}$ are the negative logarithm of the ionization constants in the r- or t-isomerization state.

It has been previously demonstrated that the equilibrium constant for the reaction of DTNB with hemoglobin sulfhydryl group whose reaction is linked to n ionizable groups is related to the pH and the ionization constant Q_{jr}, Q_{jt} and other physical parameters according to equation (1) (Okonjo *et al*., 2009; Okonjo *et al*., 2014; Fodeke, 2022):

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

Where only two amino acid side chains of the hemoglobin are ionized, n = 2, and Eq. (1) reduces to,

$$K_{equ} = \frac{K_{E3}\{1+(H^{+})^{2}(Q_{1r}Q_{2r})^{-1}+(H^{+})(Q_{2r})^{-1}+K_{rt3}[1+(H^{+})^{2}(Q_{1t}Q_{2t})^{-1}+(H^{+})(Q_{2t})^{-1}]\}}{1+K_{E3}\{(H^{+})^{2}(Q_{1r}Q_{2r})^{-1}(K_{E1})^{-1}+(H^{+})(Q_{2r})^{-1}(K_{E2})^{-1}\}} \quad (2)$$

The best fit curve of the dependence of $-\log K_{equ}$ on pH with the fitting parameters; pK_{E3}, K_{E3}/K_{E2}, K_{E3}/K_{E1}, pQir, pQit (i = 1, 2) and K_{rt3} were used to analyze the experimental data.

RESULTS AND DISCUSSION

DTNB reaction with carbon monoxide bound hemoglobin

The curves of the equilibrium constant of the reaction of DTNB with the carbonmonoxyhemoglobin F9[93]β sulfhydryl group of the different mammals as function of pH are presented in Figure 1. The affinities of the hemoglobin for DTNB in each case decreases with increasing pH over the entire pH, for all the hemoglobins. The affinity of rabbit Hb for DTNB however increases marginally above pH 7.8. Below pH 7.2, the affinity of human hemoglobin A for DTNB was the greatest. The affinity however decreases with increasing pH to a value which is comparable to that of SCFBHb above pH 7.2. The values of the affinity of rabbit carbonmonoxyhemoglobin and SCFBHb are quite comparable at pH *ca* 5.9 and at pH *ca* 8.5, but the affinities of SFBHb was greater than that of rabbit in the range $5.9 < pH < 8.5$. The affinities of rabbit hemoglobin for DTNB were lower than that of human A and SCFBHb over the entire pH range of the reaction. But the difference reduced significantly with increasing pH, above pH 7.9. It is noteworthy that the affinity of HHbSS for DTNB was at least three times less than that of hemoglobin A and at least two times less than that of rabbit hemoglobin over the entire pH range of the experiment.

pKa of ionization of r- and t-isomeric forms

It is evident from Tables 1 and 2, that two ionizable groups which exist in two conformations r- and t- are linked to the reaction of DTNB with each the CysF9[93]β of each carbonmonoxyhemoglobin and oxyhemoglobin. From the values of the pK_a of the ionization of the ionizable groups in Tables 1 and 2 (rows 2 -5) and previous assignments that had previously been made (Okonjo *et al*., 2014; Fodeke and Bolarinwa, 2024) the first ionizable group was assigned to His at position NA2[2]β and the second ionizable group was assigned to HisEF1[77]β. From Table 1, rows 2 and 3, it is evident that r ↔ t transition of the carbonmonoxyhemoglobins resulted in pK_a ($pQ_{1,r/t}$) increase of *ca.* 0.16 in SCFBHb, 1.02 in HHbSS, 1.42 in rabbit Hb and 1.20 in HHbAA. The corresponding increase in the pK_a of the second ionizable group were 0.26 in SCFBHb, 1.53 in HHbSS, 0.56 in rabbit Hb and 0.68 in HHbAA. In the oxyhemoglobin derivative (Table 2, rows 2 and 3), pK_a increase of the first ionizable group due to r ↔ t isomerization was *ca*. 0.88 in SCFBHb, 1.25 in HHbSS, 0.03 unit in rabbit Hb and 0.50 in HHbAA.

