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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

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Abstract

The equilibrium constant of the reaction of 5,5′-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulfhydryl group of hemoglobin *decreases* by 2 to 3 orders of magnitude between pH 5.6 and 9. The reaction is coupled to the ionizations of two groups on the protein. At 25 °C one group has a pK_a of 5.31±0.2 when hemoglobin is in its (tertiary) ***r*** conformation, typified by the thiolate anion form of CysF9[93]β; this changes to 7.73±0.4 in the (tertiary) ***t*** conformation, typified by the mixed disulfide form of the sulfhydryl. The second group ionizes with a pK_a of 7.11±0.4 in the ***r*** conformation; this changes to 8.38±0.2 in the ***t*** conformation. K_{rt}, the equilibrium constant for the ***r*** ⟵⟶ ***t*** isomerization process, is 0.22±0.06. The standard enthalpy and entropy changes for the isomerization are ΔH^o_{rt}=24.2 kJ mol^{-1} and ΔS^o_{rt}=68.8 JK^{-1}mol^{-1}, respectively.

Keywords: Tertiary structure transition of hemoglobin; Equilibrium and thermodynamic parameters; Ellman's reagent; Sulfhydryl groups

1. Introduction

The CysF9[93]β sulfhydryl group of hemoglobin can exist in either of two conformations: *cis* to the main chain carbonyl group or *cis* to the main chain amino group [1]. In a temperature-jump study, we demonstrated that these two sulfhydryl conformations are coupled to a dynamic isomerization process in deoxy – as well as in carbonmonoxyhemoglobin, and that this isomerization process is not identical with the normal R⟵⟶T quaternary structure transition [2]. The ionizations of certain groups on the protein are also coupled to this isomerization process, as demonstrated by the variation of the reciprocal relaxation time with pH, observed by optical absorbance changes of pH indicators present in the system [2]. We shall refer to this isomerization process as ***r*** ⟵⟶ ***t***. This isomerization is completely abolished when the CysF9[93]β sulfhydryl group of hemoglobin is modified with iodoacetamide [2]. This happens because iodoacetamide fixes the sulfhydryl group in one of its conformations, the *cis*-to-carbonyl conformation, and thus abolishes the dynamic ***r*** ⟵⟶ ***t*** isomerization [2].

Iodoacetamide reacts with a sulfhydryl group *irreversibly*. If, instead of iodoacetamide, a reagent that reacts *reversibly* with CysF9[93]β is used, an equilibrium between the ***t*** and ***r*** isomers will be established as the sulfhydryl goes back and forth from its mixed disulfide form (isomer ***t***) to its thiol anion form (isomer ***r***). A reagent that reacts reversibly with the CysF9[93]β sulfhydryl group of hemoglobin is 5,5′-dithiobis(2-nitrobenzoate) — DTNB — as we have demonstrated kinetically [3] and in equilibrium studies [4].

K_{equ}, the equilibrium constant for the reaction of DTNB with CysF9[93]β of cat hemoglobins decreases by about four orders of magnitude as the pH increases from 5.6 to 9 [4]. This strongly indicates that the DTNB reaction is coupled to the ionizations of groups on the protein whose pK_as change as the sulfhydryl reacts reversibly with DTNB. No attempt was made previously to determine the nature and the number of these ionizable groups [4]. The emphasis then was to determine the equilibrium

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constant for the DTNB reaction to enable a determination of k_r, the apparent second order reverse rate constant, a parameter that cannot otherwise be determined for this system [4].

With the aim of (i) identifying the coupled ionizable groups in the ***r*** and ***t*** tertiary isomers, (ii) determining K_{rt}, the equilibrium constant of the ***r*** $\leftrightarrow$ ***t*** isomerization, as well as (iii) preparing the ground for determining k_r, we present here a report on an equilibrium study of the reaction of DTNB with the oxy, carbonmonoxy and aquomet derivatives of human, rabbit and bovine hemoglobins as a function of pH. Under the same conditions as were employed for the cat hemoglobins [4], we find that for human and rabbit hemoglobins K_{equ} *decreases* by about three orders of magnitude over the pH range 5.6 to 9; for bovine hemoglobin K_{equ} *decreases* by about two orders of magnitude over the same pH range. Quantitative analyses of the pH dependence profiles of $-\log_{10}K_{equ}$ indicate that the reaction of DTNB with CysF9[93]β is coupled to the ionizations of two groups on the hemoglobin molecule. The pK_a of ionization of each group *increases* when the sulfhydryl group is transformed from its thiolate anion form (***r*** isomer) to its mixed disulfide form (***t*** isomer) after reacting with DTNB. At 25 °C the pK_a of one group increases from 5.3±0.2 to 7.7±0.4; for the other group, the pK_a increases from 7.1±0.3 to 8.4±0.2. K_{rt}, the equilibrium constant for the ***r*** $\leftrightarrow$ ***t*** isomerization is 0.22±0.06. The standard enthalpy and entropy changes of the transition are ΔH^o_{rt}=24.2 kJ mol^{-1} and ΔS^o_{rt}=68.8 JK^{-1}mol^{-1}.

