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Tertiary Level r ↔t isomerization significantly raises the pK_a of CysF9[93]β linked Ionizable groups of Straw-coloured Fruit Bat Hemoglobin: Variation of Apparent Forward Second-order Rate Constant with pH.

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Abstract
The pH dependence of the apparent forward second-order rate constant for the reaction of CysF9[93]β sulfhydryl group of oxy-, carbonmonoxy- and aquomethemoglobin of straw-coloured fruit bat (SCFB) hemoglobin with 5,5'-dithiobis-(2-nitrobenzoate) were determined. The profiles of the pH dependence were complex, indicating that the reaction of DTNB with CysF9[93]β of SCFB hemoglobin is coupled to some ionizable groups. Quantitative analyses of the data show that the pK_a of the first and the second ionizable groups in the (r) conformation are 6.29±0.17 and 6.50±0.21 respectively. The pK_a of the first ionizable group was raised from 6.29±0.17 in the (r) conformation to 8.17±0.29 in the (t) tertiary conformation. That of the second ionizable group was raised from 6.50±0.21 to 8.11±0.07 as a result of r ↔ t isomerization. The first ionizable group was assigned to HisH21[143]β while the second ionizable group was assigned to ValNA1[1]β. The pK_a of the sulfhydryl group was 8.02±0.15. K_{rt3} the equilibrium constant for the isomerization when all groups are ionized is 0.011±0.001. The combination of very low K_{rt3} of r ↔ t isomerization, and the increase in pK_a of between 1.61 and 1.88 unit arising from r ↔ t isomerization were discussed in view of their role in the functional adaptation of the bat hemoglobin.

Keywords: *Hemoglobin, sulfhydryl group, ionizable groups, r ↔ t isomerization*

Introduction

It has previously been demonstrated that in solution, liganded hemoglobin CysF9[93]β sulfhydryl group exist in two isomeric conformations (r) and (t) which are in dynamic equilibrium [1, 2]. These are different from the R and T structures arising from salt bridge formation in the quaternary structure. The proportion of each conformation arising from tertiary structures can be determined from quantitative analysis of the profile of the reactivity of CysF9[93]β with 5,5'-dithiobis-(2-nitrobenzoate) (DTNB) with hemoglobin. The state of ligation determines the relative proportions of R and T quaternary structures; T quaternary structure is favored in deoxyhemoglobin whereas the R structures is favored in oxyhemoglobin.

Previously, complex profiles have been reported for the reaction of DTNB with rabbit hemoglobin [3]. These complex profiles transform to simple ones resembling the titration curve of either mono or diprotic acid, in the presence of saturating amount of inositol hexakisphosphate or at high ionic strength (200 mmol. dm^{-3} NaCl) depending on the ligand bound [3]. The complex profiles obtained at 50 mmol. dm^{-3} were attributed to ValNA1[1]β, HisNA2[2]β and HisH21[143]β, the ionizable groups electrostatically linked to CysF9[93]β sulfhydryl group. Analysis of the amino acid sequences of straw colored fruit bat (SCFB) hemoglobin shows that these ionizable groups are also present in SCFB hemoglobin. The profile for the dependence of forward apparent second-order rate constant k_f, of the reaction of DTNB with CysF9[93]β of SCFB hemoglobin should therefore exhibit a complex profile similar to those reported in human and rabbit hemoglobin [2]. Analysis of such complex profile should provide important information of the affinity of SCFB hemoglobin for oxygen. Functional implications of the affinity can then be explained. Based on previous finding [3], rabbit hemoglobin reacts with DTNB over three times faster than that of human hemoglobin at an ionic strength of 50 mmol. dm^{-3}. The higher reactivity of rabbit CysF9[93]β hemoglobin sulfhydryl group with DTNB was attributed to the substitution of neutral Threonine at F3[87]β position of human hemoglobin by Lysine in rabbit hemoglobin. 3 D structure of human oxyhemoglobin shows that position F3[87]β is only 0.5 nm away from CysF9[93]β position.

The amino acid sequences of SCFB hemoglobin has not been previously reported, but many of the previously studied bat species possess lysine at F3[87]β position [4 – 7]. SCFB hemoglobin therefore provides a good opportunity to test the proposition that substitution of neutral group by positively charged group results in increased reactivity of CysF9[93]β sulfhydryl group with DTNB.
Previous findings [8] have shown that reaction of DTNB with CysF9[93]β of hemoglobins is reversible. The k_f for the reaction of DTNB with CysF9[93]β were therefore determined from the slope of the dependence of pseudo first-order rate constant k_{obs} on DTNB concentration, using at least 20 fold excess concentration of DTNB over the hemoglobin. Three derivatives; oxy, carbonmonoxy and aquomet derivatives of SCFB hemoglobins were studied.