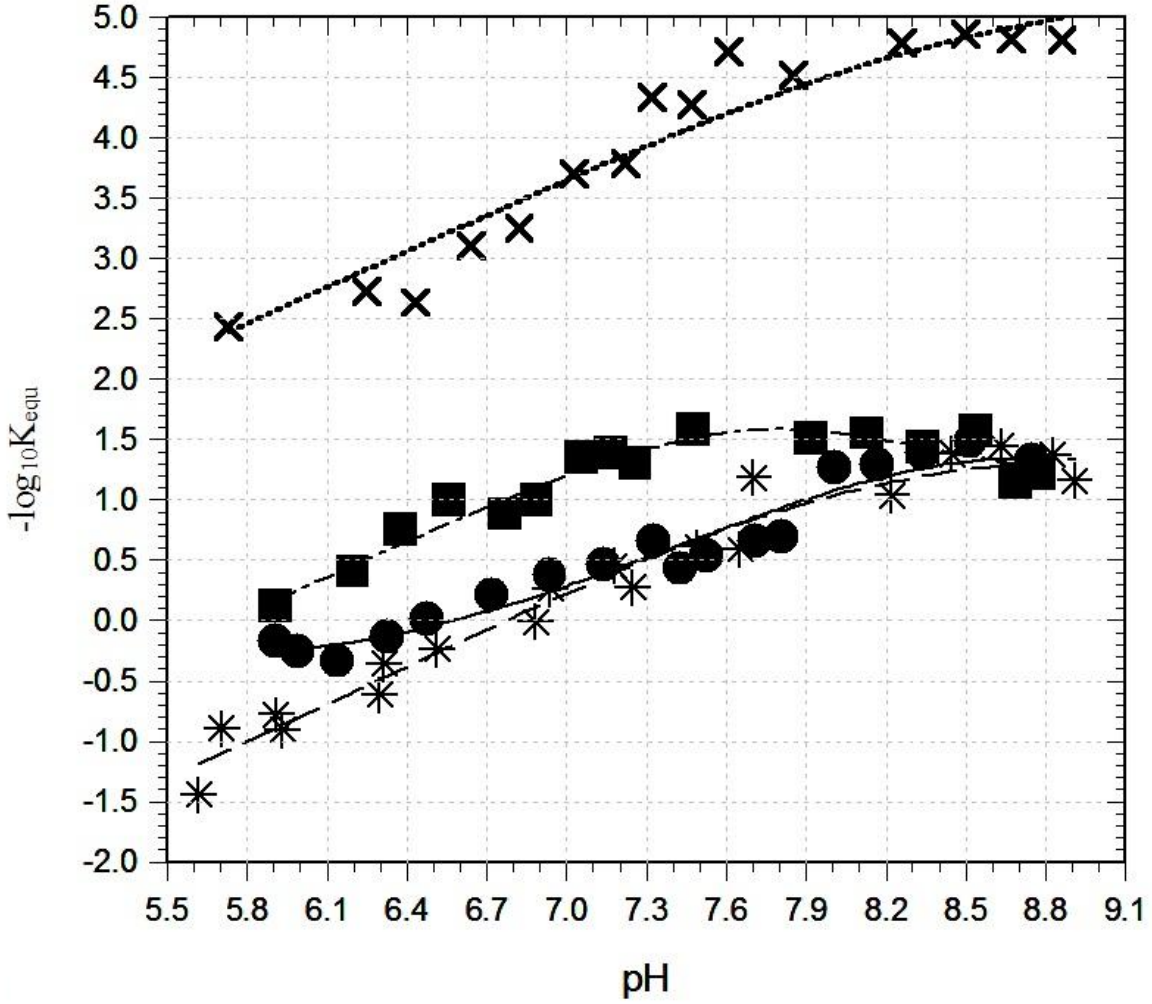

Figure 1. Dependence of the affinity of DTNB for CysF9[93]β sulfhydryl group of carbonmonoxyhemoglobin of human A (asterisk); human S (×); straw coloured fruit bat (circle) and Rabbit (square) at 25 ºC in phosphate buffer pH 5.6 – 8.0 and borate buffer pH 8.0 – 9.1. (0.05 mole dm^{-3} NaCl ionic strength). Hemoglobin concentration was 25 x 10^{-6} mole dm^{-3} in reactive SH concentration.

Whereas for the second ionizable group, from Table 2 rows 4 and 5, the same transition resulted in increase in pKa unit of ca.0.24 in SCFBHb, 1.19 in HHbSS, 0.55 in rabbit and 0.75 in HHbAA. One general observation that could be made from the change in the pK_a of the ionizable groups is that the hemoglobin with the least increase in pK_a of ionization was SCFBHb whereas the largest change in pKa of ionization occurred in HHbSS. It is significant to note that whereas the change in the pK_a values of the first and second ionizable groups of HHbSS were greater than one unit, in the oxyhemoglobin, corresponding change in HHbAA had values that were less than one. This difference underscores the difference