2. Experimental

2.1. Preparation of hemoglobin

Human blood from normal donors was obtained from the Blood Bank, University College Hospital, Ibadan. Rabbit and bovine blood were obtained from the Veterinary Teaching Hospital, University of Ibadan. Blood samples were collected in bottles containing freshly prepared acid–citrate–dextrose anti-coagulant. Hemoglobin was prepared by centrifuging blood samples at 20,000 r.p.m. for 20 min at 5 °C. The supernatant was sucked off and discarded. The red blood cells were washed three times with cold (5 °C) isotonic saline, each washing being followed by centrifugation (10,000 r.p.m. for 15 min) at 5 °C. The cells were lysed with ice-cold distilled water and the mixture centrifuged at 10,000 r.p.m. for 20 min. The oxyhemoglobin was decanted from beneath the cake of cell debris. To the hemoglobin was added NaCl (5 percent weight/volume) and the mixture was left at 5 °C for 20 min to allow time for non-heme proteins to precipitate. The hemoglobin was thereafter centrifuged at 20,000 r.p.m. for 20 min and dialyzed in a cold room (5 °C) for 3 h against a 10 mmol dm^{-3} NaCl solution, pH 6.5–7.5. The dialysis was carried out against two further changes of dialysis solution. The oxyhemoglobin so prepared was converted to carbonmonoxyhemoglobin and stored frozen under a CO atmosphere. The carbonmonoxyhemoglobin concentration was determined at 537.5 nm, assuming an absorption coefficient of 14,000 mol^{-1}(heme) dm^3 cm^{-1}. Oxyhemoglobin was made from carbonmonoxyhemoglobin by photolysis. Aquomethemoglobin was made from oxyhemoglobin by oxidation with a 2-fold molar excess of $K_3Fe(CN)_6$. Excess $K_3Fe(CN)_6$ was removed by passage through a Dintzis ion exchange column [5]. Prior to use for experiments, samples were deionized by passage through a Dintzis ion exchange column [5].

2.2. Determination of equilibrium constants

The methods employed in determining K_{equ}, the equilibrium constant of the DTNB reaction, have been described in detail

