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Contrasting effect of crowder and tertiary isomerizations on ligand binding properties of two rabbit hemoglobin derivatives

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Abstract
The need to explore the effect of molecular crowder on the tertiary structure of protein and determine how it affects the function of such protein was the motivation for this work. Reaction of 5,5'dithobis(2-nitrobenzoate) (DTNB), a reagent which reacts reversibly with hemoglobin sulfhydryl groups were carried out in dilute solution and in up to 100 mg/cm^3 Ficoll 70. The reversible binding of DTNB to hemoglobin sulfhydryl group had previously shown to be linked to some ionizable groups which are linked to the reversible binding of oxygen with hemoglobin. Reaction of rabbit oxy and carbonmonoxy hemoglobin with DTNB was studied in the range of $5.8 \leq pH \leq 8.8$. The data were fitted to a model which takes into account the ionization of the ionizable groups on the reversible binding of DTNB with the hemoglobin. Whereas, the affinity of carbonmonoxy hemoglobin for DTNB is reduced in the presence of Ficoll 70 (Fic), the affinity of oxyhemoglobin for DTNB is enhanced in the presence of the crowding agent. This finding is an indication that crowding may have possible physiological consequence of enhancing the binding of physiologically import ligand to hemoglobin.

Keywords: Ionizable groups, Ficoll 70, 5,5'-dithiobis-(2-nitrobenzoate), Tertiary structure, Isomerization

Introduction

Reaction of DTNB with hemoglobin sulfydryl group is similar to the binding of oxygen to hemoglobin in two very important ways: (i) DTNB reacts reversibly with hemoglobin sulfhydryl group just as oxygen binds reversibly with the heme group of hemoglobin; (ii) the ionizable groups which are linked to oxygen binding to hemoglobin are identical to those that are linked to DTNB reversible reaction with hemoglobin sulfhydryl group (Wray *et. al.,* 1972). As a result of these similarities, the reaction of DTNB with hemoglobin has been used as indicators of tertiary and quaternary structure changes which accompanies oxygen binding to hemoglobin. Previous studies involving reversible binding of DTNB

with hemoglobin sulfhydryl group have been carried out in dilute solutions (Okonjo and Fodeke, 2006; Okonjo *et. al.,* 2006; Okonjo *et. al.,* 2007; Okonjo *et. al.,* 2014; Fodeke, 2017). Nonetheless, under physiological conditions, the hemoglobins environment are usually crowded, due to presence of high concentration of other macromolecules. The structure and function of the hemoglobin in the real environment can therefore be qualitatively or qunatitatively different from what they were in dilute solution (Minton, 1983). It has previously been shown that high concentration of innert macromolecular crowder alter protein's functionally important structure essentially by increasing the activity of the solution containing low concentration of the protein (Fodeke and Minton, 2010; Fodeke and Minton, 2011; Fodeke and Minton, 2013). Addition of inert macromolecules at high concentration have been shown to increase the chemical potential of protein which are present at low concentration relative to when the innert macromolecules are absent (Laurent, 1971). A solution containing 200 mg/cm^3 of hemoglobin was shown to have an activity coefficient ϒ, of 10. This is about half the activity coefficient of 100 mg/cm^3 Ficoll 70 in phosphate buffered saline (Fodeke and Minton, 2010). Increasing the concentration of hemoglobin (single solute) to 300 mg/cm^3 raises the activity coefficient, ϒ, to 100 (Minton, 1983). This finding was rationalized by the finding that hemoglobin, a globular protein is much more compact than Ficoll 70 which had been successfully modelled as spherocylinder.

The molecular weight of hemoglobin (~65000 g/mol) is much greater than the size of TNB^- (198.17 g/mol). The binding of TNB^- to hemoglobin should not affect the activity of the product relative to the reactant. The implication of this is that the crowder should not distinguish between the size of hemoglobin and the product of hemoglobin reaction with DTNB (HbS-TNB). This makes it innocuous to assume that any change in activity is due to change in concentration of the crowder. Therefore, the change in tertiary and or quaternary structure of the hemoglobin could only have been due to change in concentration of the crowder.

Since the total concentration of hemoglobin in mammals is usually in the range of 100 – 200 mg/cm^3 depending on animals species and gender. It is likely that experimental result from solutions with such a remarkably high activity coefficient would be different from results from experiments that were carried out in dilute solution typically with concentration ca. 0.8 g/cm^3 l (50 μM heme). It is with the intent to determine if and how increased activity of the hemoglobin solution would affect the equilibrium constant of the reaction of DTNB with hemoglobin that the the studies of the reaction of rabbit hemoglobin with DTNB was carried out. Rabbit hemoglobin has only two DTNB reactive hemoglobin sulfhydryl groups which are located at F9[93]β position of the hemoglobin (Kleinschmidt and Sgouros, 1989). The number of rabbit hemoglobin sulfhydryl groups that are reactive towards DTNB has previously been confirmed to be two using titration and the reactivity of these sulfhydryl group have been studied (Fodeke *et al,* 2016).

