Covenant University Journals

Tertiary conformational transition constant of guinea pig haemoglobin determined from the reaction of 5,5′-dithiobis (2-nitrobenzoate) with CysF9[93]β and CysH3[125]β

Idowu Adeogun,
Olaniyi Yusuff, &
Kehinde Okonjo.

Abstract:
We have determined K_{equ}, the equilibrium constant for the reaction of 5,5′-dithiobis(2-nitrobenzoate) — DTNB — with the CysF9[93]β and CysH3[125]β sulphydryl groups of various derivatives of guinea pig haemoglobin at 25°C. In the pH range 5.6 to 9, K_{equ} decreases almost 50-fold: from a mean of 3.45 ± 0.2 to a mean of 0.073 ± 0.01. Quantitative analyses of the pH dependence profiles of K_{equ} enable the determination of K_{rt}, the equilibrium constant for the **r** $\rightleftharpoons$ **t** tertiary conformational transition of haemoglobin. The **t** isomer population is 53.9 (± 2)%. In the **r** conformation the pK_as of the amino acid residues whose ionisations are coupled to the reaction of DTNB with the sulphydryl groups are 5.74 ± 0.02 — for a combination of HisNA2[2]β and HisH21[143]β) — and 7.74 ± 0.2 for ValNA1[1]β); in the **t** conformation they are 5.88 ± 0.05 and 8.23 ± 0.1, respectively.

Keywords --- guinea pig haemoglobin; sulphydryl groups; 5,5′-dithiobis(2-nitrobenzoate); equilibrium constants; tertiary conformational transition.

1. INTRODUCTION

``The reactivities of protein sulphydryl groups are determined by their degree of nucleophilicity, which is maximized by stabilization of the thiolate anion form of the sulphydryl groups. This, in turn, depends on the nature of their microenvironments [1,2]. The reactivity of a sulphydryl group in haemoglobin is an indicator of the structure in its immediate neighborhood. Apart from those sulphydryl groups that are totally internal — and therefore completely hindered from reacting — haemoglobin sulphydryl groups seem to belong to two categories: (i) Those with high solvent accessibilities, whose microenvironments do not change with haemoglobin conformation, like CysH3[125]β in rat and guinea pig haemoglobins and CysA10[13]β in mouse haemoglobin. These sulphydryls are possibly involved in physiologically relevant redox reactions such as detoxifying functions. (ii) Those like CysF9[93]β and CysA11[13]α, which display oxygen-linked reactivity. In this second category, steric hindrance plays an important role in the sulphydryl group reactivity [2,3]. The CysF9[93]β sulphydryl group is involved in NO metabolism [4]. The CysF9[93]β sulphydryl group has been employed as an indicator for tertiary and quaternary structure change in haemoglobin [5 – 13]. In both the R and T quaternary structures of haemoglobin this sulphydryl exists in two conformations relative to the main chain: *cis*-to-amino and *cis*-to-carbonyl [12,13]. These two conformations are coupled to two tertiary isomeric forms of haemoglobin (**r** and **t**, respectively) in dynamic equilibrium [13]. Evidence for the **r** $\rightleftharpoons$ **t** isomerisation process comes from temperature-jump studies on carbonmonoxy- and deoxyhaemoglobin. We found that τ^{-1}, the reciprocal relaxation time for the isomerization, varies strongly with pH [13]. However, the pH dependence of τ^{-1} is abolished when CysF9[93]β is modified with iodoacetamide [13]. This arises because iodoacetamide binds *irreversibly* to this thiol group and tilts the **r** $\rightleftharpoons$ **t** equilibrium fully to the right, in favour of the **t** isomer.

We have demonstrated that, in contrast to the irreversibility of the iodoacetamide reaction, the reaction of CysF9[93]β with 5,5'-dithiobis(2-nitrobenzoate) — DTNB — is a *reversible* process [14-19]. We have taken advantage of this finding to determine K_{rt}, the equilibrium constant for the **r** $\rightleftharpoons$ **t** tertiary structure transition, from quantitative analyses of the pH dependence of the parameters of the DTNB reaction: (i) K_{equ}, the equilibrium constant; (ii) k_F, the apparent second order forward rate constant; and (iii) k_R, the apparent second order reverse rate constant [14-21]. These analyses also enabled us to determine the number and the nature of the amino acid residues influencing the reactivities of CysF9[93]β [14-21].

