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High crowder concentration promotes oxygen transport function of hemoglobin S - carbon monoxide binding renders it insensitive to change in crowder concentration.

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Abstract

Changes in crowding conditions affect functionally important structural changes in proteins. To understand how such changes affect the function of human hemoglobin S (HbSS), the affinity of CysF9[93]β sulfhydryl group of oxy and carbonmonoxy derivatives of HbSS for 5,5'-dithiobis(2-nitrobezoate) (DTNB) were measured in buffer 5.8 < pH < 9.0 under different crowding conditions (0 – 100 g dm^{-3} Ficoll 70). The affinities of the two hemoglobin derivatives for DTNB decreased with increasing pH at a given crowder concentration. For oxyhemoglobin, the average instantaneous gradient of the curve resulting from the fit under each crowding condition increases with increasing crowder concentration. Presence of the crowder did not significantly affect DTNB affinity of carbonmonoxyhemoglobin. Below pH 6.7, the affinity of oxyhemoglobin for DTNB increases with increasing Ficoll 70 concentration. Conversely, above pH 6.7, the affinity of oxyHbSS for DTNB decreases with increasing Ficoll 70 concentration at a fixed pH. Crowder did not significantly affect the affinity of carbonmonoxyhemoglobin for DTNB under the pH conditions of the experiment. This insensitivity of COHbSS to presence of crowder was attributed to formation of fiber by COHbSS. Hemoglobin fiber should be insensitive to high crowder concentration. The non-responsiveness of the affinity of DTNB for COHbSS to change in activity of the medium indicates that carbon monoxide binding to the hemoglobin promotes sickling or aggregation of hemoglobin. These findings were rationalized on the basis of the greater physiological relevance of oxygen binding to hemoglobin compared to carbon monoxide binding.

Keywords: Deoxyhemoglobin; Equilibrium constant; Crowder; r↔t transition; Sickle cell; Bohr effect

1 Introduction

Sickle cell is an inherited disease of hemoglobin which is caused by the sickling of the human red blood cells containing homozygous hemoglobin S. Following deoxygenation, the deoxyhemoglobin formed in the red blood cells polymerizes and forms fiber, giving rise to the sickling of the red blood cells [1, 2]. This disease affects several millions of people all over the world [3]. The difference between sickled human hemoglobin (HbSS) and normal human hemoglobin A (HbA) arises from the substitution of the negatively charged glutamic acid at position A3[6]β by a hydrophobic valine residue [2, 4]. Of all the hemoglobin variant entries in the hemoglobin database [5], HbSS is the most common and medically important hemoglobin variant [6]. This red blood cell deformity inherited by the progeny usually decreases the synthesis of beta globin chain. Different types of sickle cell disease are known: Sickle cell anemia (SS), sickle hemoglobin C (SC), sickle beta-plus (HbS beta+) and sickle beta-zero (HbS beta 0) thalassemia. The kinetics of the reaction of DTNB with CysF9[93]β of HbSS had been previously reported [4]. We have previously demonstrated that the reaction of DTNB with CysF9[93]β of hemoglobins is a reversible process, and have determined the equilibrium constant of the reaction of DTNB with various animal hemoglobins in dilute solutions [7 -12]. The data obtained from these experiments which

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were carried out at *ca*. 1 g/dm^3 total macromolecule concentration were analyzed based on the previous findings that hemoglobin CysF9[93]β sulfhydryl group exists in two conformations which are coupled to the tertiary level transition of the hemoglobin [9,10]. These two conformations were depicted by r- and t- which are quite different from the quaternary level conformations R and T which were attributed to the different ligand binding affinities to the hemoglobin. The r- and t- conformations were determined by whether the sulfhydryl group was cis- or trans- to the terminal amino group. On the other hand, R is the high oxygen affinity form of the quaternary conformation and the T is the low oxygen affinity form.

The hemoglobin concentration of normal adult human red blood cell is usually between 120 g/dm^3 to 170 g/dm^3, depending on age and sexual category. These values are more than a hundred times higher than the concentrations of hemoglobin often employed for experiments in dilute solution, the conditions under which most of the previous studies of hemoglobin were undertaken. Moreover, other high molecular weight macromolecules exist jointly with hemoglobin in animal red blood cells, thus creating a crowded conditions with the total concentration of macromolecule up to 400 g/dm^3 [13]. Such crowded conditions should result in high activity coefficients with its consequences on the tertiary structure and quaternary structure of the hemoglobin molecule [14,15]. Changes in tertiary structure have been shown to affect the functional properties of some proteins [13,15]. It would therefore be interesting to determine how crowding conditions affect the structure and function of human HbSS. The finding from such studies might provide useful information for the management of patients with hemoglobin S syndrome. Previously, a study involving the use of DTNB as indicator of tertiary and quaternary structure under different crowding conditions have been carried out using rabbit hemoglobin [16]. The use of DTNB as an indicator of the tertiary and quaternary structure of the hemoglobin relies on: (i) previous finding which shows that just as oxygen reacts reversibly with hemoglobin, the reaction of DTNB with CysF9[93]β of hemoglobin occurs reversibly (though the binding site is different in both cases); (ii) the ionizable groups linked to the binding of oxygen to hemoglobin are similar to the ones linked to the equilibrium reaction of DTNB with CysF9[93]β sulfhydryl group of hemoglobins [11]; (iii) the negligibly small size of DTNB compared to the size of both hemoglobin and the crowder used also makes it very suitable for this kind of studies. This is because the contribution of DTNB to the activity coefficient of the equilibrium system is negligible in the range of the DTNB concentration used. It should also be noted that Ficoll 70, the crowding agent used in the present study has a molar mass of *ca* 70kDa, a value which is quite comparable to that of hemoglobin. Although, hemoglobin has a molar mass of *ca* 68kDa, its concentration was kept low and constant under the experimental conditions so that its contribution to the activity coefficient would remain constant and insignificant under the experimental conditions. Previous work has demonstrated that whereas the affinity of hemoglobin sulfhydryl group for DTNB decreases with increase in pH, the affinity of hemoglobin for oxygen increases with increasing pH [17].