The two most physiologically important liganded hemoglobin derivatives are the

oxyhemoglobin and the carbonmonoxy hemoglobin also called carboxyhemoglobin. Although there are other natural derivatives which include the, carbamino hemoglobin, reduced hemoglobin (HHb) and aquomethemoglobin (hemoglobin in which the ferrous ion has been converted to the ferric ion with no capacity for oxygen binding. In normal hemoglobin, ca. 1.7 - 2.4% may occur as the methemoglobin. Catalytic efficiency of some enzymes/proteins has been reported to have been altered by presence of crowder by a process which is believed to be due to change in tertiary or quaternary structure of the protein/enzyme (Ogumoyole *et al*., 2020; Patel *et. al.,* 2002, Yamamoto, et al, 2021). Two hemoglobin derivatives oxy- and carbonmonoxy- were studied for the effect of molecular crowder, ficoll 70 on the ionization of the ionizable groups and tertiary level isomerization with a view to gaining insight on how crowding condition affects this isomerization and ligand binding affinity of the hemoglobin. To the best of our knowledge, this is the first time crowding effect on the equilibrium reaction of DTNB with any animal hemoglobin sulfhydryl is being reported.

Materials and Methods

Preparation of Hemoglobin

Rabbit blood was collected from a male rabbit purchased from a local market. The hemoglobin was prepared as described earlier (Okonjo *et. al.,* 2007). Rabbit blood was collected in to acid-citrate-dextrose anticoagulant and the blood was centrifuged at 12,000 rpm for 20 minutes at 5 ^{o}C to remove the anticoagulant. The red blood cells were washed three times with 10.5 g/dm^3 NaCl solution at 5 ^{o}C . After each washing the saline solution was decanted after centrifugation at 12000 rpm for 15 minutes. The red blood cells were then lysed by addition of ice cold distilled water to the red cells and then shaken vigorously to free the hemoglobin from the membrane. Centrifugation was done at 15,000 rpm 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 and the centrifugation was repeated at 20,000 rpm. The hemoglobin was then purified by dialysis three times, each for three hours against 10 mmol dm^{-3} of NaCl in 2 liter conical flask, followed by passing it through Dintzis column (Benesch *et al.,* 1965). The concentration of the stock hemoglobin was determined by converting the oxyhemoglobin to the carbonmonoxy hemoglobin and the absorbance measurement was carried out at 537.5 nm, assuming molar extinction coefficient of 14000 mol^{-1} dm^{-3} cm^{-1}.

Equilibrium reaction

Appropriate volume of stock hemoglobin soultion in a 10 cm^3 standard flask was diluted with the required buffer, $5.8 \leq pH \leq 9.0$ (ionic strength (I = 0.05 mol dm^3 NaCl) without crowder) to give 50 x 10^{-6} mol dm^{-3} heme concentration of hemoglobin solutions. This is the same as 25 x 10^{-6} mol dm^{-3} SH of the hemoglobin. 1 cm^3 of the 25 x +10^{-6} mol dm^{-3} SH was then measured into 10 test tubes. Increasing volume (0 – 80 μM) of 25 mM DTNB

was added to each test tube and left in a water bath which was made isothermal at 25 ^{o}C for six hours. The absorbance of the equilibrium mixture of the reaction was then determined at 412 nm. The experiment in the presence of crowding agent was carried out using buffer solutions containing 0.05 g cm^{-3} and 0.1 g cm^{-3} of Ficoll 70. The absorbance of the mixture of DTNB and buffer solution prepared exactly as the reacting mixture except that hemoglobin was excluded was also measured at 412 nm. The concentration of TNB^{--} the chromophoric product of reaction of DTNB with hemoglobin in Eq. (1) was then calculated using Eq. (2). The equilibrium constant of the reaction of DTNB with hemoglobin CysF9[93]β sulfhydryl group as a function of pH under different crowding conditions were calculated using Eq. (2). In Eq. (2) PSH is the hemoglobin sulfhydryl group, the ionized hemoglobin sulfhydry group is PS^{-}, PS.TNB is the mixed disulphide formed by the reaction of DTNB with hemoglobin sulfhydryl group. TNBH is the protonated form of TNB^{-}, the chromophoric product of the reaction of DTNB with hemoglobin sulfhydryl group. Q_{TNB} is the ionization constant of TNBH and Q_{SH} is the ionization constant of CysF9[93]β. A value of 6.27 was assumed for pQ_{TNB}, the $-\log_{10}(Q_{TNB})$ and pQ_{SH}, the $-\log_{10}(Q_{sH})$ was taken as 8.3 (Giles *et. al*., 2001; Witt *et al*, 2008; Thurlkill *et al*., 2006).