All the haemoglobins so far studied in our laboratory have had CysF9[93]β as the only sulphydryl reacting with DTNB [14-23]. Apart from CysF9[93]β, guinea pig haemoglobin has a further sulphydryl group — CysH3[125]β — that reacts with DTNB. Here we report an equilibrium study of the reaction of

DTNB with CysF9[93]β and CysH3[125]β between pH 5.6 and 9. Analyses of these data enable us, for the first time, to determine K_{rt} values for a haemoglobin containing more than one reacting sulphydryl group. We were, however, unable to determine K_{rt} values separately for the reactions of CysF9[93]β and CysH3[125]β.

2. MATERIAL AND METHODS

2.1. Preparation of haemoglobin

Guinea pig blood was obtained by decapitation of live animals. The blood was collected in bottles containing freshly prepared acid-citrate-dextrose anticoagulant. Haemoglobin was prepared as follows: The blood sample was centrifuged at 18,000 r.p.m at 5°C for 20 minutes using a refrigerated MSE HS 18 centrifuge. The red blood cells were washed thrice with isotonic saline (for guinea pig, 11.5 g NaCl dm^{-3}). After each washing, the resulting solution was centrifuged at 10,000 r.p.m. for 15 minutes. The erythrocyte sediment was lysed by shaking the centrifuge tube vigorously after the addition of a volume of ice-cold water equal to the volume of the erythrocytes. This yielded a mixture of haemolysate and red cell fragments. The mixture was centrifuged at 10,000 r.p.m for 20 minutes. The haemolysate was decanted from the cake of cell debris, after which NaCl (5% wt./vol.) was added. This mixture was left for 20 minutes at 5°C in the cold room. The haemolysate was then centrifuged at 18,000 r.p.m. for 20 minutes. Low molecular weight impurities contained in the haemolysate were removed by dialyzing it in the cold room against a dialysis solution of pH between 6.5 and 7.5. Two changes of dialysis solution were made at 3-hour intervals to obtain salt-free haemoglobin. The haemoglobin was stored as the carbonmonoxy derivative.

2.2. Preparation of stock DTNB solution for equilibrium measurements

The stock DTNB solution was prepared as previously described [14]. 25 cm^3 of a 50 mmol dm^{-3} solution of DTNB in 95% ethanol was titrated to pH 6.8 with 200 mmol dm^{-3} phosphate buffer pH 8.0. About 18 cm^3 of this buffer was usually required. The final DTNB concentration was 29 mmol dm^{-3}. This solution was employed as the stock solution for the equilibrium measurements.

2.3 Equilibrium measurements of the reaction of DTNB with haemoglobin

The method previously employed for the determination of equilibrium constants [15] was adopted. 10 cm^3 aliquots of a 50 µmol (haem) dm^{-3} haemoglobin solution in a buffer at a given pH were measured into several clean, dry tubes. Increasing volumes (between 1 and 120 mm^3) of a stock 29 mmol dm^{-3} DTNB solution, prepared as detailed in [14], were added to the different tubes. The mixtures were stirred and left to equilibrate at 25°C for about 6 h. The absorbances were determined on a Zeiss PMQ II uv-visible spectrophotometer using a 2 cm light path cuvette. The reference solution was a 50 µmol (haem) dm^{-3} haemoglobin solution to which no DTNB had been added. The transmittance of this reference solution was adjusted to 100 percent (zero absorbance) by increasing the slit width of the spectrophotometer. The absorbance change, ΔA_{412}, of each haemoglobin/DTNB mixture was then determined at 412 nm relative to the reference solution of zero absorbance. A molar absorption coefficient of 14,000 mol^{-1} dm^3 cm^{-1} was assumed for 5-thio-2-nitrobenzoate (TNB^-), the chromophoric product of the reaction. The TNB^- concentration so determined was substituted into Eq. (2) below to obtain K_{equ}, the equilibrium constant of the reaction.