We measured the affinity of human HbSS for DTNB at various pH values, under different crowding conditions, at a fixed temperature. This was with a view to understanding how crowding conditions affect the affinity of HbSS for DTNB. The results were analyzed using a scheme that was employed previously for such analyses [11].

2 Materials and Methods

2.1 Materials

DTNB, a product of Sigma Aldrich was used without further purification. Ethanol used has a purity of 95% and is a product of Lab Alley. Absorbance measurements were made using UV-1800 Spectrophotometer manufactured by **Shimadzu Europa GmbH**. Gallenkamp Oven Model Ov-160 was made in England. TDL-60B Tabletop low speed centrifuge was shipped in from China and was equipped with cooling device to ensure that the rotor is kept at room temperature throughout the duration of use. Grant Thermostated Water bath with CC-60 Cryocool immersion cooler is a product of Thermoscientific Neslab. The pH measurements were made using HI 2209 pH meter, a product of Hanna Instruments.

2.2 Methods

2.2.1 Preparation of Hemoglobin

Blood containing human hemoglobin S (HbSS) was collected from an adult sickle cell anaemia patient attending adult sickle cell patients' clinic at the Haematology Department, Obafemi Awolowo University Teaching Hospital, Ile-Ife, Nigeria. HbSS was prepared from the blood using the procedure that we have already described in detail [8]. The blood of the sickle cell patient was collected into acid-citrate-dextrose anticoagulant and was centrifuged at 6,000 rpm for 20 minutes at room temperature to remove the anticoagulant. The red blood cells were washed three times with saline solution (9.5 g/dm^3 NaCl) at 5 ^{o}C . After each washing, the mixture in the solution was centrifuged at 6,000 rpm for 15

minutes and the clear supernatant was decanted. The red blood cells were then lysed to free the hemoglobin from the membrane by vigorously shaking the mixture after adding ice cold distilled water. Centrifugation was repeated at 6,000 rpm for 30 minutes and the supernatant containing the hemoglobin was decanted from the cell debris which settles at the bottom of the centrifuge tubes. 5% weight by volume of NaCl was added to this supernatant and the mixture was kept in ice chips for 20 minutes. Centrifugation was repeated at 6,000 rpm for 30 minutes. Purification of the hemoglobin was carried out by dialysing it against 10 mmol dm^{-3} of phosphate buffer pH 6.8 in a 2 liter conical flask for three hours. The dialysis was repeatedthree times, each for three hours. The hemolysate was then passed very slowly through Dintzis column to remove endogenous impurities from the hemoglobin [18]. The concentration of the stock hemoglobin was determined by first converting the oxyhemoglobin to carbonmonoxyhemoglobin, then measuring the absorbance at 537.5 nm, using 14,000 mol^{-1} dm^{-3} cm^{-1} as the molar extinction coefficient.

2.2.2 Determination of K_{equ}, the equilibrium constant for the binding of DTNB

An appropriate volume of stock hemoglobin solution was measured into a 10 cm^3 standard flask and was diluted with buffer of known pH to give a final concentration of 50 x 10^{-6} mol (heme) dm^{-3}, that is 25 x 10^{-6} mol dm^{-3} in reactive sulfhydryl groups. This was employed as the stock solution for the measurement of K_{equ} at the specified pH. A 1 cm^3 of this stock solution was measured into each of nine test tubes. An appropriate volume (0 – 80 mm^3) of a stock DTNB solution (27.17 mmol dm^{-3} DTNB in buffered ethanol) was added to the hemoglobin in each of the nine test tubes. The content of each test tube was mixed and left to equilibrate at 25 ^oC for six hours in a thermostated water bath. The absorbance of the equilibrium mixture in each test tube was measured in a 2 mm pathlength cuvettes at 412 nm with a double beam UV-1800 spectrophotometer (Shimadzu Europa GmbH). The reference cuvette for each measurement contained buffer of the same pH and composition as the solution in the sample cuvette, without hemoglobin but with appropriate volume of stock DTNB solution added. After correcting the absorbance reading for dilution by the added DTNB, the concentration of TNB^-, the chromophoric product of the reaction of DTNB with hemoglobin was calculated and the equilibrium constant, K_{equ} was obtained with Eq. (2). The experiments undertaken in the presence of Ficoll 70 (the crowding agent) followed the same procedure as detailed above, except that the buffers used contained 0.05 g/cm^3 and 0.1 g/cm^3 Ficoll 70 respectively.

2.2.3 Calculation of K_{equ}, the equilibrium constant

The reaction of DTNB with the CysF9[93]β sulfhydryl group may be depicted as:

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

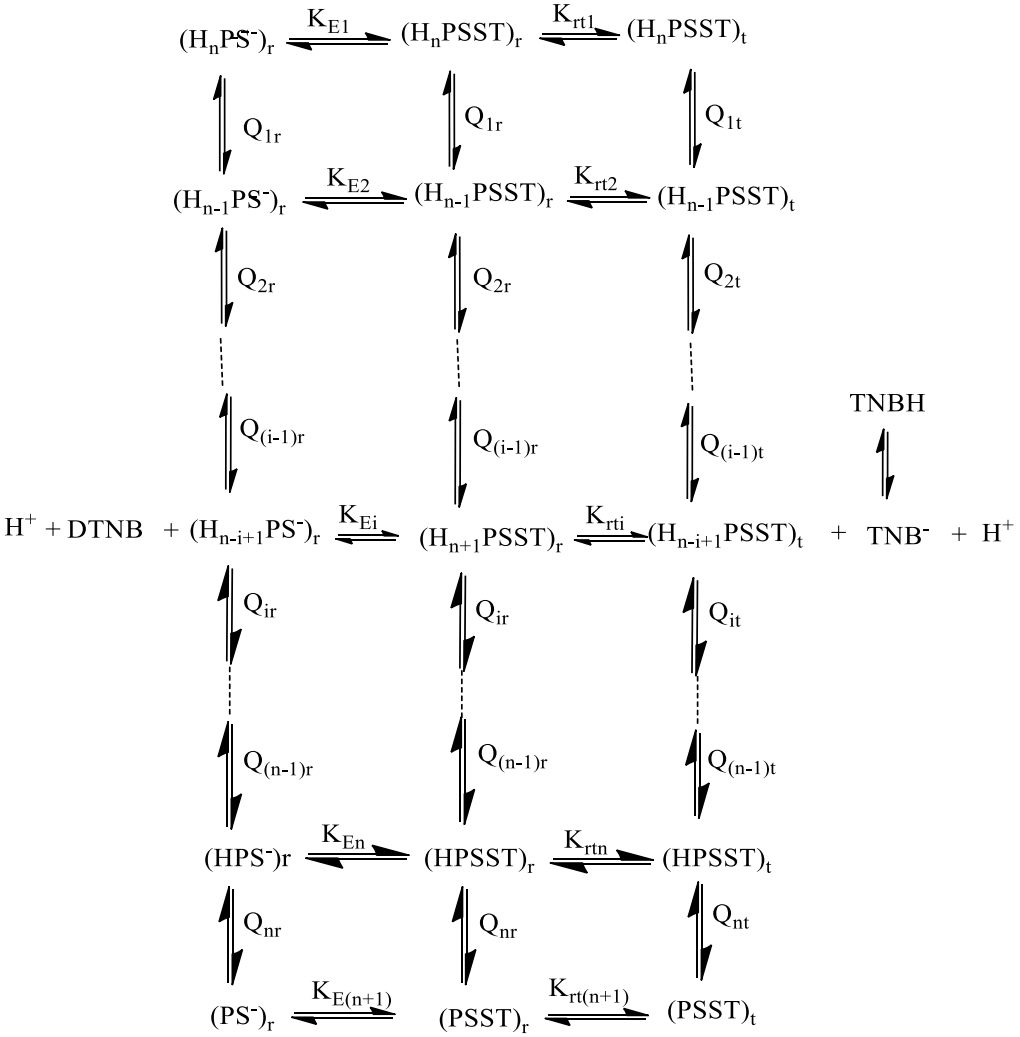
Among the species in Eq. (1), PSH, the protonated form of CysF9[93]β does not react with DTNB; the ionized form of PSH which reacts with DTNB to give the mixed disulfide PS.ST is PS^-. TNB^- is the chromophoric product of the DTNB reaction with PS^- and TNBH is the protonated form of TNB^-. Q_{SH} is the ionization constant of the Cys[F9]93β sulfhydryl group; K_{equ} is the equilibrium constant for the DTNB binding step; and Q_{TNB} is the ionization constant of TNBH. A value of 8.3 was assumed for pQ_{SH} [19-21]. We previously reported the value of pQ_{TNB} to be 5.27 [8]. The relationship between K_{equ}, the species and parameters that appear in Eq. (1) is given by Eq. (2):

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

We presented a detailed derivation of Eq. (2) in a previous report [8].

The negative logarithm of the average calculated K_{equ} values under each experimental conditions were plotted as a function of pH under the different crowding conditions; in the absence of Ficoll 70 and at two concentrations of Ficoll 70. The curves were fitted with Eq. (3) and scheme 1 assuming an n value of 2 for the number ionizable groups linked to the equilibrium reaction. In scheme 1, K_{Ei} values (for i = 1, 2,…n+1) are the various equilibrium constants of the reaction of DTNB with species in which (i - 1) protons have been ionized. If any of the groups has not been ionized i = 1. The value of i is equal to 2 after the first ionization and i = 3…n+1 after each successive ionization of the ionizable group.

n is the total number of ionizable groups. Q_{ir} and Q_{it} (i = 1, 2 … n+1) are the ionization constants of the ionizable groups that are linked with equilibrium reaction of DTNB with Cys[F9]93β sulfhydryl group of the reacting hemoglobin in the "r" and "t" isomeric forms. The subscript r- and t- indicate the tertiary isomerization states of the hemoglobin. This is important because DTNB reacts reversibly with hemoglobin CysF9[93]β sulfhydryl group. In the thiolate anion form, the hemoglobin exist as r-isomer, whereas in the mixed disulphide form it exist as the t-isomer. Since both the thiolate anion and the disulphide form occur reversibly, both r-isomer and t-isomer would exist reversibly in the equilibrium solution. Various equilibrium steps involved in the reaction of DTNB with hemoglobin had been described earlier using scheme 1 [9], and is reproduced here out of necessity. Parameters of scheme 1 have been obtained by fitting the dependence of logarithm of equilibrium constant of DTNB reaction with the hemoglobin sulfhydryl group on pH according to the complex Eq. (3) [10].



Scheme 1

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

Where only two amino acid side chains of the hemoglobin are ionized in the reaction of DTNB with CysF9[93]β, n = 2, and Eq. (3) reduces to,

$$K_{equ} = \frac{K_{E3}\left\{1+(H^+)^2(Q_{1r}Q_{2r})^{-1}+(H^+)(Q_{2r})^{-1}+K_{rt3}\left[1+(H^+)^2(Q_{1t}Q_{2t})^{-1}+(H^+)(Q_{2t})^{-1}\right]\right\}}{1+K_{E3}\{(H^+)^2(Q_{1r}Q_{2r})^{-1}(K_{E1})^{-1}+(H^+)(Q_{2r})^{-1}(K_{E2})^{-1}\}} \quad \text{.....(4)}$$