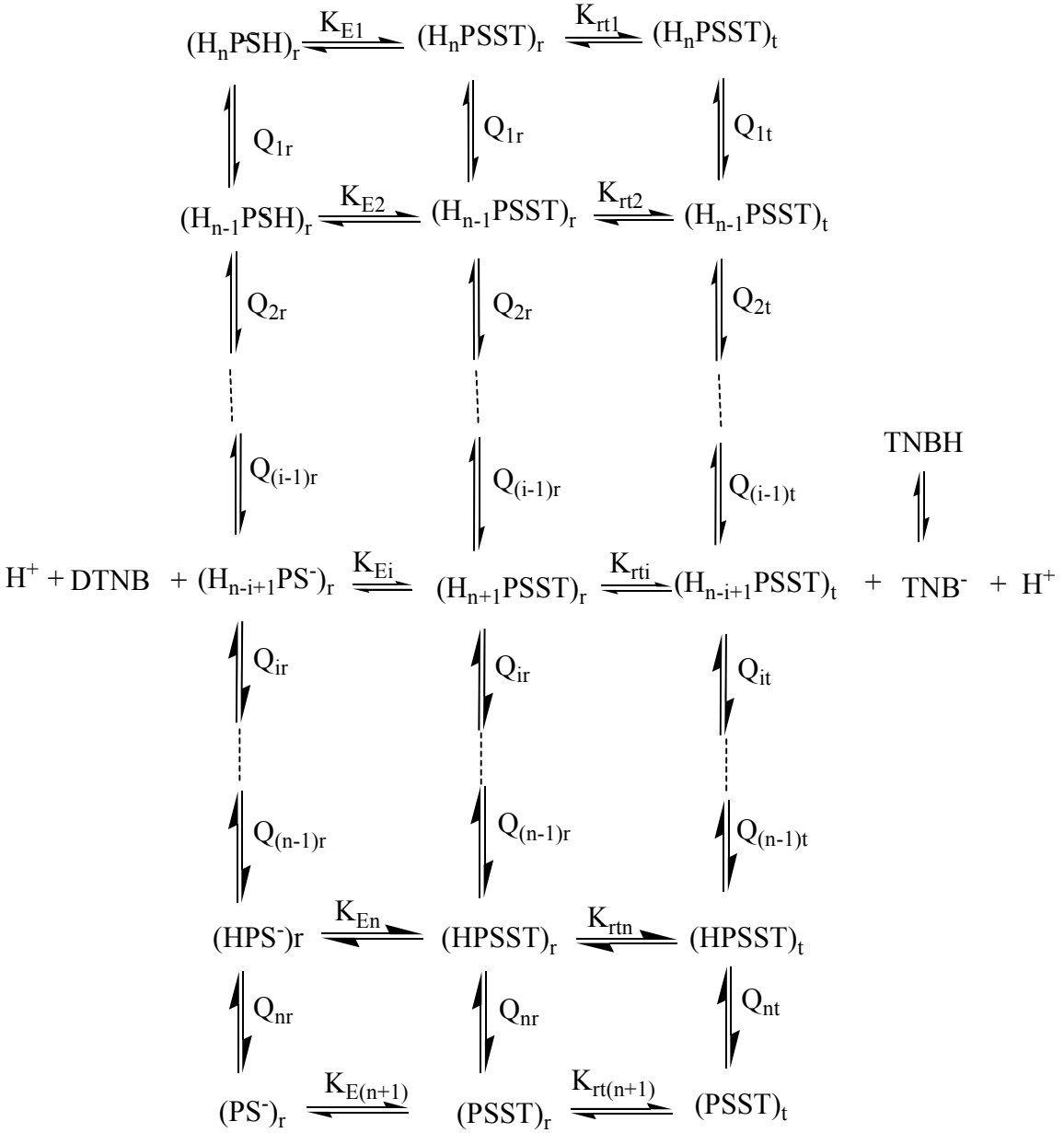
$$PSH + DTNB \overset{Q_{SH}}{\rightleftharpoons} H^{+} + PS^{-} + DTNB \overset{K_{EQ}}{\rightleftharpoons} H^{+} + PS.TNB + TNB^{-} \overset{Q_{TNB}}{\rightleftharpoons} PSTNB + TNBH \quad \text{…..(1)}$$