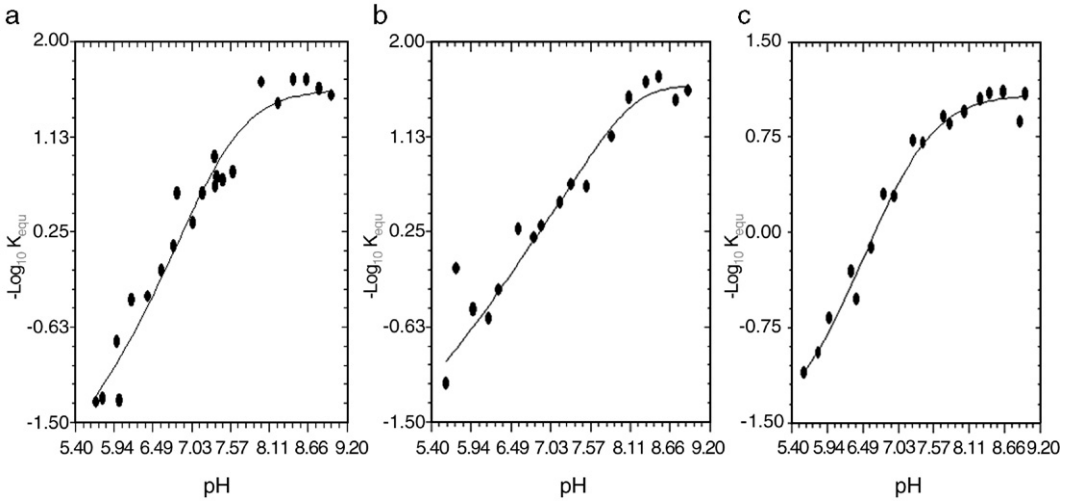


Fig. 1. Reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β sulfhydryl group of oxyhemoglobins: variation of $-\log_{10}K_{equ}$ with pH. (a) human; (b) rabbit; (c) bovine. Conditions: phosphate buffers, pH 5.6 to 8.0; borate buffers, pH 8.0 to 9.0; ionic strength, 50 mmol dm^{-3} (added salt, NaCl); hemoglobin concentration, 50 μmol (heme) dm^{-3} (25 μmol dm^{-3} in reacting sulfhydryl groups); volume of hemoglobin used, 10 cm^3; stock DTNB concentration, 29 mmol dm^{-3} in 0.2 mol dm^{-3} phosphate buffer, pH 6.8; volume of stock DTNB used, 2.5–32.5 mm^3; 25 °C. Each experimental point was calculated from the optical transmittance data (after conversion to absorbance), using Eq. (2) of the text. Each point is subject to a standard error of about 10%. The lines through the experimental points are the best-fit lines drawn with the parameters for oxyhemoglobin shown in Table 1, using Eq. (3) of the text.

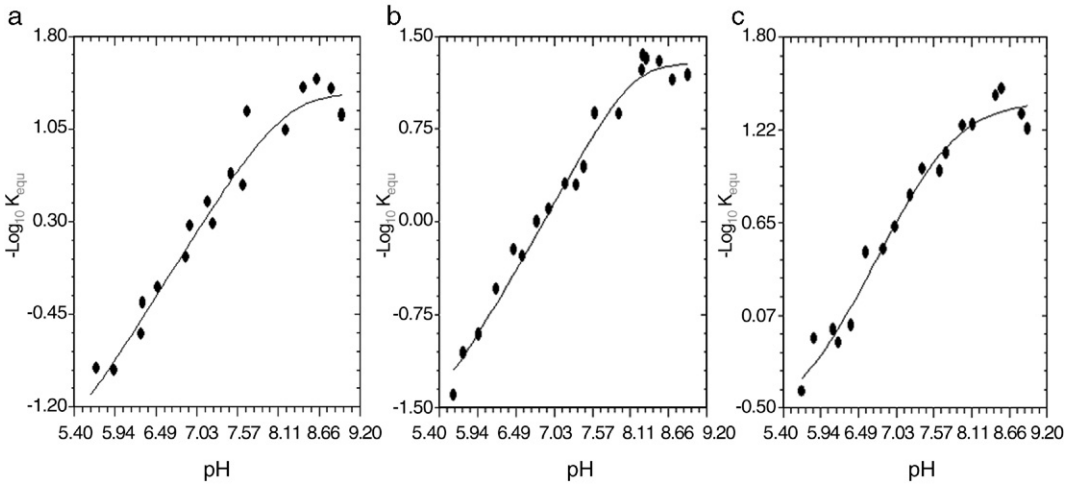


Fig. 2. Reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β sulfhydryl group of carbonmonoxyhemoglobins: Variation of $-\log_{10}K_{equ}$ with pH. (a) human; (b) rabbit; (c) bovine. Conditions as in Fig. 1. Each experimental point was calculated from the optical absorbance data, using Eq. (2) of the text, and is subject to a standard error of about 10%. The lines through the experimental points are the best-fit lines drawn with the parameters for carbonmonoxyhemoglobin shown in Table 1, using Eq. (3) of the text.

before [4]. The only difference between the previous and present determinations is that for the present report optical transmittance readings were taken on a Zeiss PMQ II UV–visible spectrophotometer and the transmittances were determined with 2 × 2 cm cuvettes. The cell compartment of the spectrophotometer was thermostated at 25 °C. The pH values of experimental solutions were determined on a Radiometer PHM 85 Research pH meter. Experiments were carried out in phosphate ($5.6 \le pH \le 8$) and borate ($pH \ge 8.0$) buffers, the ionic strengths of which were brought up to 50 mmol dm^{-3} with NaCl. Other conditions are reported in the legend of Fig. 1 (see above).

pH dependence profiles were fitted to theoretical models with computer programs written on a MicroMaths Scientist software (Salt Lake City, Utah).