3 Results

3.1 Affinity of oxyhemoglobin S for DTNB

The dependences of the affinity of CysF9[93]β of oxyHbSS for DTNB on pH under three different crowding conditions were presented in Fig. 1. Each experimental data point is the mean at least six replicate experiment subject to 10 % error. The curve through each set of the experimental data points were fitted separately by allowing each of the 8 fitting parameters in Eq. (4) vary independently under each of the different crowding conditions. The fitting parameters of the oxyHbSS experimental data were reported in Table 1. The values of pQ_{1r} and pQ_{1t} for the reaction of DTNB with oxyHbSS under the different crowding condition do not differ substantially. The mean values of pQ_{1r} and pQ_{1t} are *ca*. 6.082 and 7.074 respectively. Similar values have previously been assigned to HisNA2[2]β [11,22], the values were therefore assigned to HisNA2[2]β. The difference between the values of the average pQ_{1r} and pQ_{1t} shows that the value pQ_{1t} is greater than that of pQ_{1r} by *ca*. 0.992 units. The small change in the ionization constant might be suggestive of marginal change in the tertiary structure of the protein around the ionizable group. The second ionizable group with mean $pQ_{ir/t}$ values of 7.865 and 9.372 for pQ_{2r} and pQ_{2t} respectively was assigned to HisEF1[77]β [9]. In this ionizable group, the value of pQ_{2t} is greater than pQ_{2r} by 1.507 unit. The curves through the experimental data points which were fitted with equation (4) using the fitting parameters in Table 1 columns 2 (for experiment in dilute solution), column 3 (for experiment in 50 g dm^{-3} Ficoll 70) and column 4 (for experiment in 100 g dm^{-3} Ficoll 70) were presented together in Fig. 1. In each case, the affinity of CysF9[93]β of oxyHbSS for DTNB decreases with increasing pH under the three different crowding conditions. At low pH (pH < 6.8), the affinity of the hemoglobin was greatest in 100 g dm^{-3} Ficoll 70, which is followed by the affinity of the hemoglobin for DTNB in 50 g dm^{-3} Ficoll 70 and is least in the dilute solution. At *ca*. pH 6.8 the affinity of oxyhemoglobin for DTNB under the three crowding conditions becomes indistinguishable. However, above pH 6.8, switch of the ranking occur, such that the affinity of dilute oxyHbSS for DTNB becomes the greatest at high pH, this is followed by the affinity of the hemoglobin for DTNB in 50 g dm^{-3} Ficoll 70 and the least affinity is observed in the oxyHbSS at 100 g dm^{-3} Ficoll 70. This showed that the affinity of oxyhemoglobin for DTNB at physiological pH, *ca*. pH 7.3, decreases with increasing concentration of Ficoll 70. This is contrary to the observation at low pH, where the affinity of the oxyHbSS for DTNB increases with increasing crowder concentration.

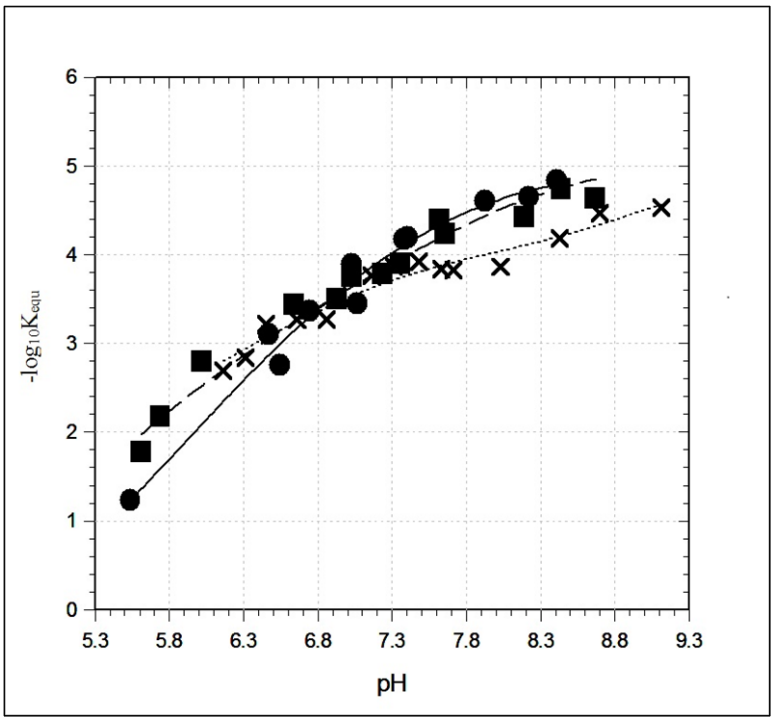


Figure 1 Dependence of the affinity of DTNB for CysF9[93]β sulfhydryl group of human S oxyhemoglobin on pH at 25 ^oC in phosphate buffer 5.6 ≤ pH ≤ 8.0 and borate buffer 8.0 ≤ pH ≤ 8.8 (ionic strength = 0.05 mole dm^{-3} NaCl). Crowding conditions and symbols: "X" with dotted curve, 0 g/dm^3 Ficoll 70; squares and broken curve, 50 g/dm^3 Ficoll 70; circle and full curve, 100 g/dm^3 Ficoll 70

Table 1 Fitting parameters for the equilibrium reaction of DTNB with CysF9[93]β of human oxyhemoglobin S, in Figure 1 according to Eq. (4)

Parameter	0 g dm^{-3} Ficoll 70	50 g dm^{-3} Ficoll 70	100 g dm^{-3} Ficoll 70
pQ_{1r}	6.110	6.124	6.011
pQ_{1t}	7.366	6.986	6.871
pQ_{2r}	8.305	7.992	7.298
pQ_{2t}	9.496	9.456	9.165
K_{E3}/K_{E2}	0.827	4.71×10^{-16}	0.00352
K_{E3}/K_{E1}	4.71×10^{-17}	4.74×10^{-17}	1.58×10^{-20}
pK_{E3}	4.809	4.767	4.991
Krt_3	0.317	0.00244	0.0727