Materials and Methods

Hemoglobin Preparation

SCFB (*Eidolon helvum*) blood was obtained from the bat colony in Obafemi Awolowo University Campus, Ile-Ife, Nigeria. This was collected into freshly prepared acid-citrate-dextrose anticoagulant.The hemoglobin was prepared according to the method described [3, 9].The blood sample was centrifuged at 8,000 r.p.m for 20 minutes at 5^oC.The supernatant was sucked off and discarded. Instead of washing with 9.5 g dm^{-3} NaCl isotonic solution, 11.5 g dm^{-3} NaCl was found to be more appropriate because some the red blood cells lysed in 9.5 g dm^{-3} NaCl isotonic solution. The blood cells were washed three times with 11.5 g dm^{-3} saline solution, centrifuging after each washing at 8,000 r.p.m for 15 minutes at 5^oC and then removing the supernatant. The cells were lysed with ice cold distilled water and the mixture centrifuged at 8,000 r.p.m for 20 minutes. The oxyhemoglobin was then decanted from the cake of cell debris. 5% weight by volume of NaCl was then added to the hemoglobin and left for 20 minutes to allow for the non-heme protein to precipitate. The hemoglobin was thereafter centrifuged at 15,000 r.p.m for 20 minutes and then dialyzed at about 5 ^oC for 3 hours against 10 $mmol.dm^{-3}$ phosphate buffer pH 6.5 - 7.5 in an ice bath. The dialysis was repeated two more times using fresh dialysis solution. The oxyhemoglobin prepared was converted to carbonmonoxyhemoglobin and stored in ice bath under CO atmosphere. When needed, the oxyhemoglobin was made from carbonmonoxyhemoglobin by photolysis. Aquomethemglobin was made by oxidation of oxyhemoglobin with 2-fold molar excess of $K_3Fe(CN)_6$. Excess $K_3Fe(CN)_6$ was removed by passage of the derivatized hemoglobin through Dintiz ion exchange column [10]. Prior to use, each derivative of the hemoglobin was deionized by passage through a Dintzis ion exchange column and the concentration of the stock solution was determined. Carbonmonoxyhemoglobin concentration was determined at 538 nm, assuming an absorption coefficient of 14,000 mol^{-1} (heme) dm^3 cm^{-1}. Concentration of aquomethemoglobin was determined at 540 nm using an absorption coefficient of 10900 mol^{-1} (heme) dm^3 cm^{-1}.

Kinetics

The kinetics of the reaction of DTNB with SCFB hemoglobin sulfhydryl groups were monitored under pseudo first-order conditions at 412 nm using a Shimadzu 1800 UV-Vis Spectrophotometer at 30^oC. This was achieved by reacting each of the hemoglobin samples with at least 20-fold excess concentration of DTNB over the reactive sulfhydryl group concentration. Previous studies have shown that SCFB hemoglobin has two reactive sulfhydryl group per hemoglobin tetramer (Fodeke and Oyedare 2016, in press). These two reactive sulfhydryl group were assigned to F9[93]β positions.

10 µmol dm^{-3} heme (5 µmol dm^{-3} in reactive sulfhydryl groups) solution of a hemoglobin derivative in a chosen buffer (phosphate buffers pH 5.6-7.8 or borate buffers pH 8.0-9.0, each at ionic strength 50 mmol dm^{-3}) was allowed to equilibrate at 30^oC in a thermostated water bath, Grant Instrument. 3 cm^3 aliquots of the hemoglobin solution were separately transferred to two 1 cm x 1 cm cuvette. The cuvettes were placed in the reference and the sample compartments of the uv-visible spectrophotometer thermostated at 30 ^oC. A few micro liters of 50 mmol dm^{-3} DTNB in 95% ethanol was placed on a glass rod, one end of which had been shaped in the form of a shallow spoon. The reaction was initiated by mixing the DTNB with the hemoglobin in the sample cuvette; the glass rod, serving as the stirrer. The final concentration of the DTNB in the reaction mixture was between 100 µmol dm^{-3} and 300 µmol dm^{-3}. To avoid a situation in which the approximations made in the rate law breaks down, the reaction was allowed to proceed to very near or to equilibrium. This procedure was repeated at least three times with different concentrations of DTNB. Each experiment was repeated at least twice for any particular concentration of DTNB. The kinetics traces (transmittance as a function of time) were then tracked using an online computer which was connected to the uv-vis spectrophotometer to obtain the value of the transmittance at the corresponding time of the reaction [4, 7, 8].