3. Results

The reaction between CysF9[93]β of hemoglobin and DTNB may be depicted [4] as:

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

In Eq. (1) PSH is hemoglobin with the CysF9[93]β sulfhydryl in its protonated, unreacting (with DTNB) form; PS^- is the corresponding (reacting) anion form; PS.ST is the mixed disulfide formed after reaction with DTNB; TNB^- is 5-thio-2-nitrobenzoate, the anionic, chromophoric product of the

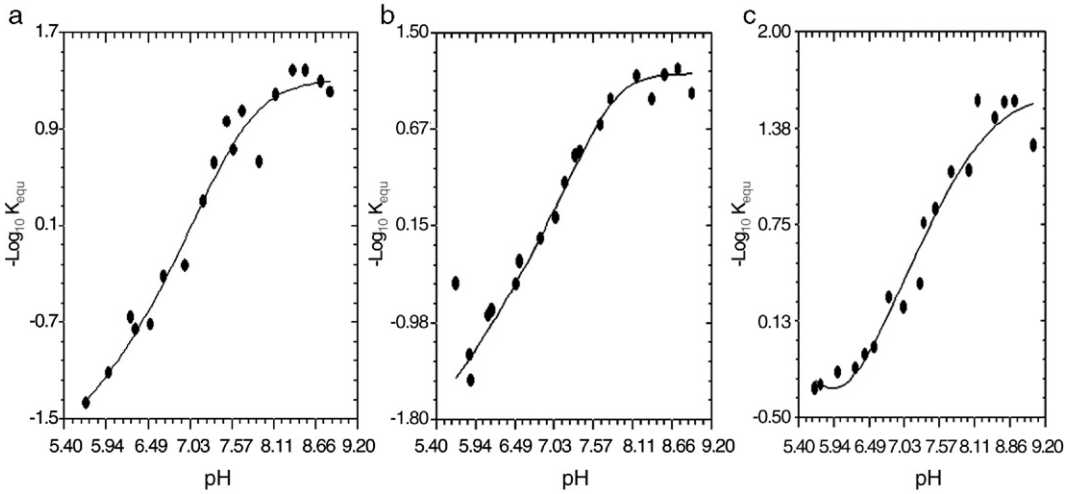
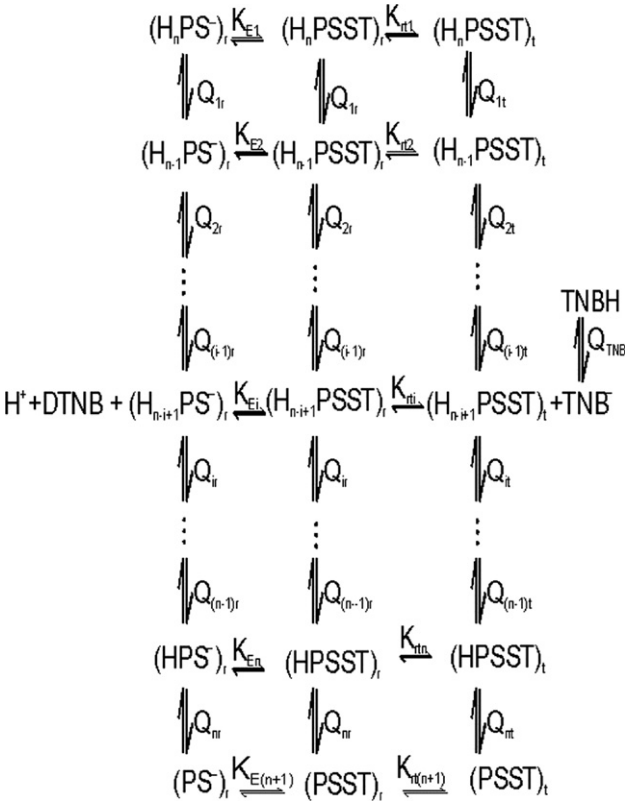


Fig. 3. Reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β sulfhydryl group of aquomethemoglobins: Variation of $-\log_{10}K_{equ}$ with pH. (a) human; (b) rabbit; (c) bovine. Conditions as in Fig. 1. Each experimental point was calculated from the optical absorbance data, using Eq. (2) of the text, and is subject to a standard error of about 10%. The lines through the experimental points are the best-fit lines drawn with the parameters for aquomethemoglobin shown in Table 1, using Eq. (3) of the text.



Scheme 1.

reaction; TNBH is the protonated form of TNB^-; Q_{SH} and Q_{TNB} are the ionization constants of CysF9[93]β and TNBH, respectively; K_{equ} is the equilibrium constant for the formation of the mixed disulfide (PS.ST), that is, the DTNB reaction step.

The equation relating K_{equ} to the parameters and species in Eq. (1) is:

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

A full derivation of Eq. (2) has been reported elsewhere [4]. In calculating K_{equ}, an absorption coefficient of 14,000 $mol^{-1}dm^3$ cm^{-1} at 412 nm was assumed for TNB^-. The standard error in the determination of K_{equ} was about 10%.