in the environment of the of the ioinizable groups owing to the single point mutation resulting in the substitution of glutamic acid at position A3[6] of the β-chain in HHbAA by valine in HHbSS.

Table 1. Fitting parameters of the curve through the experimental data points for the reaction of CysF9[93]β of carbonmonoxy hemoglobin with DTNB.

	SCFBHb	HHbSS	Rabbit Hb	HHbAA
PQ_{1r}	7.572	6.620	5.812	5.710
PQ_{1t}	7.734	7.640	7.239	6.903
PQ_{2r}	8.628	7.194	7.616	8.011
PQ_{2t}	8.884	8.724	8.176	8.694
K_{E3}/K_{E2}	7235.3	0.1240	446.7	0.0256
K_{E3}/K_{E1}	602.64	0.0583	3.975×10^{-30}	7.432×10^{-19}
pK_{E3}	1.2854	4.982	1.261	1.463
K_{rt3}	160.58	0.0885	11.616	0.130

Table 2: Fitting parameters of the curve through the experimental data points for the reaction of CysF9[93]β of oxy hemoglobin derivatives with DTNB.

	SCFBHb	HHbSS	Rabbit Hb	HHbAA
PQ_{1r}	6.911	6.110	6.682	5.701
PQ_{1t}	7.792	7.364	6.710	6.198
PQ_{2r}	8.136	8.300	7.338	7.842
PQ_{2t}	8.371	9.491	7.883	8.597
K_{E3}/K_{E2}	0.857	0.826	0.128	2.438×10^{-18}
K_{E3}/K_{E1}	0.132	4.707×10^{-17}	0.00867	1.563×10^{-19}
pK_{E3}	1.582	4.809	2.277	1.815
K_{rt3}	0.282	0.317	0.250	0.364