3.2 Affinity of carbonmonoxyhemoglobin S for DTNB

The experimental data points and the curve of the pH dependences of the affinity of CysF9[93]β of COHbSS for DTNB under the three crowding conditions were presented in Fig. 2. Each experimental data point was obtained from the mean of at least six replicate K_{equ} values subject to 10% error. It is noticeable that the experimental data points of the dependences of affinities as a function of pH under different crowding conditions were somewhat comparable. The data at the three crowding conditions were therefore fitted together with a set of fitting parameters, allowing all the 8 fitting parameters to vary independently. The curve through the experimental data points were fitted using the fitting parameters reported in Table 2. As for oxyHbSS, in the previous section, the affinity of the carbonmonoxyhemoglobin for DTNB decreases with increasing pH under the three different crowding conditions (0, 50 and 100 g dm^{-3} Ficoll 70) in a nonlinear fashion. The curve through the experimental data points were well fitted to the combined data under the different crowding conditions. The comparable values of the affinities of carbonmonxyhemoglobin for DTNB under the different crowding conditions at equivalent pH suggests that the affinity of COHbSS for DTNB might be insensitive to change in activity of the medium of interaction. This observation could be the consequence of irreversible binding of carbon monoxide to hemoglobin. It is known that hemoglobin has affinity for carbon monoxide that is more than 300 times that of oxygen in human hemoglobin. The pK_a of the first and second ionizable groups linked to the reaction of DTNB with COHbSS are in appreciable agreement with that of oxyHbSS reported in Table 1. The ionizable groups were therefore assigned to the histidine at position NA2[2]β for the first ionizable group and the second ionizable group with mean pQ_r value of 7.865 was assigned to HisEF1[77]β. The pK_a of this ionizable group in carbonmonoxyhemoglobin A had previously been determined to be 7.73 [22]. It is however remarkable that the pK_a difference due to r↔t transition led to an increase in pK_a value of *ca*. 1.67 unit in the first ionizable group and increase of *ca* 1.76 unit in the second ionizable group. This change which is comparable to the change in pK_a of the second ionizable group in the oxyHbSS, may contribute in a significant way to the negative Bohr Effect leading to loss of proton from the solution in hemoglobin. The consequence is that it stabilizes the pH as oxygen bind to hemoglobin at high pH under the different crowding conditions.

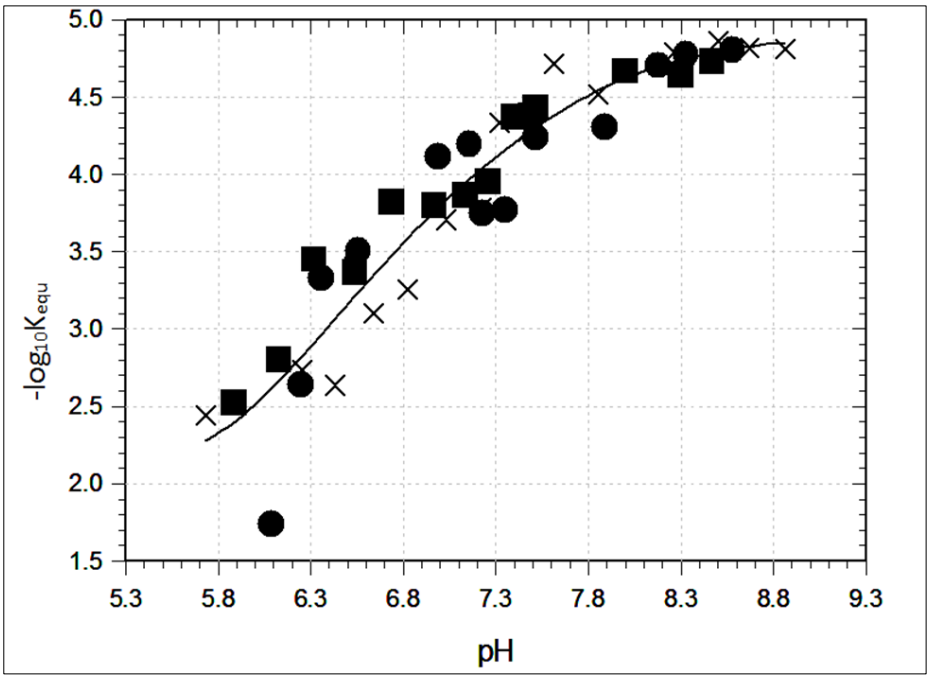


Figure 2 Dependence of affinity of DTNB for CysF9[93]β sulfhydryl group of human carbonmonoxyhemoglobin S on pH at 25 °C in phosphate buffer 5.6 ≤ pH ≤ 8.0 and borate buffer 8.0 ≤ pH ≤ 8.8 (ionic strength = 0.05 mole dm^{-3} NaCl). Crowding conditions and symbols: "X", 0 g/dm^3 Ficoll 70; squares, 50 g/dm^3 Ficoll 70; circle, 100 g/dm^3 Ficoll 70. The curve through the experimental data points is the fit using the fitting parameters of Table 2

Table 2 Fitting parameters of the reaction of DTNB with CysF9[93]β of human carbonmonoxyhemoglobin S under different crowding conditions, in Figure 2 according to Eq. (4)

Parameter	0 – 100 g dm^{-3} Ficoll
pQ_{1r}	6.064
pQ_{1t}	7.734
pQ_{2r}	7.782
pQ_{2t}	9.541
K_{E3}/K_{E2}	0.0206
K_{E3}/K_{E1}	0.00620
pK_{E3}	4.859
Krt_3	0.00266