Fig. 1 reports the variation of $-\log_{10}K_{equ}$ with pH for human, rabbit and bovine oxyhemoglobins. It is seen that, for human and rabbit hemoglobins, $-\log_{10}K_{equ}$ increases by about three orders of magnitude between pH 5.6 and 9. For bovine hemoglobin, the increase is about two orders of magnitude over the same pH range. Similar results were obtained for the carbonmonoxy and aquomet derivates (Figs. 2 and 3).

The strong pH dependences reported for $-\log_{10}K_{equ}$ in Figs. 1–3 imply that the DTNB reaction (Eq. (1)) is coupled to the ionizations of groups on the hemoglobin molecule. In order to determine the nature and the number of these groups, we propose the following reaction scheme, Scheme 1. In Scheme 1 the H^+ ions produced in the various ionization steps have been omitted for clarity. Scheme 1 is based on the experimental finding that the CysF9[93]β sulfhydryl group exists in two conformations coupled to two isomeric forms (***r*** and ***t***) of hemoglobin in dynamic equilibrium [2]. The species $H_{n-i+1}PSH$ ($i=1, 2,\ldots, n$) are hemoglobin species in which the CysF9[93]β sulfhydryl group is in its protonated, unreacting (with DTNB) form. These species are therefore not shown in Scheme 1. $H_{n-i+1}PS^-$ ($i=1, 2,\ldots, n$) are the species in which the sulfhydryl is in its thiolate anion form, the form that reacts with DTNB [6–8]; and $H_{n-i+1}PS.ST$ ($i=1, 2,\ldots, n$) are the mixed disulfide species formed after the reaction of the sulfhydryl with DTNB. Species marked with subscripts r and t are those in which CysF9[93]β is in the ***r*** and ***t*** tertiary isomeric forms of hemoglobin, respectively. The various proton ionization constants are represented as Q_{ir} and Q_{it} ($i=1, 2,\ldots, n$) to differentiate them from the equilibrium constants K_{Ei} ($i=1, 2, \ldots, n+1$) for the reaction of DTNB. K_{rti} ($i=1, 2,\ldots,n+1$) are the pH-dependent equilibrium constants for the ***r*** $\longleftrightarrow$ ***t*** isomerization. It is readily demonstrated that the relationship between K_{equ} and the parameters of Scheme 1 is given by the equation:

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

We have employed Eq. (3) to fit the data in Figs. 1–3. The best fits to all the data were obtained with an n value of 2. The lines through the data points in Figs. 1–3 are the best-fit lines drawn with the parameters reported in Table 1. It is seen that the fits are quite good. The mean values of the best-fit parameters are $pQ_{1r}=5.3\pm0.2$; $pQ_{1t}=7.7\pm0.4$; $pQ_{2r}=7.1\pm0.3$; $pQ_{2t}=8.4\pm0.2$; $K_{E3}=0.038\pm0.01$; and $K_{rt3}=0.2\pm0.03$.

Table 1
Reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β sulfhydryl group of various hemoglobin derivatives: best-fit parameters to the data reported in Figs. 1 2 3

Hemoglobin derivative	pQ_{1r}	pQ_{1t}	pQ_{2r}	pQ_{2t}	K_{E3}	K_{rt3}
Human oxy	4.97	7.48	6.53	9.00	0.026	0.050
Rabbit oxy	4.98	8.92	8.04	8.00	0.019	0.30
Bovine oxy	5.36	6.82	6.33	8.83	0.079	0.050
Human carbonmonoxy	4.97	8.90	8.59	8.42	0.035	0.33
Rabbit carbonmonoxy	4.97	9.02	8.05	8.00	0.043	0.19
Bovine carbonmonoxy	5.68	6.56	6.63	8.50	0.033	0.20
Human aquomet	5.42	7.87	6.95	8.29	0.037	0.28
Rabbit aquomet	4.98	8.89	7.30	7.37	0.053	0.32
Bovine aquomet	6.48	5.13	5.53	8.99	0.021	0.21
Mean	5.31± 0.2	7.73± 0.4	7.11± 0.3	8.38± 0.2	0.038± 0.007	0.20± 0.03

Compare with Scheme 1 and Eq. (3) of the text for $n=2$. Conditions as in Fig. 1.