Detailed derivation of Eq. (2) had been reported earlier (Okonjo *et. al.,* 2006) and used for the determination of the pK_a here refered to as pQ (to avoid confusion with the constant of other equilibrium) of the ionizable groups linked to the equilibrium reaction of DTNB with Cys[F9]93β hemoglobin sulfhydryl groups of some animal hemoglobins (Okonjo *et. al.,* 2008; Okonjo *et. al.,* 2007; Fodeke, 2017).

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

The mean calculated K_{EQ} values were plotted as a function of pH at different concentrations of Ficoll 70. The curves were fitted with Eq. (3) and scheme 1 (with n = 2). In scheme 1, K_{Ei} (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 = 2 for the first ionization and i = 3…n+1 after each successive ionization of the ionizable groups while 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 state. The subscript r- and t- indicates the tertiary isomerization state of the tertiary 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 equilibrium solution. Various equilibrium steps involved in the reaction of DTNB with hemoglobin have been previously described using scheme 1 (Okonjo *et. al.,* 2007) reproduced here for exigency. Parameters of scheme 1 can be obtained by fitting the dependence of equilibrium constant of the DTNB reaction with the hemoglobin on pH according complex equation (3).



Scheme 1

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

Where only two amino acid side chain of the hemoglobin are ionized, it means that n = 2, and Eq. (3) comes down to,

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

Results and Discussion

Affinity of Carbonmonoxyhemoglobin for ligand binding

The three data sets, plotted in Figure 1 describe the dependences of $-\log_{10}(K_{EQ})$ on pH for the reaction of DTNB with CysF9[93]β of carbonmonoxyhemoglobin at three different concentrations of Ficoll 70. Each experimental data point was obtained from the mean of at least six K_{EQ} values calculated for different concentrations of DTNB, subject to *ca*. 10 % error. The curves through the experimental data points were calculated using Eq. (4) and the fitting parameters in Table (1) column 2, for reaction in the absence of crowder; column 3, for reaction in 50 mg/cm^3 Ficoll 70 and column 4, for reaction in 100 mg/cm^3 Ficoll 70. The three data sets collected under the different crowding conditions in Fig. 1 were fitted to common set of ionization constant values, whereas other parameters were allowed to vary independently. The justification for this is that the ionization constant being an intrinsic property of the molecule should not depend on the crowding conditions. The curve through the experimental data sets were therefore fitted by allowing the parameters which depend on the of tertiary isomerization state of the hemoglobin to vary independently. Under all crowding conditions, the affinity of carbonmonoxyhemoglobin for DTNB is highest at the lowest pH of each experiment. It is seen that in the absence of Ficoll 70 the affinity of carbonmonoxyhemoglobin for DTNB decreases with increasing pH, and attains a minimum at pH ca. 7.7. Above pH 7.7, the affinity of the hemoglobin begins to increase until maximum pH is attained. It is noteworthy that at physiological pH (ca. 7.2), in dilute solution, the affinity of carbonmonoxyhemoglobin for DTNB (binding ligand) is greater than its affinity in either of the crowding conditions, Figure 1. In the absence of crowding agent, because of this high affinity, carbonmonoxide would much more readily bind to hemoglobin and would be more difficult to displace by oxygen or any other ligand.