DTNB reaction with oxyhemoglobin

Figure 2 shows the dependence of the affinity of DTNB for oxy-derivative of human A, SCFB, rabbit and human S hemoglobins. The affinity of each of the hemoglobin species decreases with increasing pH. Whereas, the affinity of human A and SCFBHb were comparable above pH 7.3, below this pH, affinity of HHbAA gets increasingly higher with decreasing pH (affinity of HHbAA for oxygen gets increasingly lower than that of SCFBHb with decreasing pH). It is noticeable that the average gradient of the dependence of the affinity of HHbAA is somewhat higher than that of SCFBHb. This suggests higher sensitivity of HHbAA affinity for DTNB (oxygen) compared to SCFBHb. The higher average gradient of the curve through HHbAA data is also suggestive of the difference in the Bohr effect of the hemoglobins at high and at low pH. This Bohr effect differences could be used to deduce the oxygen loading and unloading capacity (Zhang *et al*, 2006). At low pH, hemoglobin affinity for oxygen are generally lower than at high pH. The consequence of this is that whereas the affinity of Hb for oxygen is comparable in human and SCFB, at high pH, the affinity of HHbA for oxygen is lower at low pH. This results in higher oxygen transport efficiency of HHbAA hemoglobin compared to SCFBHb. Previously, it has been shown that the affinity of hemoglobin for oxygen increases with increase in pH, not necessarily in linear way up to pH *ca* 7.4, whereas the affinity of oxyhemoglobin for DTNB decreases with increase in pH (Fang *et al*., 1991; Fodeke and Okonjo 2006). The affinity of rabbit oxyhemoglobin for DTNB were generally lower than those of human HbA and SCFBHb over the entire pH of experiment. The low oxygen affinity of rabbit hemoglobin compared to HHbA and SCFBHb may not be unconnected to the oxygen requirement of each mammal for their activities. It should be noted that sickled hemoglobin is an abnormal case with pathophysiological consequences leading to premature death (Malowany and Butany, 2012; Sundid *et al*. 2019). The affinity of human A and SCFBHb for DTNB becomes

more significantly greater than that of rabbit, between pH 6.7 and 7.3, with the size of the affinity of human HbA for DTNB approaching twice that of rabbit Hb. This is qualitatively similar to what was observed with carbonmonoxyhemoglobins (Fig. 1). On the other hand, the affinity of OxHHbSS for DTNB is at least three times lower than the affinity of OxyHHbAA over the entire pH range. This finding is consistent with the much lower affinity of HHbSS for oxygen at both high and low pH values. The implication of this is that HHbSS can only bind small amount of oxygen which is released to the cell at low pH as the carbon dioxide concentration in blood increases due to combustion of glucose (Lawrence and Meier, 2021). On the other hand the much higher affinity of HHbAA for DTNB suggests that it is able to bind much more oxygen which is efficiently released for cellular activities as the pH reduces due to pH reduction by CO_2 from cellular activities.

Carbonmonoxyhemoglobin r-conformation

In other to determine the proportion of r- and t-conformations of the carbonmonoxyhemoglobins, K_{rt3} values of Table 1, (row 5, columns 2 – 5) were used to calculate the percentage r- and hence t-conformations. Percentage r- and t-conformation for the oxyhemoglobins were calculated using the K_{rt3} values of Table 2, (row 5, columns 2 – 5). The proportion of r-conformation of carbonmonoxy derivatives of human A and S hemoglobins at high pH were quite high (88.51 % in human A and 91.87 % in human S). The high proportion of r-conformation in both COHHbSS and COHHbAA might be an indication that the single amino acid substitution at position A6 which account for the difference between HHbAA and HHbSS did not significantly affect the hemoglobin tertiary conformation. Interestingly, this single point mutation was sufficient to bring about at least three fold increase in the affinity of HHbAA for DTNB at any pH, compared to HHbSS. An interesting finding from the comparison of the proportion of the hemoglobin conformations of the HHbs in the oxy and carbonmonoxy derivatives is that the proportion of r-conformation in the carbonmonoxyHb derivative is 88.51 % for COHHbAA and 91.87 % for COHHbSS. These values are higher compared to the proportion of r-conformation in the oxy-derivative which are 77.93 % for oxyHHbAA and 75.92 % for oxyHHbSS. This finding suggests that carbon monoxide favour more of r-conformation compared to oxyhemoglobin. The r-conformation is the non-liganded conformation, whereas, the t-conformation is formed after ligation of the hemoglobin. These findings suggest that COHHbAA is prone to reversible binding of ligand unlike COHbSS which is more resistant to ligand binding.