3. RESULTS

3.1. Variation of K_{equ} with pH

The reversible reaction between a sulphydryl group and DTNB may be depicted as:

$$PSH+DTNB \rightleftharpoons^{Q_{SH}} H^+ + PS^- + DTNB \underset{k_R}{\overset{k_F}{\rightleftharpoons}} H^+ + PS.ST + TNB^- \rightleftharpoons^{Q_{TNB}} PS.ST + TNBH \quad \ldots (1)$$

In Eq. (1) PSH is haemoglobin with its sulphydryl group in the protonated form, which does not react with DTNB; PS^- is the corresponding (reacting) anion form in the *cis*-to-amino conformation; PS.ST is the mixed disulfide formed after reaction with DTNB, and it is in the *cis*-to-carbonyl conformation [12,13]; TNB^- is 5-thio-2-nitrobenzoate, the anionic, chromophoric product of the reaction; TNBH is the protonated form of TNB^-; Q_{SH} and Q_{TNB} are the ionization constants of the sulphydryl group and TNBH, respectively; k_F and k_R are the apparent second order forward and reverse rate constants, respectively; K_{equ} ($=\frac{k_F}{k_R}$) is the equilibrium constant for the formation of the mixed disulfide, that is, the DTNB reaction step..

Eq. 2, the equation relating K_{equ} to the concentrations of the species in Eq. (1), has been derived in detail previously [15]. In Eq. (2) $[P]_{total}$ refers to the total haemoglobin concentration in terms of reacting sulphydryl groups.

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

A computer programme aided the calculation of K_{equ} from Eq. (2) using the experimental absorbance data. The standard error in the determination of K_{equ} was about 10 percent. The values of pQ_{SH} and pQ_{TNB} used in these calculations were 8.30 and 5.27, respectively, and the extinction coefficient of TNB^- was assumed to be 14,000 mol^{-1} dm^3 cm^{-1} [15].

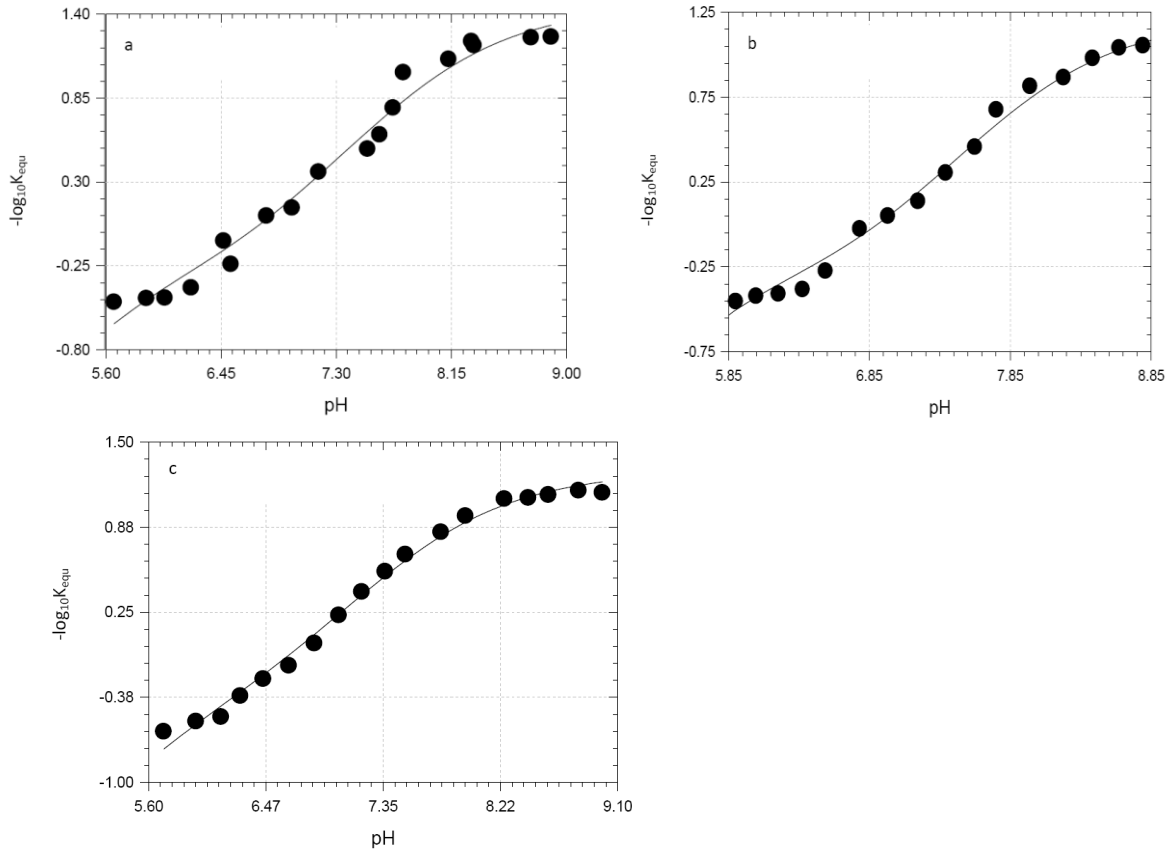
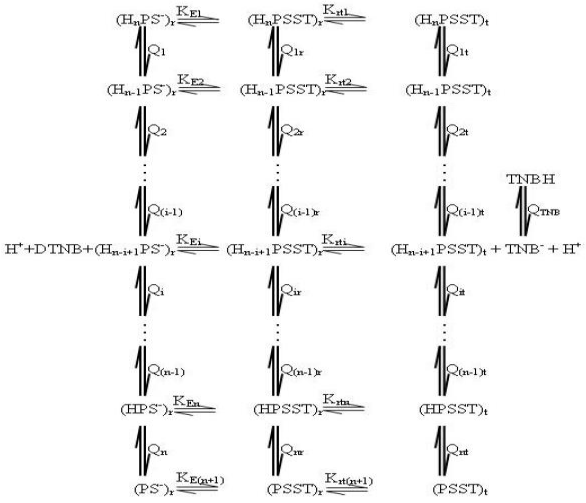