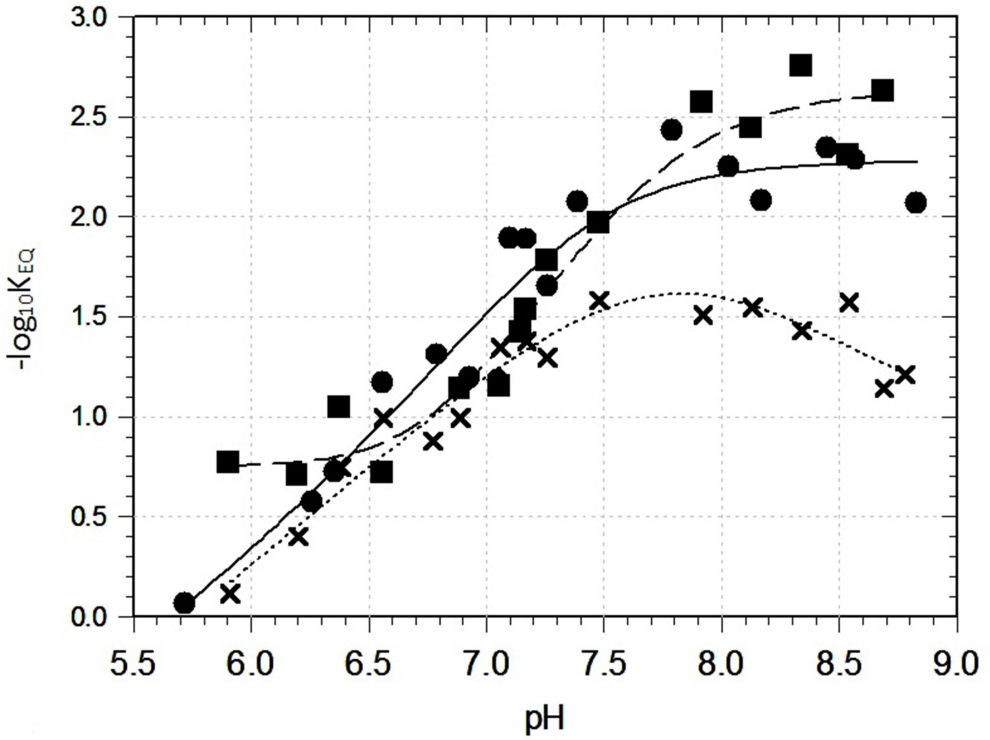


Figure 1: Dependence of negative logarithm of equilibrium constant of DTNB for CysF9[93]β sulfhydryl group of carbonmonoxyhemoglobin on pH at 25^{o}C in phosphate buffer 5.6 ≤pH≤8.0 and borate buffer 8.0≤ pH≤ 8.8 at 0.05 mole dm^{-3} NaCl (ionic strength). Crowding conditions and symbols: "X" and dotted curve, no crowder; squares and broken curve, 50 mg/cm^{3} Ficoll 70; circle and full curve, 100 mg/cm^{3} Ficoll 70.

Table 1: Fitting parameters for dependence of equilibrium constant on pH for DTNB reaction with CysF9[93]β of Rabbit carbonmonoxyhemoglobin

Parameter	No Fic	(+) 50 mg/cm^{3} Fic	(+) 100 mg/ cm^{3} Fic
pQ_{1r}	4.970	4.970	4.97
pQ_{1t}	7.923	7.923	7.92
pQ_{2r}	6.364	6.364	6.36
pQ_{2t}	7.556	7.556	7.56
K_{E3}/K_{E2}	226.463	$1.51x10^{-15}$	16.684
K_{E3}/K_{E1}	$4.48x10^{-57}$	155.615	$5.73x10^{-63}$
PK_{E3}	2.201	3.350	3.230
K_{rt3}	15.152	4.472	8.012

In the presence of 50 mg/cm^3 Ficoll 70, the sigmoidal shape of the curve shows that in the range 5.8≤ pH≤ 6.5 the affinity of DTNB for carbonmonoxyhemoglobin reduces slowly with increasing pH. Above pH 6.5, it decreases more sharply with increasing pH until the pH 7.8 is attained. Above pH 7.8, the affinity of DTNB for CysF9[93]β of carbonmonoxyhemolgolobin in 50 mg/cm^3 Ficoll 70 decreases more slowly again until pH 8.8 is reached. Under this crowding condition, at high pH, the equilibrium constant of isomerization is only slightly in favour of t-isomer (K_{rt3} = 4.47). Under physiological pH condition (ca. 7.2), the affinity of carbonmonoxyhemoglobin for DTNB in 100 mg/cm^3 Ficoll 70 is less than the affinity in 50 mg/cm^3 Ficoll 70 which is also slightly less than the affinity in dilute solution. A cursory comparison of the affinities and the value of K_{rt3} of the carbonmonoxyhemoglobin under the different crowding conditions at high pH (Figure 1) shows that affinity of the carbonmonoxyhemoglobin for DTNB at high pH increases as the value of K_{rt3} increases. The implication of this is that crowding might be performing the role of regulating the affinity of hemoglobin for carbonmonoxide by altering the tertiary structure of the hemoglobin by shifting the equilibrium in favour of the t-conformation. The consequence of this is that the affinity of carbonmonoxyhemoglobin for ligand in the absence of Ficoll 70 is much higher than under other crowding conditions. This means that at physiological pH (7.2 – 7.4), the presence of Ficoll 70 up to 50 mg/cm^3 reduces the affinity of hemoglobin for carbonmonoxy compared to solution containing no Ficoll 70.
In the presence of 100 mg/cm^3 Ficoll 70, the affinity of carbonmonoxyhemoglobin for DTNB decreases sharply with increasing pH in the range 5.8≤ pH≤ 7.8. Above pH 8.0, the affinity of the hemoglobin for DTNB in the presence of 100 mg/cm^3 Ficoll 70 begins to decrease very slowly with increasing pH. The maximum value of $-\log K_{EQ}$ for carbonmonoxyhemoglobin at 100 mg/cm^3 was ca. 2.50 at pH 8.8. This value is significantly greater than the maximum value of $-\log K_{EQ}$ in the absence of crowder which is ca.1.6 at pH ca.7.5. Therefore, the maximum affinity of carbonmonoxyhemoglobin for DTNB in dilute solution is significantly greater than the affinity in either 50 mg/cm^3 or 100 mg/cm^3 Ficoll 70.