The transmittance at the end of the reaction, the so-called infinity reading (T_{eq}) and the pseudo-first order rate constant, k_{obs} were calculated from the fit of the kinetics traces using a written Matlab program of single exponential decay. (4, 7, 8). Standard error in the pseudo-first order rate constant was about 5 %.

Data analyses

More recent studies show that the reaction of DTNB with some animal hemoglobins is reversible [8,9, 13]. We therefore assumed that the reaction of DTNB with bat hemoglobin might be reversible and that DTNB reacts only with the anion form of a sulfhydryl group [14–16].

The reaction steps previously formulated are used as follows:

$$PSH + DTNB \underset{}{\overset{K_{SH}}{\rightleftharpoons}} H^+ + PS^- + DTNB \quad \text{(i)}$$

$$H^+ + PS^- + DTNB \underset{k_r}{\overset{k_f}{\rightleftharpoons}} H^+ + PS.ST + TNB^- \overset{K_{TNB}}{\rightleftharpoons} PS.ST + TNBH \quad \text{(ii)} \qquad (1)$$

In step (i) of equation 1, PSH is hemoglobin with the CysF9[93]β sulfhydryl in its protonated (unreactive) form, the reactive thiolate anion form of PSH is PS^-; in step (ii) PS.ST is the mixed disulfide formed after the reaction of hemoglobin with DTNB; TNB^- is 5-thio-2-nitrobenzoate, the anionic, chromophoric product of the reaction; TNBH is the protonated form of TNB^-; k_f and k_r are the apparent second-order rate constants for the forward and reverse reactions respectively. In what follows, it is assumed that the protolytic steps in the above equation are several order of magnitude faster than the forward and reverse reaction steps [17, 18].

Let 'a' depict the total concentration of the sulfhydryl groups (DTNB-reactive), b the total DTNB concentration, and x the concentration of TNB produced at time t. The rate of the reaction for the above equation is given by:

$$-\frac{d(a-x)}{dt} = k_f(a-x)(b-x) - k_r x^2 \qquad (2)$$

Before equilibrium,

$$-\frac{da}{dt} = k_f(a)(b) \qquad (3)$$

$$-\frac{d(a-x)}{dt} = k_f(ab - x(a+b) + x^2) - k_r x^2 \qquad (4)$$

$$-\frac{da}{dt} + \frac{dx}{dt} = k_f ab - k_f x(a+b) + k_f x^2 - k_r x^2 \qquad (5)$$

$$\frac{dx}{dt} = -k_f ab + k_f ab - k_f x(a+b) + k_f x^2 - k_r x^2 \qquad (6)$$

$$\frac{dx}{dt} = -k_f x(a+b) + k_f x^2\left(1 - \frac{k_r}{kf}\right) \qquad (7)$$

$K_{eq} = \frac{k_f}{k_r}$, then

$$\frac{dx}{dt} = -k_f x(a+b) + k_f x^2\left(1 - \frac{1}{K_{eq}}\right) \qquad (8)$$

Under pseudo-first order conditions, b>>a, therefore, Eq. (8) becomes;

$$\frac{dx}{dt} = -k_f bx + k_f x^2\left(1 - \frac{1}{K_{eq}}\right) \qquad (9)$$

$$\frac{dx}{dt} = -k_f\left(bx - x^2\left(1 - \frac{1}{K_{eq}}\right)\right) \qquad (10)$$

If $x^2\left(1 - \frac{1}{K_{eq}}\right)$ <<bx, equation above gives

$$\frac{dx}{dt} = -k_f bx \qquad (11)$$

Over a given time range

$$-\ln x = k_f bt \qquad (12)$$

In terms of transmittance changes, equation (12) becomes

$$-\ln(T - T_{eq}) = k_f bt \quad (13)$$

or using the single exponential decay curve

$$T = T_{eq} + \exp-(k_f.b.t) \quad (14)$$

Where; T_{eq} is the transmittance at equilibrium, T is the transmittance at any time t and k_{obs} (k_f.b) is the product of k_f and the concentration of DTNB. The slope of the plot of $-\ln(T - T_{eq})$ against time using Eq. (13) corresponds to the best fit value of k_{obs} in the single exponential decay curve described by the data using Eq. (14).

The forward second-order rate constant k_f, at each pH of the reaction can therefore be obtained from the slope of the dependence of k_{obs} on concentration of DTNB.