Fig. 1
Reaction of guinea pig haemoglobin with 5,5´-dithiobis(2-nitrobenzoate. Variation of the equilibrium constant, K_{equ}, with pH: plots of $-\log_{10}K_{equ}$ against pH. (a) oxyhaemoglobin; (b) carbonmonoxyhaemoglobin; (c) aquomethaemoglobin. Conditions: 25°C; phosphate buffers, pH 5.6-8.0; borate buffers, pH 8.0-9.0. Total ionic strength, 50 mmol dm^{-3} (added salt, NaCl). The lines through the data points were fitted with the parameters in Table 1 (compare with Scheme 1 and Eq. (3) for n = 2.)

In Fig. 1a we report the variation of K_{equ} with pH for oxyhaemoglobin. It is seen that K_{equ} *decreases* by about two orders of magnitude as the pH increases from about 5.6 to 9. Similar results are seen in Fig. 1b and 1c for the carbonmonoxy and aquomet derivatives, respectively.

3.2. Theoretical analysis of pH dependence of K_{equ} data

As can be seen in Fig. 1, K_{equ} decreases strongly as the pH of the reaction medium increases. This behaviour strongly indicates that the reaction of DTNB with guinea pig haemoglobin sulphydryl groups is linked to the ionization of certain amino acid residues on the protein. If this were not so, K_{equ} would have remained constant as the pH increased. In order to determine the nature and the number of such linked groups we propose the following reaction scheme:



Scheme 1.

In Scheme 1 the protons arising from the various ionization steps have been omitted for clarity. The species $H_{n-i+1}PSH$ (i = 1, 2,…, n) are haemoglobin species in which the sulphydryl group is in its protonated form, which does not react with DTNB. These species are therefore omitted from Scheme 1. $H_{n-i+1}PS^-$ (i = 1, 2, …., n) are species in which the sulphydryl group is in its thiolate anion form, the form that reacts with DTNB [14–23]; $H_{n-i+1}PS.ST$ (i = 1, 2, …., n) are the mixed disulfide species formed after the reaction of the sulphydryl with DTNB. Species marked with subscripts r and t are those in which the sulphydryl is in the **r** and **t** tertiary isomeric form of haemoglobin, respectively. The various proton ionization constants are represented as Q_i, Q_{ir} and Q_{it} (i = 1, 2, …, n) to differentiate them from the equilibrium constants K_{Ei} (i = 1, 2, …., n + 1) for the reaction of DTNB; and $K_{rt(n+1)}$ is the equilibrium constant at high pH for the $\mathbf{r} \rightleftharpoons \mathbf{t}$ transition. The relationship between K_{equ} and the parameters of Scheme 1 that is most amenable to a computer analysis of the K_{equ} versus pH data is [18]

Eq. (3) was employed to fit the data reported in Fig. 1. The fitting procedure has been described in detail before [18]. The value of n that gave the best fit to the data was 2. Table 1 reports the values of the best-fit parameters used to draw the theoretical lines reported in Fig. 1 for the three haemoglobin derivatives.