Affinity of oxyhemoglobin for ligand binding

The three data sets presented in Fig. 2 describe the variation of $-\log_{10}(K_{EQ})$ on pH for the reaction of DTNB with CysF9[93]β of rabbit oxyhemoglobin under different crowding conditions. The curves through Figure 2 were fitted using Eq. (4) together with the fitting parameters in Table 2. Parameters of column 2 were used for fitting the theoretical curve through the experimental data points for the reaction in dilute solution. Solution containing 50 mg/cm^3 and 100 mg/cm^3 Ficoll 70 were fitted with the fitting parameters in columns 3 and 4, respectively. Since pQ_{ir} and pQ_{it} are not expected to vary with ligand changes, the values of ionization constants obtained for the carbonmonoxyhemoglobin were used for fitting the curve. Also for the same reason as in the previous section, the theoretical line

through the three data sets obtained under different crowding conditions was fitted with the same values of pQ_{ir} and pQ_{it} obtained previously for the carbonmonoxyhemoglobin. The other fitting parameters were however allowed to vary independently under different crowding conditions. Crowding is expected to alter the isomerization process (Winzor and Wills, 2006; Hu *et al*., 2007; Zhou *et. al*, 2008). Like in previous cases, the affinity of DTNB for oxyhemoglobin in the absence of Ficoll 70 is highest at low pH, but reduces sharply with increasing pH in the range $5.7 \leq pH \leq 7.5$ after which it decreases slowly with increasing pH until the maximum pH was reached. At high pH, the equilibrium constant of tertiary isomerization ($K_{rt3} = 0.182$) is slightly in favour of the r-isomer, suggesting that in oxyhemoglobin, at high pH the r-isomer is slightly favoured. Under this condition, except at very low pH, the affinity of oxyhemoglobin for DTNB is generally lower than in the presence of Ficoll 70.

However, in the presence of 50 mg/cm^3 of Ficoll 70 the affinity of oxhyhemoglobin for DTNB at low pH is also high but reduces gradually with increasing pH until pH 7.8 was reached. At this pH the $-\log K_{EQ}$ value is ca. 1.2. Above pH 7.8 the affinity of oxyhemoglobin in 50 mg/cm^3 Ficoll 70 begins to increase gradually with increasing pH until the highest pH of the reaction is reached. The gradual reduction in affinity with increasing pH may be the consequence of the relatively high value of K_{rt3} at high pH compared to the value in dilute solution. This is an indication that the t-isomer favours high affinity of ligand binding to oxyhemoglobin. Similar increase in affinity could be seen in carbonmonoxyhemoglobin (in the previous section, Table 1 column 2 and Figure 1) where the value of K_{rt3} at high pH was highest in the absence of Ficoll 70.

In the presence of 100 mg/cm^3 Ficoll 70 the affinity of DTNB for CysF9[93]β oxyhemoglobin sulfhydryl group decreases slightly with increasing pH in the range $5.8 \leq pH \leq 6.5$. It then decreases a little more rapidly with increasing pH over the the range $6.5 \leq pH \leq 7.5$. Above pH 7.5 the decrease in affinity of the oxyhemoglobin for DTNB with increasing pH decline, leading to a sigmoildal overall profile. A cursory comparison of the affinity of the oxyhemoglobin under the different crowding conditions (Figure 2) and the value of K_{rt3}, the equilibrium constant of isomerization r↔t at high pH (Table 2) shows that affinity of the hemoglobin for DTNB at high pH increases as the the value of K_{rt3} increases. This findings contrast in a very significant way from the what was observed with carbonmonoxyhemoglobin in which the affinity of hemoglobin for DTNB increases as the value of K_{rt3} decreases. It is significant to note that whereas the affinity of carbonmonooxyhemoglobin for the ligand decreases with increasing crowder concentration, the affinity of oxyhemoglobin for the ligand increases with increasing crowder concentration.