On the other hand, only 0.62 % of SCFBHb and 8.0 % of rabbit carbonmonoxyhemoglobin existed in the r-conformation. This finding suggests the tertiary conformation adopted by hemoglobin is essentially a function of the hemoglobin amino acid composition and sequences. Whereas, the tertiary structure of both A and S hemoglobins which differ at a single position are not too different in the conformation they adopt in solution in both oxy- and carbonmonoxy- forms. Hemoglobins of rabbit and SCFB which exhibit significant difference in their hemoglobin sequences from human hemolobins, are quite different in the proportion of r-conformation they adopt in the carbonmonoxy derivative. High proportion of t-isomer in carbonmonoxy derivatives of SCFBHb and rabbit hemoglobins indicate that both hemoglobin have higher tendency to bind ligand in the carbonmonoxy form and less tendency to release such ligand (Ahmed *et al*., 2020). It is evident from the result that carbonmonoxy derivative of hemoglobin might be more sensitive to amino acid sequence changes than oxyhemoglobin.

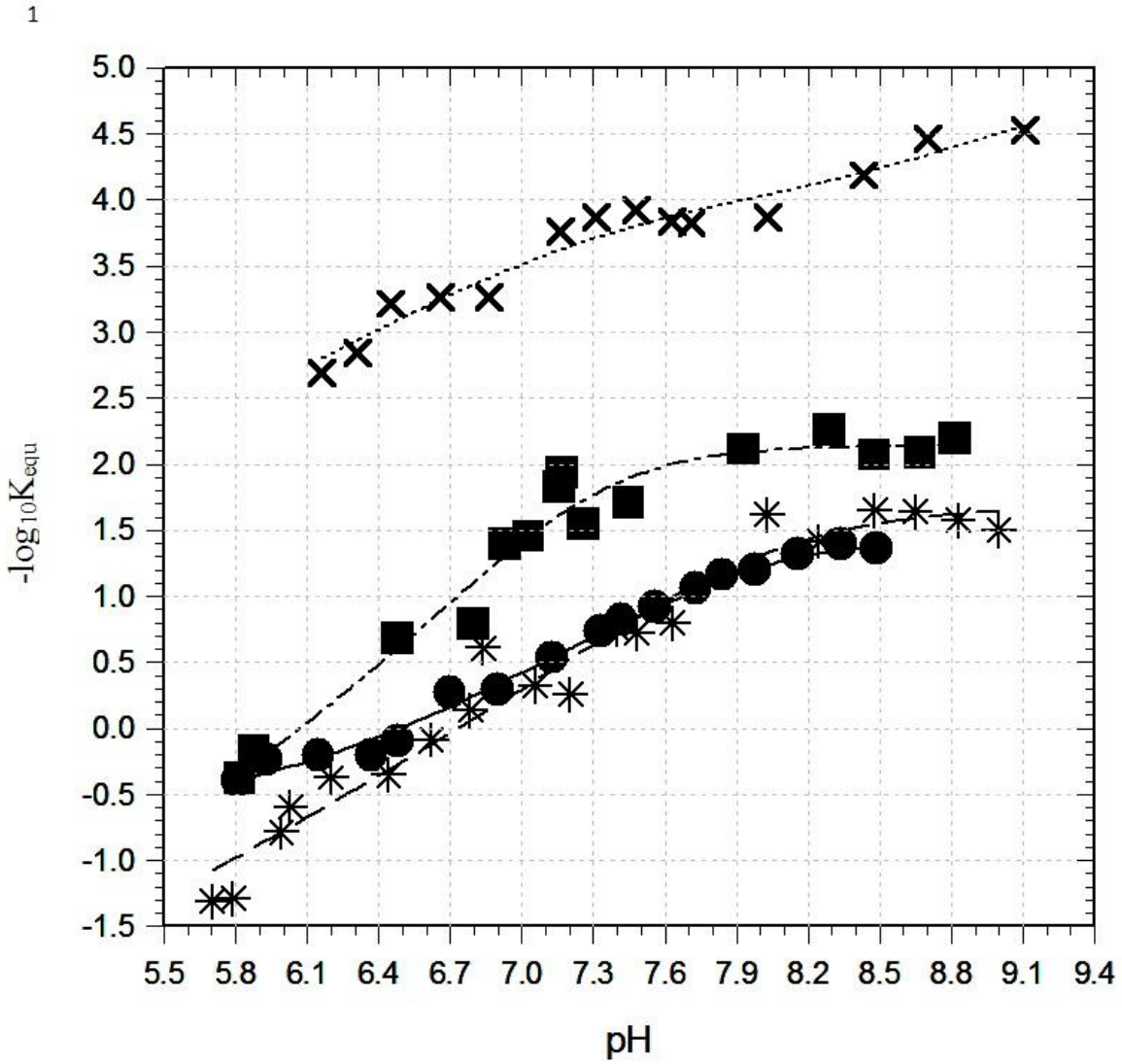

Figure 2. Dependence of the affinity of DTNB for CysF9[93]β sulfhydryl group of oxyhemoglobin of human A (asterisk); human S (×); straw coloured fruit bat (circle) and Rabbit (square) at 25°C in phosphate buffer pH 5.6 – 8.0 and borate buffer pH 8.0 – 9.1. (0.05 mole dm^{-3} NaCl ionic strength). Hemoglobin concentration was 25 x 10^{-6} mole dm^{-3} in reactive SH concentration.

Oxyhemoglobin r-conformation

All the four mammalian oxyhemoglobin presented in this report have preponderance of r-conformation over the t-conformation: OxyHHbAA has 73.35 %, SCFB oxyHb has 78.04 %, rabbit oxyHb has 79.97 % and oxyHHbSS has 75.92 % of r-conformation. This may be due to the formation of hydrogen bond between Oxygen and the N of distal histidine of the α-chain of oxyHb. The distal histidine of α-chain is much closer to the heme axis in OxyHb than in COHb. This may be responsible for fixing the oxyhemoglobin essentially in the r-conformation unlike COHb where tertiary change is conformation is freer. COHb conformation of SCFBHb and rabbit Hb however have 0.62% and 7.93% r-conformation respectively in solution. This indicates that carbonmonoxide bound liganded hemoglobin undergo conformational changes without restriction unlike oxyHb. Drawing from the finding that whereas in COHb, the hemoglobins are either more than *ca*. 88% r-conformation or more than 88% t-conformation unlike the oxyhemoglobin which exist as at least 80% t-conformation (which is adopted in DTNB bound Hb) and 20% r-conformation (adopted in the absence of DTNB binding to the Hb) we posit that the reversible