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

Table 1: *Reaction of 5,5´-dithiobis(2-nitrobenzoate) with CysH3[125]β and CysF9[93]β of guinea pig haemoglobin. Best-fit parameters employed to fit the –log_{10}Kequ versus pH data reported in Fig. 1 (compare with Scheme 1 and Eq. (3) of the text. The best value of n is 2.*

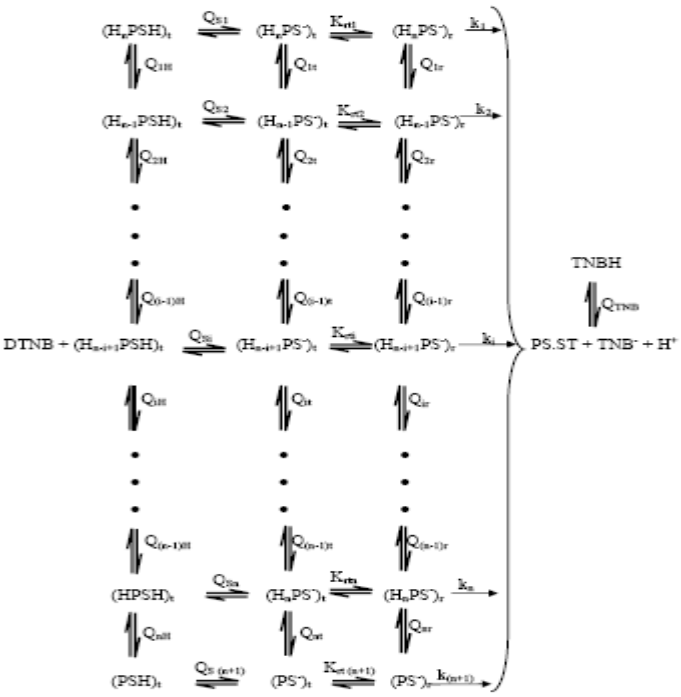
Derivative	pQ_{1r}	pQ_{1t}	pQ_{2r}	pQ_{2t}	K_{E3}/K_{E2}	K_{E3}/K_{E1}	K_{E3}	K_{rt3}
Oxy	5.72	5.86	7.83	8.32	0.13	0.030	0.051	1.20
Carbonmonoxy	5.78	5.81	7.96	8.29	0.14	0.030	0.088	0.99
Aquomet	5.72	5.96	7.43	8.09	0.19	0.011	0.067	1.33
Mean	5.74 ± 0.02	5.88 ± 0.05	7.74 ± 0.2	8.23 ± 0.1	0.15 ± 0.02	0.024 ± 0.01	0.069 ± 0.01	1.17 ± 0.1

Table 1 gives the values of K_{rt3}, the constant for the **r** $\rightleftharpoons$ **t** tertiary transition. The mean value of this parameter is 1.17 ± 0.11. Calculation from this value yields a **t** isomer population of 53.9 (± 2)%. This value is comparable to that calculated for sheep haemoglobins [18, 19]. Thus, roughly half of the haemoglobin molecules are in the **t** tertiary conformation at 25°C. It should be noted that the tertiary **r** $\rightleftharpoons$ **t** transition referred to here is for the mixed disulphide PS.ST (see Eq. 1). It is not possible, using an equilibrium method, to determine the parameters of this transition for the PS^- species, that is, for haemoglobin that has not reacted with DTNB.

Measurements of equilibrium constants for molecular systems containing more than one type of reacting site for a given ligand are notoriously incapable of distinguishing between the sites. Such sites can usually be distinguished kinetically, especially if their rates of reaction differ by up to an order of magnitude. This is the case with the CysH3[125]β and CysF9[93]β sulphydryl groups of guinea pig haemoglobin: CysH3[125]β is two orders of magnitude faster-reacting with DTNB than CysF9[93]β [24]. In order to determine K_{rt} for haemoglobin that has not reacted with DTNB, it is necessary to perform a kinetic study. Unfortunately, the kinetic data available [24] were obtained at 20°C, not the 25°C at which we performed our equilibrium experiments. We therefore cannot employ them to do separate analyses for each of the two sulphydryl groups in the manner we reported recently for sheep haemoglobins [19]. Furthermore, we cannot perform the kinetics experiments ourselves because the reaction rates for the two sulphydryl groups are best studied with a fast mixing instrument, that is, a stopped-flow machine. This is currently not available to us. Instead, with the kinetic data obtained at 20°C in mind, we present here a theoretical analysis of the data that could be won in a future stopped-flow experiment at 25°C.