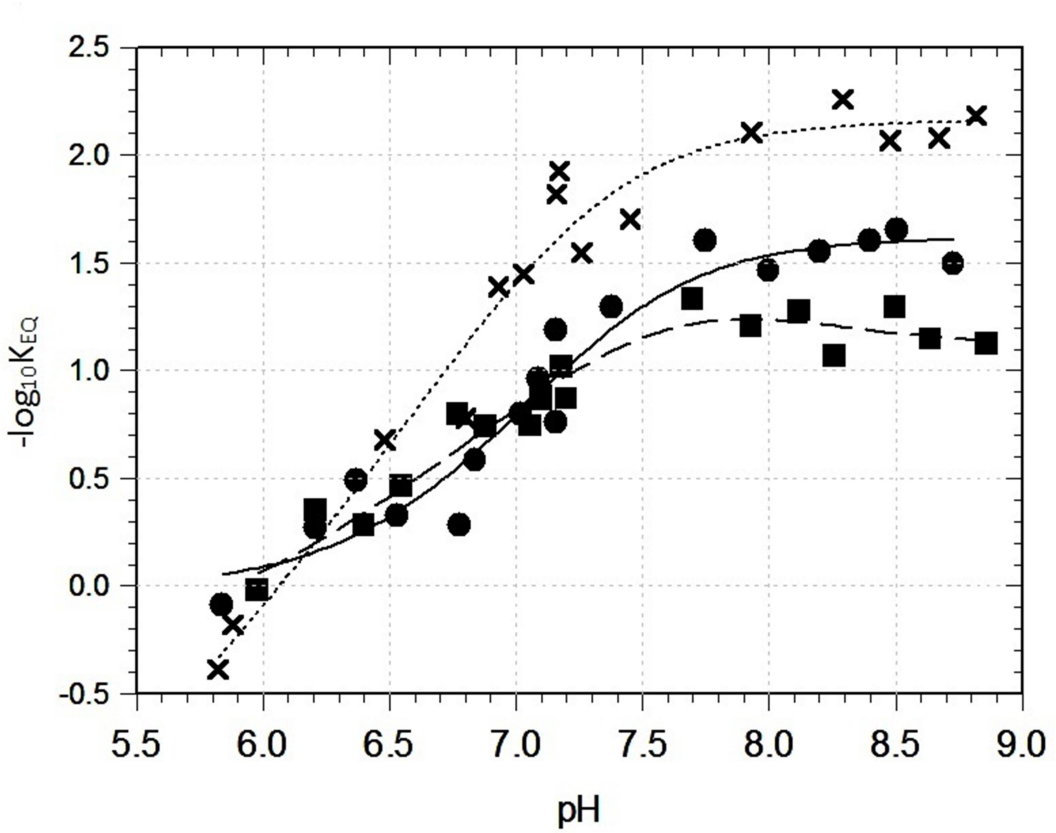


Figure 2: Dependence of negative logarithm of equilibrium constant of the affinity of DTNB for CysF9[93]β sulfhydryl group of oxyhemoglobin at 25 ^{o}C in phosphate buffer 5.6 ≤ pH ≤ 8.0 and borate buffer 8.0 ≤ pH ≤ 8.8 at 0.05 mole dm^{-3} NaCl (ionic strength). Symbols and crowding condition: "X" and dotted curve, no crowder; square and broken curve, 50 mg/cm^3 Ficoll 70; circle and full line curve, 100 mg/cm^3 Ficoll 70.

Table 2: Fitting parameters for the dependence of equilibrium constant of DTNB reaction with CysF9[93]β of rabbit oxyhemoglobin

Parameter	No Fic	(+) 50 mg/cm^3 Fic	(+) 100 mg/cm^3 Fic
pQ_{1r}	4.970	4.970	4.970
pQ_{1t}	7.923	7.923	7.923
pQ_{2r}	6.364	6.364	6.364
pQ_{2t}	7.556	7.556	7.556
K_{E3}/K_{E2}	0.757	47.720	2.579
K_{E3}/K_{E1}	$2.21x10^{-72}$	439.608	107.35

PK_{E3}	2.218	1.578	1.768
K_{rt3}	0.184	2.107	0.463

An important aim of this study is to determine how crowding conditions affect structural changes which have functional consequences. Bearing in mind that physiological pH is ca. 7.2, we computed the the values of $-\log_{10}(K_{EQ})$ and K_{EQ} at pH 7.2 under each of the crowding conditions used in the experiment and the results are presented in Table 3.