4 Discussion

4.1 Effect of Crowder on the affinity of hemoglobin

This study was embarked upon to understand how high activity of the hemoglobin solution due to presence of crowder affect its affinity for ligands. Studies have shown that the affinity of DTNB for F9[93]β sulfhydryl group decreases with increasing pH [7-10], whereas the affinity of oxygen for hemoglobin increases with increasing pH [22], though similar ionizable groups are linked to both processes. This suggests that high DTNB affinity for hemoglobin F9[93]β sulfhydryl implies low oxygen affinity for hemoglobin under identical pH conditions. It is therefore possible to semi-quantitatively gauge relative affinities of hemoglobin for oxygen as a function of pH from the affinities of its CysF9[93]β sulfhydryl for DTNB. This understanding was used in discussing the physiological consequence of the findings of our experiment. It is

obvious from the results that higher activity of the medium increases the affinity of oxyHbSS for DTNB (decreases the affinity of hemoglobin for oxygen) at pH below 6.8, but reduces the affinity of DTNB (increases its affinity for oxygen) at pH > 6.8. The consequence of this is that at low pH, high crowder concentration would stimulate the release oxygen bound to hemoglobin for the cells thereby reducing the amount of oxygen that are bound to hemoglobin at low pH. On the other hand, under conditions of high pH (pH above 6.8), the affinity of hemoglobin for oxygen increases. This enhances the uploading of oxygen on hemoglobin in the lung where the pH is within the physiological range, *ca*. 7.3. The consequence of this is that high activity should favour physiological function of HbSS compared to when the activity is low.

The findings of this work show that increased concentration of Ficoll 70 enhanced the affinity of hemoglobin for oxygen binding in the lung (at physiological pH). On the other hand, the affinity of hemoglobin for oxygen at low pH (due to release of carbon (IV) oxide) would be lower. Around cell tissue, the pH is low, therefore reduced affinity of hemoglobin for ligand should favour unloading of oxygen to the cells. Whereas, at high pH, increased affinity of hemoglobin for ligand should favour uploading of oxygen in the lung. This finding suggests that in addition to increasing the number of molecules of oxygen carried when the concentration of hemoglobin in the red blood cells is increased, enhancement of the loading of oxygen in the lung by HbSS is also a consequence of the increased activity caused by increased concentration of hemoglobin. The unloading of oxygen to the cell is also assisted by increase in the activity of the medium.

The situation of a sickle cell patient is made worse in two fronts when sickled hemoglobins are destroyed in red blood cell of a living host: by reduction in the amount of oxygen carrying molecules (HbSS) and reduction in the activity of the medium, both of which reduce the efficiency of HbSS oxygen uploading and unloading. The enhanced performance of hemoglobin under crowded conditions may indicate that high activity of the medium stabilizes the native structure of hemoglobin compared to hemoglobin in dilute solution.

4.2 Effect of tertiary isomerization

In an attempt to rationalize the consequence of the equilibrium constant of r ↔ t transition on the affinity of hemoglobin for ligand, we compared the K_{rt3} values which are a measure of the proportion of r- and t- isomers of the hemoglobin that are formed under each crowding condition. The proportion of t- isomer is greatest in the dilute solution and least in the solution containing 50 g/dm^3 Ficoll 70. Since there is no correlation between the proportion of each isomer and the affinity, we posit that though tertiary isomerization may be important in determining the affinity of hemoglobin for ligand, it is not unlikely that the difference in affinity between the hemoglobin in different concentration of Ficoll 70 may also be due to the effect of the crowding agent on the quaternary structure.

The response of the affinity of oxyHbSS for DTNB to change in concentration of Ficoll 70 here reported, contrast with that of rabbit hemoglobin. In rabbit hemoglobin, at low pH (6.3 – 6.8) the affinity of rabbit oxyhemoglobin for DTNB was increased with increasing activity of the solution. At pH *ca.* 7.3 however, the affinity of the oxyhemoglobin for DTNB was least in the absence of Ficoll 70 but highest in 50 gdm^{-3} Ficoll 70 [16]. On the other hand, the affinity of human HbSS for DTNB at high pH decreases with increasing Ficoll 70 concentration and increases at low pH with increasing Ficoll 70 concentration. This findings suggests that whereas the affinity of HbSS for oxygen increases with activity of the solution at high pH, it decreases with activity at low pH. On the other hand, the affinity of rabbit hemoglobin for DTNB decreases with increasing Ficoll 70 concentration at low pH. Unlike rabbit hemoglobin, whose efficiency of oxygen transport is hampered by increased activity coefficient of the medium between *ca*. 10 (at 50 g/dm^3) and 90 (at 100 g/dm^3), efficiency of oxygen transport by oxyHbSS at activity coefficient up to 10 favours oxygen transport function of the hemoglobin. It may therefore be necessary to keep the activity of oxyHbSS solution in vivo reasonably high for proper functioning of the sickled hemoglobin.

Change in the $pQ_{r/t}$ values of r↔t isomerization of the tertiary structure of oxyHbSS, Table 3, were calculated from the data in Table 1. For oxyhemoglobin, r- to t-isomerization resulted in *ca*. 1.256, 0.862 and 0.860 unit increases in the pK_a value of the first ionizable group in 0 g dm^{-3}, 50 g dm^{-3} and 100 g dm^{-3} Ficoll 70 solutions respectively. The corresponding change in pK_a values of the second ionizable group gave 1.191, 1.464 and 1.867 increase under the respective crowding conditions. Notable trend in the pK_a of ionization reveals that whereas the change in pK_a of the first ionizable group on r↔t transition decreases with increasing concentration of Ficoll 70 that of the second ionizable group increases with increasing activity of the medium. It is obvious that the two ionizable groups make negative contribution to the Bohr effect as ligands are bound to the hemoglobin sulfhydryl group. The amount of negative contribution by the isomerization process is a measure of the change in pK_a of the ionizable group as a result of the isomerization process. The values of the change in the pK_a of ionization of the first ionizable group indicate that the contribution of the first ionizable group to the negative Bohr effect decreases with increasing activity of the medium. Conversely the

contribution of the second ionizable group to the negative Bohr effect increases with increasing activity of the medium (increased Ficoll 70 concentration).