Results

Validity of k_{obs} data

An important aspect of the work is to determine the pH range of the reaction over which determination of k_f using the method reported here is valid. Figs. 1A - C present the semi-logarithmic plot of transmittance against time for the reaction of DTNB with carbonmonoxyhemoglobin at pHs 6.0, 7.2 and 8.6 respectively. It is seen that within experimental errors, Figs.1A and 1B are linear and that Fig. 1C is non-linear. Plots similar to those reported in Figs. 1A and 1B were obtained in the range $5.6 \leq pH \leq 8.4$ for all the hemoglobin derivatives. Above pH 8.4 the semi-logarithmic plot of the experimental data was non-linear. Similar results were obtained for the other two derivatives (oxy- and aquomethemoglobin) at high pH. This shows that above pH 8.4, use of Eq. (13) for analyzing the experimental data is not valid. The experimental data for dependence of k_f on pH for the reaction of DTNB with SCFB hemoglobin sulfhydryl group were therefore limited to the range $5.6 \leq pH \leq 8.4$.

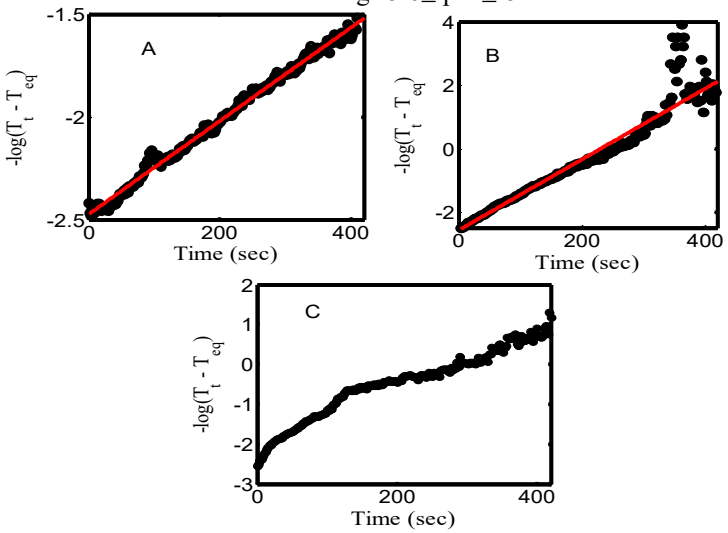


Fig. 1 Semi-logarithmic plots of the time courses of the reaction of DTNB with carbonmonoxyhemoglobin of SCFB at 30 °C: (A) pH 6.0 phosphate buffer, ionic strength 50 mmol dm^{-3} NaCl; (B) pH 7.2 phosphate buffer, ionic strength 50 mmol dm^{-3} NaCl; (C) pH 8.6 borate buffer, ionic strength 50 mmol dm^{-3} NaCl. Conditions: 5 µmol. dm^{-3} in reactive sulfhydryl group; 100 µmol. dm^{-3} DTNB and wavelength 412 nm.

Forward second-order rate constant k_f

Fig. 2 presents the dependence of k_{obs} on concentration of DTNB for the reaction of SCFB oxyhemoglobin with DTNB at pH 7.0. Similar plots were obtained in the range $5.6 \leq pH \leq 8.4$ for all the derivatives. For each plot the square of the correlation coefficient, R^2 was at least 0.90. The k_f values were obtained from the slope of the linear plots. It should be noted that each of the plot (not shown) has a non-zero intercept, further evidence that the reaction of DTNB with SCFB is reversible.