It is known that the CysF9[93]β sulphydryl group exists as a mixture of two tertiary conformations, **r** and **t**, in dynamic equilibrium [8,9]. It is not so clear whether CysH3[125]β also exists in two tertiary conformations and, if it does, what the relative populations of the **r** and **t** conformations are. Furthermore, we demonstrated that, for CysF9[93]β, DTNB preferentially reacts with the **r** conformation. It is necessary to determine which of the two possible conformations CysH3[125]β DTNB preferentially reacts with.

We first assume that DTNB preferentially reacts with the **r** conformation of both sulphydryl groups. On the basis of this assumption, and because of the usually complex nature of the pH dependence of k_F, the apparent second order forward rate constant [19], we propose the following reaction scheme (Scheme 2) to explain (i) the relative magnitudes of k_F for CysH3[125]β and CysF9[93]β and (ii) the pH dependence of k_F:



Scheme 2

In Scheme 2, $(H_{n-i+1}PSH)_t$ (i = 1, 2, … n+1) are haemoglobin species in which the sulphydryl groups are protonated, and they are in the **t** conformation; $(H_{n-i+1}PS^-)_t$ are the corresponding thiol anion forms; species $(H_{n-i+1}PS^-)_r$ are the thiol anion species in the **r** conformation; Q_{si} are the dissociation constants of the sulphydryl group; K_{rti} are the tertiary conformation transition

constants; and k_i are the second-order forward rate constants for the reaction of DTNB with the various $(H_{n-i+1}PS^-)_r$ species in the **r** tertiary conformation. The equation relating k_F to the parameters of Scheme 2 is [19]:

$$k_F = \frac{k_{n+1} + \sum_{i=1}^{n} k_i \left[H^+\right]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jr}\right)^{-1}}{1 + \sum_{i=1}^{n} [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jr}\right)^{-1} + K_{rt(n+1)} \left[1 + [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jt}\right)^{-1} + \frac{[H^+]}{Q_{s(n+1)}} \left\{1 + \sum_{i=1}^{n} [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jH}\right)^{-1}\right\}\right]} \quad \ldots. (4)$$

Eq. (4) will enable the determination of several parameters that will make it possible to explain the large difference in DTNB reaction rates observed (at 20°C) between CysH3[125]β and CysF9[93]β [24].

We are currently studying, at 25°C, the kinetics and equilibrium of the reaction of DTNB with the two haemoglobins of the domestic chicken, each of which has two sulphydryl groups per molecule reacting with DTNB. Unlike guinea pig haemoglobin, the chicken haemoglobin sulphydryls react at rates that can be monitored conveniently with a simple uv-visible spectrophotometer.

If DTNB reacts preferentially with the sulphydryl groups in the **t** rather than in the **r** tertiary conformation, the appropriate reaction scheme will be similar to Scheme 2, except that all the Q_{jr} terms in the latter are replaced by Q_{jt} terms, and vice versa; and $K_{rt(n+1)}$ in the latter is replaced by $K_{tr(n+1)}$: $K_{tr(n+1)} = 1/K_{rt(n+1)}$. The relationship between K_F and the parameters of the new scheme will be [19]:

$$k_F = \frac{k_{n+1} + \sum_{i=1}^{n} k_i \left[H^+\right]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jt}\right)^{-1}}{1 + \sum_{i=1}^{n} [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jt}\right)^{-1} + \frac{1}{K_{rt(n+1)}} \left[1 + [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jr}\right)^{-1} + \frac{[H^+]}{Q_{s(n+1)}} \left\{1 + \sum_{i=1}^{n} [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jH}\right)^{-1}\right\}\right]} \quad \ldots. (5)$$

Eq. (5) is similar to Eq. (4), except that all the Q_{jr} terms in the latter are replaced by Q_{jt} terms , and vice versa; and $\frac{1}{K_{rt(n+1)}}$ appears in the denominator of Eq. (5) in place of $K_{rt(n+1)}$ in Eq. (4).