Table 3: Values of K_{EQ} estimates at pH 7.2

	Carbonmonoxyhemoglobin			Oxyhemoglobin		
Ficoll 70 (mg/cm^3)	0	50	100	0	50	100
$-\log_{10}(K_{EQ})$	1.35	1.55	1.74	1.67	0.95	1.03
K_{EQ}	0.0447	0.0282	0.0182	0.0214	0.112	0.0933

From Table 3, it is clear that in the absence of crowder, the affinity of CysF9[93]β for DTNB at physiological pH is greater than the affinity in the presence of crowding agent. This implies that the affinity of carbonmonoxyhemoglobin for ligand binding is greater than the affinity in the presence of crowding agent. It is also clear that at 100 mg/cm^3 Ficoll 70 the affinity of carbonmonoxyhemoglobin for ligand binding is least. The implication of this is that in dilute solution, ligand binding to carbonmonoxyhemoglobin is a more feasible process than it would be in crowded solution.

On the other hand, in oxyhemoglobin, the presence of crowding agent the affinity of CysF9[93]β for DTNB is greater than in the absence of Ficoll 70. The implication of this is that the affinity of oxyhemoglobin for ligaoxyhemond binding is least in dilute solution (K_{EQ} ca. 0.0214 at pH 7.2). Increasing the concentration of the Ficoll 70 to 50 mg/cm^3 result in ca. 5 fold increase in the affinity of the oxyhemoglobin for ligand binding. When the concentration of Ficoll 70 was increased to 100 mg/cm^3, the affinity of the oxyhemoglobin for DTNB dropped slightly from what it was in 50 mg/cm^3 Ficoll 70 (K_{EQ} *ca* 0.112) to *ca* 0.093 in 100 mg/cm^3 Ficoll 70.

Relating the affinities of the two hemoglobin derivatives for ligand binding under the different crowding conditions with K_{rt3} values suggest that absence of crowder favour t-isomerization of carbonmonoxyhemoglobin, a conformation which is favourable for ligand binding to the hemoglobin. On the other hand, absence of crowder favour r-tertiary isomer in oxyhemoglobin. It is also clear that ligand binding to both hemoglobin derivatives are favoured when hemoglobin is in the t-isomer conformation. This finding is in qualitative agreement with our previous finding that altering the crowding condition of glutathione

transferase alters the refolding yield and the catalytic efficiency of the enzyme (Ogunmoyole *et. al.,* 2019; Ogunmoyole *et. al.,* 2020). In the presence of 50 mg/cm^3 Ficoll 70 the proportion of t-isomer of carbonmonoxyhemoglobin decreases compared to the proportion in dilute solution and affinity for ligand binding increases. Increasing the concentration of Ficoll 70 from 50 mg/cm^3 to 100 mg/cm^3 only marginally reduces the proportion of t-isomer in the carbonmonoxyhemoglobin polymer mixture. The effect is the opposite when the oxyhemoglobin was exposed to increasing concentration of Ficoll 70.
It is noteworthy that the K_{rt3} values of rabbit carbonmonoxyhemoglobin under all crowding conditions were generally higher than that of oxyhemoglobin. This may not be unconnected with the high affinity of carbonmonoxide for hemoglobin compared to oxyhemoglobin.

Conclusions

The findings in this study showed that the presence of crowder reduces the affinity of carbonmonoxyhemoglobin for ligand binding, whereas it increases the affinity of oxyhemoglobin for ligand binding. This was evidenced in the higher affinity of oxyhemoglobin for ligand binding in the presence of 50 mg/cm^3 Ficoll 70 than the affinity of carbonmonxyhemoglobin derivative for ligand binding under the same crowding condition. Similar observation was made for the effect of 100 mg/cm^3 Ficoll 70 on the affinity of the oxyhemoglobin for DTNB when compared with the affinity of carbonmonoxyhemoglobin for DTNB.
The different K_{rt3} values obtained at high pH for the same hemoglobin derivative under different crowding conditions suggest that crowding affects the physiological function of hemoglobin by altering the state of isomerization of the tertiary and quaternary structures of the hemoglobin derivatives. It could therefore be posited that the differences in affinity of the hemoglobin derivatives might be due to difference in equilibrium constant of $r \leftrightarrow t$ isomerization. The implication of our findings is that crowding increases the affinity of hemoglobin for oxygen, a physiologically important ligand compared to the binding of the carbonmonoxide. It would therefore be interesting to carry out a more comprehensive study of this nature using different animals with CysF9[93]β as the only reactive sulfhydryl group for a more categorical conclusion to be made.

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