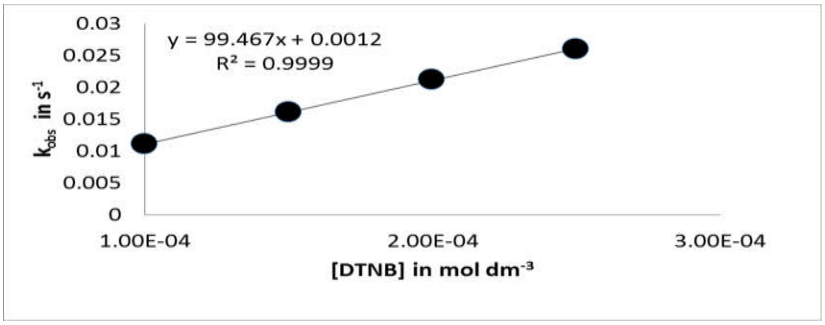
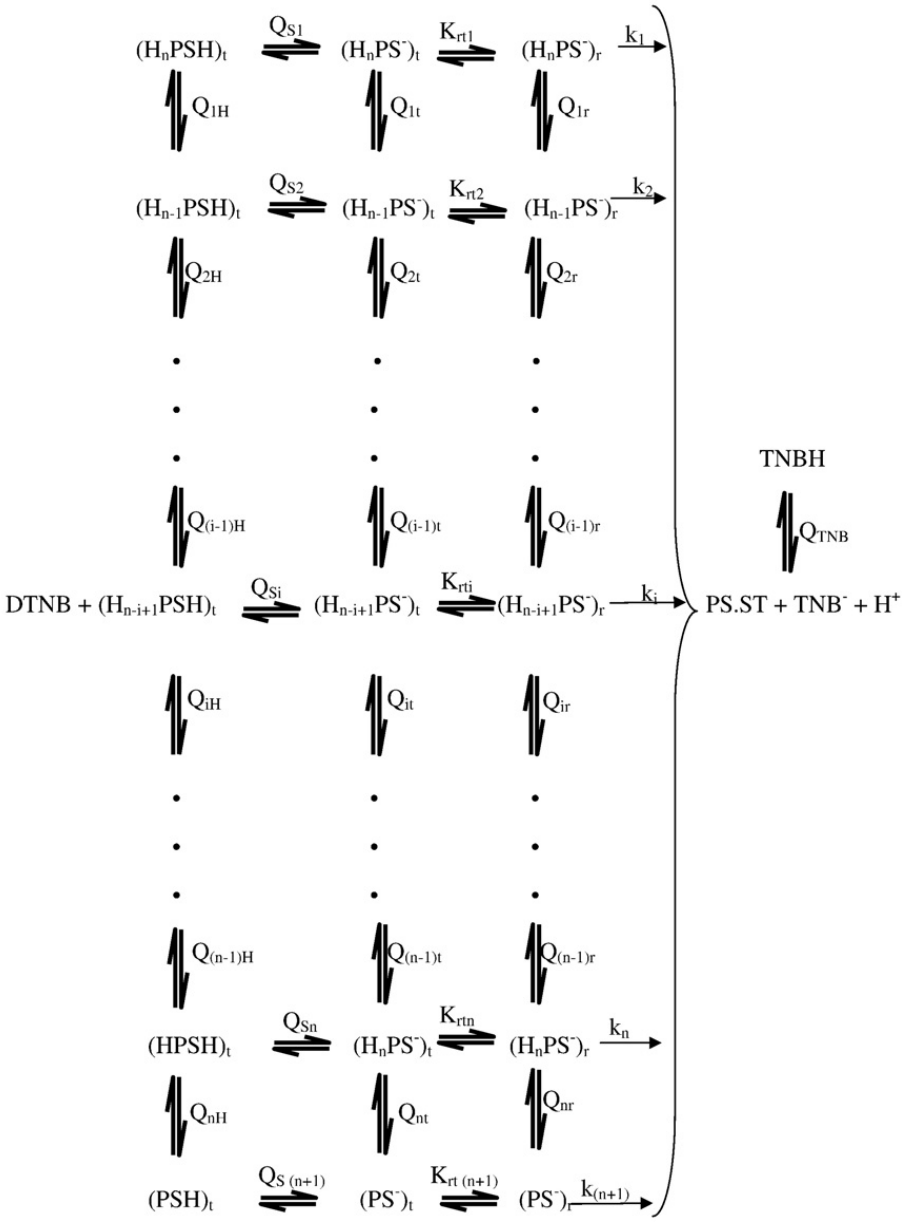


Fig 2: The Dependence of the pseudo-first order rate constant, k_{obs} on the DTNB concentration for the reaction of DTNB with the CysF9[93]β of SCFB oxyhemoglobin. Each data point is the mean of at least three replicate experiments at 30 °C.

Conditions: Phosphate buffer pH 7.0 (ionic strength 50 mmol dm^{-3} NaCl); hemoglobin concentration, 5µmol dm^{-3} in reacting sulfhydrylgroups; wavelength = 412 nm.

Analysis of pH dependence profile of k_f

The CysF9[93]β sulfhydryl group exists as a mixture of two tertiary conformations, r (cis to amino) and t (cis to carbonyl) in dynamic equilibrium [1, 2], on the assumption that DTNB preferentially reacts with the r isomer, scheme 1 (shown below) was proposed. The scheme has previously been used to account for complex profiles of the reaction of DTNB with sheep hemoglobin [13]. In this scheme, n ionizable groups electrostatically linked to the CysF9[93]β site are; $(H_nPSH)_t$, $(H_nPSH)_r$, $(H_{n-1}PSH)_t$, $(H_{n-1}PSH)_r$,. . . , $(H_{n-i+1}PSH)_t$, $(H_{n-i+1}PSH)_r$,. . . , $(HPSH)_t$, $(HPSH)_r$, and $(PSH)_t$, $(PSH)_r$ each of these species has its thiol group protonated. $(H_nPS^-)_t$, $(H_nPS^-)_r$, $(H_{n-1}PS^-)_t$, $(H_{n-1}PS^-)_r$, . . . , $(H_{n-i+1}PS^-)_t$, $(H_{n-i+1}PS^-)_r$, . . . , $(HPS^-)_t$, $(HPS^-)_r$, $(PS^-)_t$ and $(PS^-)_r$ are the corresponding thiolate anion forms of the various species having n, (n-1), . . . , (n-i+1), . . . , 1 and 0 protons bound to the electrostatically thiol-linked ionizable groups in r and t conformations. The various Q terms signify ionization constants K_a of the various ionizable species linked to the reaction of the DTNB with CysF9[93]β. $pQ_{ir/t}$ are the corresponding $pK_{air/t}$ (where i = 1 – (n+1)). This scheme is an extended form of reaction steps i and ii (Eq. (1)), in which the ionization of groups on the various hemoglobin derivatives are taken into consideration. We therefore propose to use the scheme to analyze the results.