binding of DTNB with change in pH would be more reversible in all four mammalian oxyHbs and less so in the COHb in agreement with earlier findings about oxygen and carbon monoxide binding to Hb (Benner *et al.*, 2023). It was also noted that structural studies involving the determination of the effect of amino acid sequences or pH on the tertiary structure of hemoglobin should include carbon monoxide liganded Hb rather than the oxy-derivative only.

Four mammalian hemoglobins were studied. The proportion of r-isomer in the tertiary structure were between 75.92 – 92.00 % in the four mammalian oxy derivatives as well as in COHHb. However, the proportion of r-conformation in rabbit and SCFBHb were 8.0% and 0.62 % respectively. These proportions indicate that t-conformation is the dominant conformation of the tertiary structure of both rabbit and straw coloured fruit bat COHbs, whereas r-conformation is dominant in both human A and S hemoglobins. Noteworthy is the finding that the proportion of the r-conformation in the carbonmonoxy derivative of both HHbAA and HHbSS hemoglobins is greater than the proportion of r-conformation in their respective oxy-derivative. Since the values of r- and t- tertiary isomers of COHb at high pH varies more remarkably from one mammalian hemoglobin to another compared to oxyhemoglobin, it should be safe to postulate that the COHb would be better used in structural studies involving the effect of amino acid sequences on the tertiary structure of hemoglobins. It appears the carbonmonoxyHb is more sensitive to variation in amino acid and hemoglobin sequences than oxyHb. The inability of oxyhemoglobin to freely undergo significant r↔t isomerization is attributable the closeness between the distal histidine and of the α-subunit to the heme axis due to the formation of hydrogen bond between O and N of the distal histidine in oxyhemoglobin. The distal histidine in the α-subunit is much closer to the heme axis in OxyHb than in COHb (Shannan 1983; Safo and Abraham 2001). This could be responsible for the restriction of the tertiary structure to isomerization in oxyhemoglobin compared to the carbonmonoxyhemoglobin. The result here presented for HHbSS is consistent with previous report in which the r-isomer in COHHbSS is more than 99% and that in oxyHHbSS is ca. 76% (Fodeke and Bolarinwa, 2024).