4. DISCUSSION

4.1 Equilibrium results

The equilibrium data reported in Fig. 1 are similar in magnitude to those reported previously for sheep haemoglobins [18]. As in the previous report [18], there is no marked difference in the magnitude of K_{equ} between the various haemoglobin derivatives: oxy, carbonmonoxy and aquomet. Clearly, equilibrium measurements do not appear to be useful for distinguishing between the various derivates of a given haemoglobin. They also do not appear to be able to distinguish between haemoglobins from different animal species. The only possible exception is found with the cat haemoglobins for which, at high pH, K_{equ} values are an order of magnitude *lower* than for other haemoglobins [15]. Nevertheless, K_{equ} values are very useful for determining the apparent second order reverse rate constant, k_R, from k_F and K_{equ} values, since $k_R = \frac{k_F}{K_{equ}}$. At present k_R cannot be determined directly by experiment [14-19].

4.2. Assignment of pQ_i values

Examination of Table 1 shows that the mean value of pQ_{1r} is 5.74 ± 0.03; that of pQ_{1t} is 5.88 ± 0.05. Following our previous assignments [14-19], we attribute the first value to both HisNA2[2]β and HisH21[143]β in the **r** tertiary conformation; the value 5.88 is assigned to the same histidines in the **t** conformation. The mean pQ_{2r} value is 7.74 ± 0.17 and that of pQ_{2t} is 8.23 ± 0.08. We assign these values to ValNA1[1]β in the **r** and **t** conformations, respectively. It is clear that for the groups whose ionizations are linked to the reaction of DTNB with the sulphydryl groups the mean pQ_{it} values are higher than the mean pQ_{ir} values. Thus, these groups appear to ionize more readily in the **r** than in the **t** tertiary conformation, as previously observed for other haemoglobins [16-19].

HisNA2[2]β, HisH21[143]β and ValNA1[1]β are the ionizable groups at the organic phosphate binding site of haemoglobin [26, 27]. We have already demonstrated [20, 21] that these groups are electrostatically linked to the reaction of haemoglobin with DTNB. This linkage forms the basis of the above assignment of the pQ values.

Acknowledgements

We give all glory to the Lord God Almighty, the Author of all wisdom and knowledge, for inspiring this work. We are grateful to the Alexander von Humboldt Foundation, Bonn, Germany, to the Postgraduate School, University of Ibadan, and to Covenant University, Ota for financial support.

References

[1] Miranda, J. J., Highly reactive cysteine residues in rodent hemoglobins, *Biochem. Biophys. Res. Commun.* 275 (2000) 517–523.

[2] Kleinman, W. A. Komninou, D., Leutzinger, Y., Colosimo, S., Cox, J., Lang, C. A., Richie, J. P., Protein glutathiolation in human blood, *Biochem. Pharmacol.* 65 (2003) 741–746.

[3]. Rossi, R., Barra, D., Bellelli, A., Boumis, G., Canofeni, S., Di Simplicio, P., Lusini, L., Pascarella, S., Amiconi, G. Fast-reacting thiols in rat hemoglobins can intercept damaging species in erythrocytes more efficiently than glutathione, *J. Biol. Chem.* 273 (1998) 19198–19206.

[4] Chan, N. L., Rogers, P. H., Arnone, A. Crystal structure of the S-nitroso form of liganded human hemoglobin, *Biochemistry* 37 (1998) 16459–16464.

[5] Guidotti, G. The rates of the reaction of the sulphydryl groups of human haemoglobin, *J. Biol. Chem.* 240 (1965) 3924-3927.

[6] Antonini, E., Brunori, M. On the rate of conformation change associated with ligand binding in haemoglobin, *J. Biol. Chem.* 244 (1969) 3909– 3912.

[7] Gibson, Q. H. p-Mercuribenzoate as an indicator of conformation change in hemoglobin, *J. Biol. Chem.* 248 (1973) 1281–1284.

[8] Perutz, M.F., Fersht, A.R., Simon, S.R., Roberts, G.C.K. Influence of globin structure on the state of the heme. II. Allosteric transitions in methemoglobin, *Biochemistry* 13 (1974) 2174– 2286.

[9] Hensley, P., Edelstein, S.J., Wharton, D.C., Gibson, Q.H. Conformation and spin state in methemoglobin, *J. Biol. Chem.* 250 (1975) 952– 960.