Table 2
Reaction of DTNB with derivatives of the major and minor cat hemoglobins: best-fit parameters to the data reported in Figs. 1 and 2 of [4]

Hemoglobin derivative	pQ_{1r}	pQ_{1t}	pQ_{2r}	pQ_{2t}	K_{E3}	K_{rt3}
Major oxy	5.04	7.14	5.27	8.68	0.00084	0.30
Minor oxy	4.99	6.24	4.99	8.76	0.00179	0.29
Major carbonmonoxy	6.70	8.09	5.53	8.48	0.00063	0.145
Minor carbonmonoxy	4.97	6.42	4.97	8.75	0.00175	0.24
Major aquomet	4.86	8.93	6.71	7.94	0.00066	0.29
Minor aquomet	5.04	8.88	4.87	7.03	0.00170	0.175
Mean	5.27± 0.3	7.62± 0.5	5.39± 0.3	8.27± 0.3	0.00123± 0.0002	0.24± 0.03

Compare with Scheme 1 and Eq. (3) of the text for $n=2$.

A close examination of Scheme 1 reveals that DTNB first reacts with CysF9[93]β in the hemoglobin **r** isomer. After the mixed disulfide has been formed, the hemoglobin molecule undergoes an **r**→**t** transition. This is in line with our previous finding [2]: that after reacting with CysF9[93]β, iodoacetamide transforms the **r** to the **t** isomer and fixes it irreversibly in the latter isomeric form. The difference in the present case, though, is that DTNB reacts *reversibly* with the sulfhydryl [3,4] and, therefore, the **r** isomer is in dynamic equilibrium with the **t** isomer, even though the sulfhydryl group has been modified. We had initially attempted to fit the data with another scheme: one in which the **r**→**t** transition first occurs in the thiol anion form of the sulfhydryl, followed by the reaction of DTNB with the sulfhydryl in the **t** isomer. The equation developed from such a scheme (the equivalent of Eq. (3)) gave very poor fits to all the data. The fits were particularly poor in the low pH range. These results indicate that DTNB does not react with CysF9 [93]β in the **t** isomer, only in the **r** isomer. This may be why sulfhydryl reagents react more slowly with deoxyhemoglobin, which favors the **t** isomer [1], than with liganded hemoglobin, which favors the **r** isomer.

We previously presented equilibrium data for the reaction of DTNB with the major and minor hemoglobins of the domestic cat (Figs. 1 and 2, respectively, of [4]). At that time we had not yet developed a method for quantitatively analyzing the pH dependence profiles of equilibrium data. In Table 2 we present the fitting parameters for the cat hemoglobin data [4], as analyzed with Eq. (3) for $n=2$, also the best-fit value of n for the cat hemoglobins. A remarkable difference is found between the mean values of K_{E3} in Tables 1 and 2: 0.038±0.007 and 0.0012±0.0002, respectively. These values show that at high pH, when all the linked ionizable groups are deprotonated, the hemoglobins reported in Table 1 (human, rabbit and bovine) react about 32 times more favorably with DTNB than the cat hemoglobins.

In order to determine the effect on the reaction of DTNB when the linked groups are protonated, we have to calculate the parameters K_{E1} and K_{E2}, the equilibrium constants for the reaction of DTNB with species H_2PS^- and HPS^- (compare Scheme 1, with $n=2$). Similarly, in order to determine the pH dependence of the equilibrium constant for the **r**→**t** tertiary structure transition, we have to determine K_{rt2} and K_{rt1}. To achieve these ends we must use the relationships:

$$-\log_{10}K_{rt2}-\log_{10}K_{E2} = -\log_{10}K_{E3}-pQ_{2t} + pQ_{2r}-\log_{10}K_{rt3} \quad (4)$$

$$-\log_{10}K_{rt1}-\log_{10}K_{E1} = -\log_{10}K_{E2}-pQ_{1t} + pQ_{1r}-\log_{10}K_{rt2} \quad (5)$$

All the parameters on the right hand side of Eq. (4) can be obtained from a fit of the experimental data to Eq. (3) with $n=2$. Unfortunately, it is not possible from knowledge of these parameters to determine K_{rt2} and K_{E2} independently of each other. For this reason we now make a simplifying assumption: either (i) K_{rt} for the tertiary structure transition is invariant with pH or (ii) K_E for DTNB binding is invariant with pH. The second option is clearly unreasonable, given the pH dependence of K_{equ} seen in Figs. 1–3. We therefore assume that K_{rt} is invariant with pH. Consequently, Eqs. (4) and (5), with $K_{rt1}=K_{rt2}=K_{rt3}=K_{rt}$, become

$$-\log_{10}K_{E2} = -\log_{10}K_{E3}-pQ_{2t} + pQ_{2r} \quad (6)$$

$$-\log_{10}K_{E1} = -\log_{10}K_{E3}-pQ_{2t} + pQ_{2r}-pQ_{1t} + pQ_{1r} \quad (7)$$

We have used Eqs. (6) and (7) to calculate K_{E1} and K_{E2}. The values of these DTNB binding parameters so calculated are collected in Table 3. The parameters K_{E1}, K_{E2} and K_{E3} shown in Table 3 are the equilibrium constants for the reaction of DTNB with the species H_2PS^-, HPS^- and PS^-, respectively, to form the corresponding mixed disulfides (compare with Scheme 1, for $n=2$). It is clear from Table 3 that, with respect to DTNB binding, the greatest difference between the three hemoglobins of Table 1 and the cat hemoglobins is found in species PS^-, the species in which all the protons on the linked groups have been ionized off. As the linked groups become protonated, the differences between the non-feline and feline hemoglobins diminish.