CONCLUSION

In the oxy-derivative, all the four mammalian hemoglobin have high proportion of the r-tertiary conformation (between 73 to 80 %). Significant difference in the proportion of the tertiary isomeric form is noticeable from the analysis of the data of the reaction of DTNB with the carbonmonoxy form of the mammalian hemoglobin (r-conformation ranging from 0.62 – 91.87%). Carbon monoxide derivative provides more useful information about the conformational state of the hemoglobin than the oxy derivative. The studies show that tertiary isomerization state of the carbonmonoxy derivative of hemoglobin is more sensitive to change in amino acid sequences and composition than of oxy derivative.

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REFERENCES

Ahmed, M. H., Ghatge, M. S., Safo, M. K., (2020) Hemoglobin: Structure, Function and Allostery. *Sub-cellular Biochemistry.* 94:345-382.

Benner A, Patel AK, Singh K, Dua A. (2023) Physiology, Bohr Effect. StatPearls [Internet]. *StatPearls Publishing*; Treasure Island (FL).

Carrico, R.J., Blumberg, W.E., Peisach. J., 1978. The reversible binding of oxygen to sulfhemoglobin. *Journal of Biological Chemistry*, 253(20): 7212 - 7215

Fang, T-Y., Zou, M., Simplaceanu, V., Ho, N.T., Ho, C., 1999. Assessment of roles of surface histidyl residues in the molecular basis of the bohr effect and of β143 histidine in the

binding of 2,3-bisphosphoglycerate in human normal adult hemoglobin. *Biochemistry,* 38: 13423-13432

Fitzsimmons, R., Amin, N., Uversky, V.N., 2016. Understanding the roles of intrinsic disorder in subunits of hemoglobin and the disease process of sickle cell anemia. *Intrinsically Disordered Proteins*, 4(1): e1248273

Fodeke, A.A., 2022. Contrasting effect of crowder and tertiary isomerizations on ligand binding properties of two rabbit hemoglobin derivatives. *Ife Journal of Science and Technology*, 6(1): 112–125

Fodeke, A.A., Oyedare, K.O., Babalola, J.O., (2016a). Tertiary level r ↔t isomerization significantly raises the pK_a of CysF9[93]β linked ionizable groups of straw-coloured fruit bat hemoglobin: Variation of apparent forward second-order rate constant with pH. *International Journal of Life Sciences,* 5(3): 171–179.

Fodeke, A. A., Babalola, J. O. and Aboluwoye, C. O., (2016b). Effect of high ionic strength and inositol hexakisphosphate on the reactivity of CysF9[93]β of rabbit haemoglobin. *International Journal of Basic Applied Sciences*, 5(3): 86–95.

Fodeke, A.A., 2017. Organic phosphate binding inhibits high pH t-isomerization of the β-chain in straw-coloured fruit bat (Eidolon helvum) haemoglobin. *South African Journal of Chemistry*, 70: 181–188.

Fodeke, A. A. and Bolarinwa R. A., (2024). High crowder concentration promotes oxygen transport function of hemoglobin S - carbonmonoxide binding renders it insensitive to change in crowder concentration. *International Journal of Chemical and Pharmaceutical Research Updates*, 03: 001–011.