Table 3 Change in $pQ_{r/t}$ values of the ionizable groups of oxyHbSS as a result of tertiary level r↔t isomerization

pQ_{it} - pQ_{ir}	No Ficoll	+ 50 g/dm^3 Ficoll 70	+ 100 g/dm^3 Ficoll 70
i = 1	1.256	0.862	0.860
i = 2	1.191	1.464	1.867

The size of the contributions of the two ionizable groups to the negative Bohr effect were determined by comparing the pK_a increase on r↔t isomerization of the first ionizable group with that of the second. It is noteworthy that in the crowded solutions, HisEF1[77]β of oxyHbSS contributes more to the negative Bohr effect compared with HisNA2[2]β. The implication of this, is that increase in Ficoll 70 concentration promotes the oxygen exchange function of hemoglobin by increasing the affinity of hemoglobin for oxygen at physiological pH. In the presence of high concentration of Ficoll 70, unloading of oxygen to the cell is facilitated by change the pK_a of the first ionizable group.

Unlike oxyhemoglobin which shows sensitivity to changes in the concentration of the crowding agent (change in activity of the medium), carbonmonoxyhemoglobin was insensitive to presence of crowder. This finding may be rationalized by the previous report [23] which shows that once nuclei are formed by deoxyhemoglobin S, co-polymerization of COHbSS with deoxyhemoglobin for the formation of aggregates occur very readily. This could be responsible for the small value of r↔t isomerization constant at high pH as seen in Table 2. To confirm the validity of our claim, since the difference between pQ_{ir} and pQ_{it} is somewhat high (greater than 1 unit), we calculated the values of K_{rt1}, the equilibrium constant of isomerization at low pH, from the values K_{rt3} and pQ_{ir} and pQ_{it} values using Eq. (5) [9,12]:

$$pK_{rti} = pQ_{ir} - \log(Krt_{i+1}) - pQ_{it} \quad \text{.....(5)}$$

At low pH, the value of 7.143 obtained for K_{rt1}, suggests a significant r ↔ t isomerization is consistent with large change in pK_a value of the first ionizable group on isomerization. This may be an indication that low pH promotes aggregation of COHbSS, as more rigid structure of the co-polymer involving COHbSS is not expected to allow for significant isomerization of COHbSS.

Since hemoglobin is known to have high affinity for carbon monoxide compared to oxygen, the similarity in the data for the affinities of COHbSS for DTNB as a function of pH at different concentration of Ficoll 70 can be rationalized by assuming that carbon monoxide promotes polymerization leading to fiber formation just the way it does in deoxyhemoglobin. This formation of aggregates is independent of concentration of crowder. Therefore, fibers and protein aggregate are less likely to be anymore affected by increase in activity of the medium when fibers are already formed in the absence of or at low crowder concentration. It may therefore be safe to assume that the affinities of DTNB for the sulfhydryl group of COHbSS are not significantly different under the different crowding conditions, because presence of crowder did not significantly lead to alteration of the tertiary level isomerization of the COHbSS due to fiber formation.

Though, similar studies at high activity coefficient have not been studied with human hemoglobin (HbA) for comparison to be made with HbA, comparison with rabbit hemoglobin shows that in addition to rabbit hemoglobin having much greater affinity for DTNB than COHbSS, the affinity of rabbit COHb for DTNB changes with change in activity coefficient of the medium. The equilibrium constant of r↔t transition at high pH Krt_3 is also much higher in rabbit COHb. These differences between the data for rabbit COHb and human COHbSS for DTNB might be an indication that the HbSS impedes r↔t transition of the tertiary structure of hemoglobin S in addition to abolishing the effect of activity coefficient changes of the medium on the affinity of the hemoglobin for ligand. This might be a further piece of evidence that the insensitivity of the tertiary structure to change in activity coefficient in human COHbSS might have more to do with the sickling of the hemoglobin in the presence of carbon monoxide rather than with the affinity of hemoglobin for carbon monoxide. Previous studies [24] have shown that voxelotor, a promising agent in the management of sickle cell crisis inhibits polymerization of HbSS by stabilizing it in the oxygenated form. It is significant to note that like rabbit oxyhemoglobin [16], the change in affinities of DTNB for human OxyHbSS (caused by change in crowder concentration) at low pH are small compared to the effect of crowding agent on the oxyHbSS at high pH. Nonetheless, OxyHbSS contrast with rabbit oxyhemoglobin [16], in that at high pH, addition of 50 g dm^{-3} of Ficoll 70 to the dilute solution of rabbit oxyHb results in nearly a unit increase in the $\log(K_{equ})$, suggesting significantly reduced affinity of rabbit oxyHb for DTNB due to high activity coefficient at physiological pH. Whereas, under the same pH condition, the same change in

activity results in slight decrease in the affinity of oxyHbSS for DTNB. This may be due to low sensitivity of HbSS to the presence of DTNB compared to rabbit OxyHb. The consequence of these responses to increase in activity of oxyHbSS solution is that high activity enhance both ligand binding affinity of hemoglobin in the lung at physiological pH, and unloading of oxygen to the cell at low pH. It is expected that HbA would behave in a manner similar to what was reported for rabbit oxyHb. However, since HbA differ from HbSS only at only position 6 of the β chain, it would be interesting to carryout similar studies with HbA to determine if the single point mutation could account for the insensitivity of COHbSS to change in crowder concentration or if other factors were at play.