Scheme 1

The relationship between k_f and the parameters in Scheme 1 is given by Eq. (15)

$$k_f = \frac{k_{n+1} + \sum_{i=1}^{n} k_i \left(H^+\right)^{n-i+1} \left(\prod_{j=i}^{n} Q_{jr}\right)^{-1}}{\left\{1 + \sum_{i=1}^{n} \left(H^+\right)^{n-i+1} \left(\prod_{j=i}^{n} Q_{jr}\right)^{-1} + K_{rt(n+1)} \left\langle 1 + \sum_{i=1}^{n} \left(H^+\right)^{n-i+1} \left(\prod_{j=i}^{n} Q_{jt}\right)^{-1} + \frac{\left(H^+\right)}{Q_{s(n+1)}} \left[1 + \sum_{i=1}^{n} \left(H^+\right)^{n-i+1} \left(\prod_{j=i}^{n} Q_{jH}\right)^{-1}\right] \right\rangle \right\}} \quad (15)$$

The dependence of k_f on pH for the reaction of bat hemoglobin with DTNB was analyzed using Eq. (15) with the aid of a program written on MatLab software. The experiments carried out in this study were restricted to pH values around 8.4, because above 8.4 the k_f values obtained are not reliable; increased hydrolysis of disulfide bonds results in serious complications [12, 20] leading to uncertainty in the integrity of DTNB above pH 8.4. At low pH (below pH 5.6), the hemoglobin is highly protonated and does not react with DTNB. At high pH however, it is ionized, resulting in the formation of thiolate anion which reacts with DTNB forming mixed disulfide bond in agreement with previous results [8, 16, 19, 20, 21]. Only the thiolate anion forms are reactive towards DTNB.

Data of k_f against pH

Fig. 3A, 3B and 3C reports the dependence of k_f on pH for the oxy-, carbonmonoxy and aquomet- derivatives of SCFB hemoglobin respectively. The curves through the experimental data points were fitted using Eq. (15) with n = 2 and fitting parameters reported in Table 1. It is obvious that the reaction of DTNB with all SCFB hemoglobin derivatives shows strong pH dependence. The profiles are also complex, like that of the reaction of DTNB with human hemoglobin [3,16]. The profiles are also bell-shaped like those previously reported for oxy- and carbonmonoxy-derivatives of major sheep hemoglobin, and minor sheep oxyheomoglobin [13]. It has previously been demonstrated that complex profile such as obtained, arises from electrostatic linkage of a number of ionizable groups to the CysF9[93]β site [3, 13, 16]. In human and sheep hemoglobins these groups are the organic phosphate binding groups; they are ValNA1[1]β, HisNA2[2]β, HisH21[143]β and LysEF6[82]β [20, 21]. However, only three of these groups; ValNA1[1]β, HisNA2[2]β, HisH21[143]β are ionizable in the range 5.6≤ pH ≤8.4. It is impossible for LysEF6[82]β with pK_a value in the range 10.5 to be ionized in the pH range of the experiment, therefore, n = 3 was considered. Attempt made to fit the data with n = 3 gave a fairly good fit but the fitting parameter were physically unreasonable. Assuming the possibility that HisNA2[2]β and HisH21[143]β might have closely similar pK_a values, the data was therefore analyzed with n = 2. The curve through the experimental data points and the fitting parameters were found to be very good. Poor fit were obtained when attempt was made to fit the data with n = 1.