Lawrence, C. and Meier, E. R. (2021). Chapter 17 - Erythrocyte disorders, Biochemical and Molecular Basis of Pediatric Disease (Fifth Edition), *Academic Press*, 529-560.

Malowany, J. I. and Butany, J (2012). Pathology of sickle cell disease, *Seminars in Diagnostic Pathology* 29: 49 – 55.

Okonjo, K.O., Aken'ova, Y., Aboluwoye, C.O., Nwozo S, Akhigbe, F.U., Babalola, J.O., Babarinde, N.A., 1996. Effect of the A3[6] $\beta^{Glu \rightarrow Val}$ mutation on the reactivity of the CysF9[93]β sulphydryl group of human haemoglobin. S. *Journal of Chemical Society. Faraday Transactions*, 92: 1739-1746

Okonjo, K.O., Fodeke, A.A., 2006. Reversible reaction of 5,5'-dithiobis(2-nitrobenzoate) with the hemoglobins of the domestic cat: Acetylation of NH_3^+ terminal group of the β chain transforms complex pH dependence of the forward apparent second order rate constant to a simple form. *Biophysical Chemistry,* 119(2): 196–204.

Okonjo, K.O., Bello, O.S., Babalola, J.O., 2008. Transition of hemoglobin between two tertiary conformations: The transition constant differs significantly for the major and minor hemoglobins of the Japanese quail (Cortunix cortunix japonica) *Biochimica et Biophysica Acta*, 1784: 464-471.

Okonjo, K.O., Adediji, T.A., Fodeke, A.A., Adeboye, O. & Ezeh, C.V., 2007. Transition of hemoglobin between two tertiary conformations: Determination of equilibrium and thermodynamic parameters from the reaction of 5,5'-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulfhydryl group. *Biophysical Chemistry,* 128: 56-62

Okonjo, K. O., Adeogun, I., Babalola, J. O., 2009. Transition of haemoglobin between two tertiary conformations: Inositol hexakisphosphate increases the transition constant and the affinity of sheep haemoglobin for 5,5′-dithiobis(2-nitrobenzoate), *Biochimica et Biophysica Acta, Proteins Proteomics* 1794: 398 – 409.

Okonjo, K.O., Olatunde, A.M., Fodeke, A.A., Babalola, J.O., 2014. Bohr effect of human hemoglobin A: Magnitude of negative contributions determined by the equilibrium between two tertiary structures. *Biophysical Chemistry*, (190–191): 41 – 49.

Okonjo, K.O., Bello, O.S., Babalola, J.O., 2008. Transition of hemoglobin between two tertiary conformations: The transition constant differs significantly for the major and minor hemoglobins of the Japanese quail (Cortunix cortunix japonica) *Biochemica et Biophysica Acta,* 1784, 464–471

Shannan, B., 1983. Structure of human oxyhemoglobin at 2.1Å resolution. *Journal of Molecular. Biology,* 171: 31 – 57

Safo, M.K., Abraham, D.J., 2001. The X-ray structure determination of bovine carbonmonoxy hemoglobin at 2.1 Å resolution and its relationship to the quaternary structures of other hemoglobin crystal forms. *Protein Sciences,* 10: 1091–1099.

Sundid, P. Gladwin, M. T and Novelli, E. M., (2019). Pathophysiology of sickle cell disease, *Annual Review Pathology: Mechanisms of Disease* 14: 263 – 292.

Thomas, C., Lumb, A.B., 2012. Physiology of haemoglobin. *Continuing Education in Anesthesia, Critical Care & Pain,* 12: 251 – 256.

Zhang, Y., Kobayashi, K. Kitazawa, K. Imai, K. Kobayashi M. 2006. Contribution of cooperativity and the Bohr effect to efficient oxygen transport by hemoglobins from five mammalian species. *Zoological Science,* 23(1): 45 – 55.