As seen in Figs. 1–3, K_{equ} is reduced by about 2 to 3 orders of magnitude as the pH increases from 5.6 to 9. This is reflected in the values of K_{E1} and K_{E3}, the equilibrium constants for the

Table 3
Mean equilibrium parameters for the reaction of 5,5′-dithiobis(2-nitrobenzoate), DTNB, with the CysF9[93]β sulfhydryl groups of (i) cat hemoglobins; (ii) human, rabbit and bovine hemoglobins

Hemoglobin	K_{E1}	K_{E2}	K_{E3}
Major and minor cat	208.8	0.93	1.2×10^{-3}
Human, rabbit, bovine	186.1	0.71	3.8×10^{-2}
Reacting species	H_2PS^-	HPS^-	PS^-

Values of K_{E3} for human, rabbit and bovine hemoglobins were obtained from Table 1; K_{E3} values for the cat hemoglobins were obtained from Table 2. K_{E1} and K_{E2} values were obtained from K_{E3} values by calculation, using Eqs. (4) and (5) of the text.

reactions (with DTNB) of species H_2PS^-, the low pH species, and species PS^-, the high pH species: K_{E1} is greater than K_{E3} by about 4 orders of magnitude (see Table 3). In Figs. 1 and 2 of [4], K_{equ} is reduced by about 4 orders of magnitude over the same pH range, and K_{E1} is greater than K_{E3} by about 5 orders of magnitude (see Table 3).

A further remarkable difference between the data in Tables 1 and 2 is that the values of pQ_{2r} are quite different: 7.1±0.3 for the three hemoglobins and 5.4±0.3 for the cat hemoglobins. They obviously cannot arise from the same type of ionizable groups. Apart from the differences noted for K_{E3} and pQ_{r2}, the parameters obtained for the non-feline (Table 1) and feline hemoglobins (Table 2) appear to be the same.

4. Discussion

We embarked on a determination of the equilibrium constant for the DTNB reaction (Eq. (1)) for three reasons. The first is that it enables us to prepare the way for the determination of the apparent second order reverse rate constant, k_r, once the apparent second order forward rate constant has been determined. It is not feasible to directly determine k_r by initiating the reaction (Eq. (1)) from the product side [4]. A second and equally important reason — and the reason for doing a pH dependence study — is the desire to determine the nature and the number of those ionizable groups that are linked to the reactions of DTNB with CysF9[93]β in its *cis*-to-amino and *cis*-to-carbonyl conformations. For reasons to be stated below, these sulfhydryl conformations are associated with the **r** and **t** tertiary isomeric forms of hemoglobin, respectively. A third reason for determining the equilibrium constant for the DTNB reaction is that it gives us an opportunity to determine K_{rt}, the equilibrium constant for the **r**⟷**t** isomerization without necessarily doing a temperature-jump study.

4.1. Nature of the r⟷t isomerization process

We reported previously that in carbonmonoxyhemoglobin the isomerization, **r**⟷**t**, has an equilibrium constant k_t/k_r=0.136 at 11 °C (see Table 2 of [2]). This gives ΔG_{rt}=+4.71 kJ mol^{-1} as the free energy of the **r**→**t** transition at 11 °C. The corresponding values for deoxyhemoglobin are k_t/k_r=0.31 (see Table 1 of [2]) and ΔG_{rt}=+2.76 kJ mol^{-1}, respectively. These results show that in solution the **t** conformation is favored in deoxyhemoglobin compared to carbonmonoxyhemoglobin. (Indeed, in deoxyhemoglobin *crystals* the CysF9[93]β sulfhydryl is entirely in the **t** conformation (Fig. 10a of [1])). Since deoxyhemoglobin has the T quaternary structure and carbonmonoxyhemoglobin has the R quaternary structure, we have called the *cis*-to-carbonyl conformation **t** and the *cis*-to-amino conformation **r**. Calculations from the k_t/k_r values (Tables 1 and 2 of [2]) show that in carbonmonoxyhemoglobin the **r** isomer constitutes 88% of the total isomer population in solution at 11 °C. In deoxyhemoglobin in solution this is reduced to 76%.