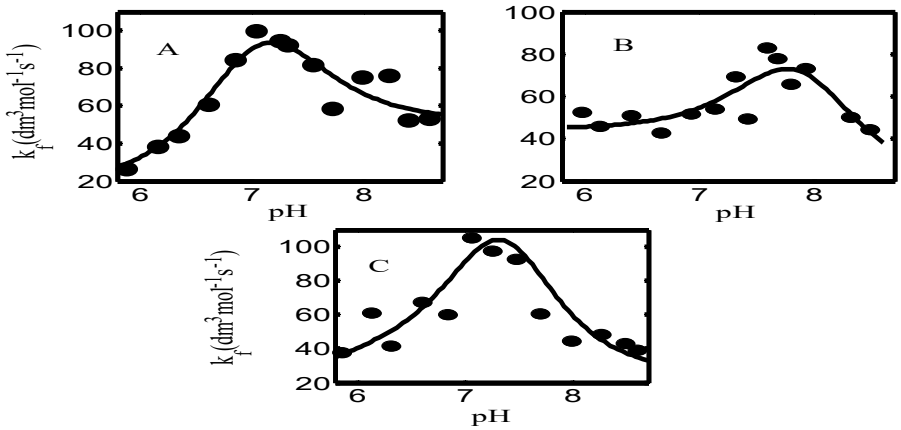


Fig. 3. Dependence of k_f on pH for the reaction of CysF9[93]β sufhydryl group of SCFB hemoglobin with DTNB at 30°C: (A) oxyhemoglobin; (B) carbonmonoxyhemoglobin; (C) aquomethemoglobin. The curve through the experimental data points are the best fit curves using Eq. (15) of the text with n = 2 together the fitting parameters reported in Table 1. Each point is the mean of three determinations subject to a standard error of about 20% Conditions: Phosphate buffers (pH 5.6 – 8.0); borate buffers (pH ≥8.0); hemoglobin concentration 10 µmol (heme) dm^{-3} (5 µmol dm^{-3} in reactive sulfhydryl groups). The DTNB concentration was between 100 and 300 µmol dm^{-3} final concentration. The total ionic strength of each buffer was adjusted to 50 mmol dm^{-3} with NaCl.

The profile of pH dependence of k_f for oxyhemoglobin (Fig 3A) exhibited a maximum k_f value (about 95 $dm^3 mol^{-1}. s^{-1}$) at about pH 7.2. Aquomethemoglobin (Fig. 3C) had a maximum k_f value at about the same pH as oxyhemoglobin, but the k_f maximum is somewhat higher (about 105 $dm^3 mol^{-1} s^{-1}$). This suggests that the coupling effect of the CysF9[93]β sulfhydryl to the ionizable groups of these two derivatives might be maximum at c.a. pH 7.2 and that the coupling effect might be stronger in aquomethemoglobin than in oxyheomoglobin. In carbonmonoxyhemoglobin however, the maximum k_f occur at a pH of about 7.7 (k_f value of about 85 $dm^3 mol^{-1} s^{-1}$). The reduction in the maximum value of k_f of carbonmonoxyhemoglobin compared to either oxyhemoglobin or aquomethemoglobin suggests that coupling between the ionizable groups and the CysF9[93]β position might be weakest in carbonmonoxyhemoglobin. It is noteworthy, that similar shift in the pH of maximum k_f towards higher pH in carbonmonoxyhemoglobin compared to the other two derivatives has been previously observed in sheep major hemoglobin [13]. This

suggests that extent of coupling might depend on the nature of the bound ligand. This finding is consistent with the observation that these three ligand behaves differently toward hemoglobin.

Table 1. Reaction of DTNB with hemoglobin: best-fit parameters used to fit k_f data in Fig.3 using scheme 1 and Eq. (15) of the text with n = 2

Parameters	Oxy	Carbonmonoxy	Aquomet	Mean
k_1	1182.84	1585.46	1957.27	1575.19 ± 316.24
k_2	943.72	489.14	807.19	746.68±190.45
k_3	54.39	37.08	26.19	39.22±11.61
pQ_{1r}	6.06	6.47	6.33	6.29±0.17
pQ_{2r}	6.21	6.61	6.68	6.50±0.21
pQ_{s3}	8.04	8.19	7.82	8.02±0.15
pQ_{1H}	-	-	6.78	6.78
pQ_{2H}	7.50	7.88	7.34	7.57±0.23
K_{rt3}	0.010	0.012	0.011	0.011±0.001
pQ_{1t}	7.83	8.14	8.53	8.17±0.29
pQ_{2t}	8.03	8.19	8.12	8.11±0.07

Discussion

The semi logarithmic plot of the experiment clearly shows that it will be illogical to carry out the kinetic experiment involving SCFB hemoglobin and DTNB above pH 8.4 since it becomes non-linear above pH 8.4. We therefore limited the pH range of the experiment to $5.6 \leq pH \leq 8.4$. The finding that the dependence of k_{obs} on concentration of DTNB all have a non-zero intercept at all pH conditions of the experiment and for all hemoglobin derivatives also shows that the reaction of DTNB with hemoglobin is reversible under the experimental conditions.