5 Conclusion

The affinity of HbSS CysF9[93]β sulfhydryl group for DTNB decreases with increasing pH in a nonlinear manner in both oxy- and COHbSS throughout the entire pH range of the experiment. Though the binding site of hemoglobin for oxygen is quite different from that of DTNB and the affinity of hemoglobin for DTNB is known to decrease with increasing pH whereas the affinity of oxygen for hemoglobin increases with increasing pH, the ionizable groups linked with both reactions are similar. The affinity of oxyHbSS for DTNB is affected by change in Ficoll 70 concentration, but COHbSS is insensitive to crowder concentration changes. The effect of Ficoll 70, on the affinity of CysF9[93]β sulfhydryl group of HbSS for DTNB shows that COHbSS readily undergoes aggregation. The formation of this aggregates might be responsible for the very low sensitivity of the affinity of COHbSS for DTNB to changes in crowding conditions. There are evidence to suggest that formation of aggregates by COHbSS increases with increasing pH. The results of this experiment show that high crowding condition might prove helpful for the functional property of human oxyHbSS and is therefore desirable to keep the activity of oxyhemoglobin high for optimum physiological function of OxyHbSS. Since copolymerization, and aggregation of COHbSS are readily initiated by deoxyHbSS, sickle cell patient are multiple times at risk of fatality in an environment containing carbon monoxide compared to humans with normal hemoglobin.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

References

[1] Nakagawa A, Ferrari M, Schleifer G, Cooper MK, Liu C, Yu B, Berra L, Klings ES, Safo RS, Chen Q, Musayev FN, Safo MK, Abdulmalik O, Bloch DB, Zapol WM. A triazole disulfide increases the affinity of hemoglobin for oxygen and reduces the Sickling of Human Sickle Cells. Molecular Pharmaceutics. (2018) 15(5):1954–1963

[2] Henry ER, Cellmer T, Dunkelberger EB, Metaferia B, Hofrichter J, Li Q, Ostrowski D, Ghirlando R, Louis JM, Moutereau S, Galactéros F, Thein SL, Bartolucci P, Eaton WA. Allosteric control of hemoglobin S fiber formation by oxygen and its relation to the pathophysiology of sickle cell disease. Proceedings of National Academy of Science. (2020) 117(26):15018–15027

[3] Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. Lancet. (2010). 376:2018–2031

[4] Okonjo KO, Aken'ova Y, Aboluwoye CO, Nwozo S, Akhigbe, FU, Babalola JO, Babarinde NA. Effect of the A3[6] βGlu→Val mutation on the reactivity of the CysF9[93]β sulphydryl group of human haemoglobin. S. Journal of Chemical Society Faraday Transactions. (1996) 92:1739-1746

[5] Giardine BM, Joly P, Pissard S, Wajcman H, Chui DHK, Hardison RC, Patrinos GP. Clinically relevant updates of the HbVar database of human hemoglobin variants and thalassemia mutations. Nucleic Acids Research (2021) 49(D1): D1192–D1196

[6] Thorm TC, Dickson CF, Gell DA, Weiss MJ. Hemoglobin Variants: Biochemical Properties and Clinical Correlates. Cold Spring Harbor Perspectives in Medicine (2013)3:a011858

[7] Okonjo KO, Fodeke AA. Reversible reaction of 5,5'-dithiobis(2-nitrobenzoate) with the hemoglobins of the domestic cat: Acetylation of NH3+ terminal group of the β chain transforms complex pH dependence of the forward apparent second order rate constant to a simple form. Biophysical Chemistry (2006) 119(2):196–204

[8] Okonjo KO, Fodeke AA, Kehinde AT. Reversible reaction of 5,5'-dithiobis(2- nitrobenzoate) with the CysF9[93]β sulfhydryl groups of the hemoglobins of the domestic cat: Variation of the equilibrium and reverse rate constants with pH. Biophysical Chemistry. (2006) 121(1):65–73

[9] Okonjo KO, Adediji AT, Fodeke AA, Adeboye O. and Ezeh CV 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. (2007)128:56–62

[10] Okonjo KO, Bello OS, Babalola JO. 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. (2008) 1784:464–471

[11] Okonjo KO, Olatunde AM, Fodeke AA, and Babalola, JO. Bohr effect of human hemoglobin A: Magnitude of negative contributions determined by the equilibrium between two tertiary structures. Biophysical Chemistry. (2014) (190–191): 41 – 49.

[12] Fodeke AA. 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. (2017)70:161–168

[13] Minton AP. The effect of volume occupancy upon the thermodynamic activity of proteins: some biochemical consequences. Molecular and Cellular Biochemistry (1983) 55:119-140

[14] Christiansen A, Wang Q, Cheung MS, Wittung-Stafshede P. Effects of macromolecular crowding agents on protein folding in vitro and in silico. Biophysical Reviews 5(2): 137–145

[15] Ogunmoyole T, Fodeke AA, Adewale IO. Denaturation studies of Clarias gariepinus glutathion transferase in dilute and crowded solutions. European Biophysics Journal. (2019) 48(8): 789– 801

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

[17] Malte H, Lykkeboe G. The Bohr/Haldane effect: a model-based uncovering of the full extent of its impact on O2 delivery to and CO2 removal from tissues. Journal of Applied Physiology. (1985) 125(3):916-922

[18] Benesch R, MacDuff G, Benesch RE. Determination of oxygen equilibria with a versatile new tonometer. Analytical Biochemistry. (1965) 11(1):81-87

[19] Giles G I, Tasker KM, Jacob C. Hypothesis: The role of reactive sulfur species in oxidative stress. Free Radical Biology and Medicine. (2001) 31:1279–1283

[20] Witt AC, Lakshminarasimhan M, Remington BC, Hasim S, Pozharski E, Wilson MA. Cysteine pKa Depression by a Protonated Glutamic Acid in Human DJ-1. Biochemistry. (2008) 47(28):74307440

[21] Thurlkill RL, Grimsley GR, Scholtz JM, Pace, CN. pK values of the ionizable groups of proteins. Protein Sciences (2006) 15(5):1214–1218

[22] Fang T-Y, Zou M, Simplaceanu V, Ho NT, Ho C 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 (1999) 38:13423-13432

[23] Adachi K, Asakura T. Effect of liganded hemoglobin S and hemoglobin A on the aggregation of deoxy-hemoglobin S. Journal of Biological Chemistry (1982) 257(10):5738-5744

[24] Tsitsikas DA, Kamal M, Braimoh A, Benson S, Abukar J. Hb S (HBB: c.20A>T) Characteristics by High Performance Liquid Chromatography in Patients with Sickle Cell Disease Receiving the Novel Agent Voxelotor. Hemoglobin. (2021) 45(6):355-357