[10] Heidner, E. J., Lardner, R.C., Perutz, M.F. Structure of horse carbonmonoxy- haemoglobin, *J. Mol. Biol.* 104 (1976) 707–722.

[11] Baldwin, J. M. The structure of human carbonmonoxyhaemoglobin at 2.7Å resolution, *J. Mol. Biol.* 136 (1980) 103–128.

[12] Shaanan, B. Structure of human oxyhaemoglobin at 2.1 Å resolution, *J. Mol. Biol.* 171(1983) 31– 59.

[13] Okonjo, K.O., Vega-Catalan, F.J., Ubochi, C.I. Temperature-jump studies on haemoglobin: Kinetic evidence for a non-quaternary isomerization process in deoxy- and carbonmonoxyhaemoglobin, *J. Mol. Biol.* 208 (1989) 347– 354.

[14] Okonjo, K.O., Fodeke, A.A. 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 the complex pH dependence of the forward apparent second order rate constant to a simple form, *Biophys. Chem.* 119 (2006) 196–204.

[15] Okonjo, K.O., Fodeke, A.A., Kehinde, A.T. Reversible reaction of 5,5′ dithiobis(2-nitrobenzoate) with the hemoglobins of the domestic cat: Variation of the equilibrium and reverse rate constant with pH, *Biophys. Chem.* 121 (2006) 65 – 73.

[16] Okonjo, K. O., Adediji, T. A., Fodeke, A. A., Adeboye, O., Ezeh, C. V. 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, *Biophys. Chem.* 128 (2007) 56–62.

[17] Okonjo, K.O., Bello, O.S., Babalola, J.O. 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*), *Biochim. Biophys. Acta* 1784 (2008) 464–471.

[18] Okonjo, K. O., Adeogun, I., Babalola, J. O. 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), *Biochim. Biophys. Acta* 1794 (2009) 398–409.

[19] Okonjo, K.O., Adeogun, I., Babalola, J.O. Tertiary conformational transition in sheep haemoglobins induced by reaction with 5,5′-dithiobis(2-nitrobenzoate) and by binding of inositol hexakisphosphate, Biophys. Chem. 146 (2010) 65-75.

[20] Okonjo, K.O., Aboluwoye, C.O., Babalola, J.O., Usanga, I.A. Organic phosphate binding groups electrostatically linked to the reactivity of the CysF9[93]β sulphydryl group of haemoglobin, *J. Chem. Soc., Faraday Trans.*, 91 (1995) 2095–2100.

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

[22] Okonjo, K.O., Okia, T.O. Hemoglobins with multiple reactive sulphydryl groups: The reaction of pigeon hemoglobin with 5,5'-dithiobis(2-nitrobenzoic acid), *J. Protein Chem.* 12 . (1993) 639-646.

[23] Okonjo, K.O., Adejoro, I.A. Hemoglobins with multiple reactive sulphydryl groups: The reaction of dog hemoglobin with 5,5'-dithiobis(2-nitrobenzoic acid), *J. Protein Chem.* 12 (1993) 33-37.

[24] Babalola, J.O., Uko, L.U., Okonjo, K.O., an Eldik, R. Hemoglobins with multiple reactive sulphydryl groups:

Reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with guinea pig hemoglobin, *Bulletin of the Chemical Society of Ethiopia* 16 (2002) 175-186.

[25] Yusuff, K.O. Equilibrium studies of the reaction of 5,5′-dithiobis(2-nitrobenzoate) with guinea pig haemoglobin, *MSc thesis*, University of Ibadan (2005).

[26] Arnone, A. X-ray diffraction study of binding of 2,3-diphosphoglycerate to human deoxyhaemoglobin, *Nature* 237 (1972) 146-148.

[27] Arnone, A., Perutz, M.F Structure of inositol hexaphosphate-human deoxyhaemoglobin complex, *Nature* 249 (1974) 34-36.

About the Authors:

Idowu Adeogun[1], Olaniyi Yusuff[1], Kehinde Okonjo[2*]: *[1]Department of Chemistry, University of Ibadan, Ibadan, NIGERIA,*
[2]Department of Chemistry, Covenant University, Ota, NIGERIA.*
** Corresponding author: Kehinde.okonjo@covenantuniversity.edu.ng*