An important motivation for this work is to establish the role of tertiary r↔t isomerization in the regulation of Bohr effect and its possible consequence on oxygen binding to SCFB hemoglobin. It must be noted that in changing from the r tertiary isomer to t isomer the pK_a of the first ionizable group increased by a mean value of 1.88 (see Table 1) while the pK_a of the second ionizable group is increased by 1.61. In similar bell-shaped profiles obtained for the reaction of DTNB with major sheep oxy- and carbonmonoxyhemoglobin[13] the mean pK_a of the first ionizable group changes from 6.05 in the r isomer to 7.43 in the t isomer (an increase of 0.98 pK_a unit arising from r→t transition). The mean pK_a of the second ionizable group in sheep changes from 8.63 in the r isomer to 7.43 in the t isomer (corresponding to 1.20 unit reduction in pK_a). It should be noted that the maximum k_f for SCFB hemoglobin is about twice the maximum k_f value reported for sheep hemoglobin [13]. This suggests that the ionizable group of SCFB hemoglobin might be twice as responsive/sensitive to events at F9[93]β position as that of sheep hemoglobin. The low reactivity of DTNB with carbonmonoxyhemoglobin compared to the other two derivatives may be the consequence of previous finding that the affinity of carbonmonoxide for hemoglobin is about 210 folds that of oxygen hemoglobin. Higher reactivity observed in aquomet hemoglobin compared to oxy- and carbonmonoxyhemoglobin might be due to the increased charge on the heme iron (i.e iron (III) compared to iron (II) on oxy- and carbonmonoxy derivatieves) on the hemoglobin molecule. The presence of additional positive charge is expected to increase the reactivity of negatively charged DTNB with CysF9[93]β sufhydryl group of the hemoglobin. The marginal increase of the rate of aquomethemoglobin reaction with DTNB compared with oxyhemoglobin might be indicative of the proximity of the heme iron on which additional charge is conferred to the F9[93]β reaction site.

It is remarkable that the K_{rt3} the equilibrium constant of transition of SCFB is about two orders of magnitude less than the mean of what was reported for major sheep oxy and carbonmonoxyhemoglobin, (see columns 1 and 2 of Table 1 of reference 13). This is the first time such a low value of K_{rt3}, the equilibrium constant for the r↔t isomerization of sulfhydryl group at high pH when ionization is complete, is being reported. The closest to what is being reported in this work was obtained from equilibrium studies of human hemoglobin. For human hemoglobinmean K_{rt3} value of 0.05 was reported [8].

Significantly higher pK_a of the ionizable groups in the t isomer compared to r isomer

It is remarkable that the pK_as of ionization of the two ionizable groups whose ionization are linked to the reaction of DTNB with SCFB hemoglobin in the t isomer, are almost 2 pK_a units higher than the pK_a of the corresponding r isomer. This should ensure high capacity of the hemoglobin for oxygen loading as the hemoglobin releases proton in in the isomerization process leading to formation of t isomer, to take oxygen in the lung where the pH is relatively higher. This should enhance positive Bohr effect, in which case, higher concentration of proton can be released for efficient uptake of oxygen accompanied by formation of t isomer with higher pK_a. The implications of this, is that high affinity of the hemoglobin for oxygen at high pH. This is made available for release to the organs where metabolic activities requiring oxygen is carried out at low pH. It is therefore expected that this might be an important adaptation strategy for SCFB at high altitude where oxygen density is lower.

Low K_{rt3}

The K_{rt3} value of 0.011 obtained for the stripped SCFB hemoglobin at 50 mmol dm^{-3} ionic strength showed that at high pH when all the ionizable groups are completely ionized only about 1.09% of the t tertiary isomer are present in solution. This indicates that at high pH, protons are readily taken by the hemoglobin to favor the r conformation. This results in negative Bohr effect (accompanied by oxygen release) to ensure reverse isomerization process leading to formation of r isomer from the t isomer. This ensures that the r isomer with lower pK_a of ionization is formed in a process leading to proton uptake and also ensuring efficient release of oxygen to the cells for metabolic activities.

Conclusion

It was demonstrated using the reaction of DTNB with SCFB hemoglobin that the significant increase in the pK_a of ionization of the two ionizable groups as a result of transformation from r to t isomer enhances oxygen loading at high altitude by SCFB hemoglobin. And that the low value of equilibrium constant of isomerization K_{rt3} is important for the efficient release of hemoglobin bound oxygen for metabolic activities in the bat specie. It would however be necessary to work with other species of bat for a valid generalization to be made.

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