It is remarkable that the equilibrium constant for the **r**⟷**t** isomerization process is the same for the non-feline and feline hemoglobins. From Tables 1 and 2, the mean value of K_{rt} is 0.22±0.06 at 25 °C. Calculation shows that the **r** isomer constitutes 82% of the isomer population at this temperature. Comparison with the value 88% calculated above for 11 °C indicates that the population of the **r** isomer decreases as the temperature increases. Substitution of the value 0.22 obtained for K_{rt} at 25 °C and the value 0.136 — obtained [2] for carbonmonoxyhemoglobin at 11 °C — into the van't Hoff equation gives ΔH°_{rt}=24.2 kJ mol^{-1} as the enthalpy change for the **r**→**t** transition. The entropy change for the **r**→**t** transition is ΔS°_{rt}=68.8 J K^{-1}mol^{-1}.

The rate of the **r**⟷**t** transition, k_t+k_r, is about 5×10^4 s^{-1} at 11 °C [2]. This is about an order of magnitude faster than the 11 °C estimate for the rate of the normal R⟷T quaternary transition obtained from the data of Sawicki and Gibson [9]. It is also about two orders of magnitude faster than the iron spin-state transition [10,11].

4.2. Nature of the ionizable groups in the r and t isomers

As can be seen in Table 1 (compare with Scheme 1 for n=2), there are two ionizable groups in the **r** isomer with pQ_{1r}=5.3±0.1 and pQ_{2r}=7.1±0.3. Table 1 also shows that there are two ionizable groups in the **t** isomer. The two groups have pQ_{1t}=7.7±0.4 and pQ_{2t}=8.4±0.2. Thus in the transition from the **r** to the **t** isomer the first ionizable group has its pQ increased from 5.3 to 7.7; and the second group has it pQ increased from 7.1 to 8.4. While such pK_a increases may arise from the formation of salt bridges, there is no structural evidence for such salt bridge formation on the transition from the **r** to the **t** isomer. On the other hand, there is considerable evidence for the specific binding of inorganic anions (Cl^- and phosphate) to hemoglobins [12–24]. Since deoxyhemoglobin binds these anions more tightly than liganded hemoglobin [17,21], it is reasonable to expect that the **t** isomer binds these anions more tightly than the **r** isomer. Consequently, the anion-binding ionizable groups should have a higher pQ in the **t** than in the **r** isomer, as we have observed (see Tables 1 and 2).

There is some uncertainty about the identity of the anion-binding sites in *liganded* hemoglobin. According to one view [17], they consist of imidazole groups of histidines only. Studies have excluded HisNA2[2]β and HisH21[143]β as possible binding sites [22,23]. According to another view [21], the anion-binding sites consist of amino groups only. In the latter view, ValNA1[1]β and LysEF6[82]β are Cl^- binding sites in *liganded* human and, presumably, rabbit hemoglobin. The corresponding sites in bovine hemoglobin are MetNA1[1]β and LysEF6[81]β [21].

The mean pQ_{1r} value of 5.31 (Table 1) suggests that imidazole groups of histidine residues form one set of Cl^- binding sites. The mean pQ_{2r} value of 7.11 (Table 1) suggests that terminal amino groups (ValNA1[1]β in human and rabbit hemoglobin; MetNA1[1]β in bovine hemoglobin) provide a second inorganic anion-binding site. While LysEF6[82]β in human hemoglobin (LysEF6[81]β in bovine hemoglobin) may be an anion-binding site, its pQ value, *ca* 10, would be too high for its effect to be observable in the pH range (5.6 to 9) of our experiments.

The cat hemoglobin data are not as easily interpreted as those of human, rabbit and bovine hemoglobins. In Table 2 it is seen that pQ_{1r} and pQ_{2r} are practically the same (*ca* 5.4). They must therefore belong to the same type of ionizable group. However, in the ***t*** isomer the two groups are in different environments and therefore have different p*Q*s.

5. Conclusion

When the equilibrium constant for the reaction of DTNB with the CysF9[93]β sulfhydryl group of hemoglobin is measured as a function of pH, it becomes a powerful tool for the determination of the ionization constants of groups linked to the reaction of DTNB with CysF9[93]β in the ***r*** and ***t*** hemoglobin tertiary isomers. It also enables the determination of K_{rt}, the equilibrium constant for the ***r*** → ***t*** transition.

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