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Reversible reaction of 5,5′-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulfhydryl groups of the hemoglobins of the domestic cat: Variation of the equilibrium and reverse rate constants with pH

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Abstract

We have determined for the first time the equilibrium constant, K_{eq}, for the reaction of Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoate), with the CysF9[93]β sulfhydryl groups of the hemoglobins of the domestic cat. In the pH range 5.6 to 9.0 K_{equ} varies over four orders of magnitude — between ca 10 and 10^{-3} — for all hemoglobin derivatives. Using these K_{equ} values and published data on the dependence of the apparent second order *forward* rate constant, k_f, on pH we have calculated the apparent second order *reverse* rate constant, k_r, as a function of pH. This parameter increases strongly with pH, particularly above pH 7.5. Quantitative analyses of the pH dependence profiles of $\log_{10}k_r$ indicate that the reverse reaction is coupled to the ionization of two groups on the protein with pK_as of 7.2±0.2 and 9.4±0.1 in the major hemoglobin and 6.7±0.3 and 8.4±0.1 in the minor hemoglobin.

Keywords: Cat hemoglobins; CysF9[93]β sulfhydryl group; 5,5′-dithiobis(2-nitrobenzoate); pH dependence of equilibrium constant; Reverse rate constant

1. Introduction

In a recent report [1] we presented kinetic evidence indicating that the reaction of 5,5′-dithiobis (2 nitrobenzoate) — DTNB — with the CysF9[93]β sulfhydryl groups of the hemoglobins of the domestic cat might be a reversible process. If this is so, then it should be possible to determine its equilibrium constant and, hence, the apparent second order reverse rate constant, a parameter that cannot be directly determined for this system.

DTNB is known to react only with the thiolate anion form of sulfhydryl groups, not with the neutral unionized form [2–4]. One of the products of the reaction, 5-thio-2-nitrobenzoate (TNB^-), is an anion and can react with a proton to establish an equilibrium with its protonated form. The whole series of reaction steps may be depicted as in Eq. (1):

$$\begin{aligned}\mathrm{HbSH} + \mathrm{DTNB} \overset{K_{SH}}{=\!=} \mathrm{H^+} + \mathrm{HbS^-} + \mathrm{DTNB} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{H^+} \\ + \mathrm{HbS\cdot TNB} + \mathrm{TNB^-} \overset{K_{TNB}}{=\!=} \mathrm{HbS\cdot TNB} + \mathrm{TNBH} \end{aligned} \quad (1)$$

In Eq. (1) HbSH is hemoglobin with the CysF9[93]β sulfhydryl in its protonated (unreacting) form; HbS^- is the corresponding (reacting) anion form; HbS·TNB 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_{SH}, K_{TNB} are equilibrium constants for the ionizations of CysF9[93]β and TNBH, respectively; k_f and k_r are the apparent second order rate constants for the forward and reverse of the DTNB reaction, respectively. The equilibrium constant of the reaction, K_{equ}, is given by the ratio of k_f and k_r, that is, $K_{equ} = \frac{k_f}{k_r}$.

The DTNB reaction step in Eq. (1), $\mathrm{HbS^-} + \mathrm{DTNB} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{HbS\cdot TNB} + \mathrm{TNB^-}$, is an example of two opposing second-order processes in which two products, HbS·TNB and TNB^-, result from the initial forward reaction. Under pseudo-first order conditions (with DTNB in excess), it is possible to determine the apparent second order *forward* rate constant, k_f, by plotting the observed pseudo-first order rate constant, k_{obs}, against the DTNB concentration [1]. It is typically difficult to directly determine the apparent *reverse* rate constant, k_r, for such a system unless the products of the forward reaction can both be

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isolated. Since HbS·TNB and TNB^- are produced in situ and cannot both be isolated, a direct determination of k_r by initiating the reaction from the right-hand-side is not feasible. However, if the forward rate constant, k_f, is known, the reverse rate constant, k_r, can be determined through a knowledge of the equilibrium constant, K_{equ}. Although our kinetic data [1] strongly indicate that the reaction of DTNB with the hemoglobins of the domestic cat is reversible, this can be confirmed only by attempting to carry out an equilibrium study.

We have undertaken a comprehensive investigation of the pH dependence of K_{equ} for the oxy, carbonmonoxy and aquomet derivatives of the major and minor hemoglobins of the domestic cat. We find that K_{equ} decreases considerably with increase in pH. Between pH 5.6 and 9, K_{equ} varies from ca 10 to ca 10^{-3}, a decrease of ca four orders of magnitude. Values of k_r calculated from k_f and K_{equ} increase by about 3 to 4 orders of magnitude between pH 5.6 and 9. Theoretical analyses of the pH dependence profiles of $\log_{10}k_r$ indicate that the reverse process is coupled to the ionizations of two groups with pK_as of 7.2±0.2 and 9.4±0.1 in the major hemoglobin and 6.7±0.3 and 8.4±0.1 in the minor hemoglobin.

2. Experimental

The methods employed for preparing the cat hemolysate and for separating the major and minor hemoglobin components have been reported elsewhere [1]. Prior to its use for an experiment, each hemoglobin component was passed through a Dintzis ion exchange column [5] to remove endogenous organic phosphates and other undesired ions.

2.1. Preparation of stock DTNB solution for equilibrium studies

The stock DTNB solution employed for the equilibrium studies was prepared as follows: 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 constant determinations.

2.2. Determination of pK_{TNB}

As can be seen in Eqs. (1) and (13) (see below) the equilibrium between TNB^- and its protonated form, $H^+ + TNB^- \leftrightarrow TNBH$, is important in any consideration of the reaction of DTNB with hemoglobin sulfhydryl groups. In particular, knowledge of K_{TNB}, the dissociation constant of TNBH, is required for the calculation of K_{equ}. We determined K_{TNB} using citric acid buffers between pH 2.2 and 5.6 [6], phosphate buffers between pH 5.6 and 8.0, and borate buffers between pH 8.0 and 10. A stock solution of TNB^- was prepared by mixing 1 cm^3 of a 50 μmol dm^{-3} solution of DTNB in buffer with 18 mm^3 of mercaptoethanol. The solution became intensely yellow, indicating the breakdown of DTNB to TNB^-. (The absorbance of a diluted sample of this solution was checked to ensure that there was complete conversion of DTNB to TNB^-). A 1 mm^3 aliquot of this stock solution was added to 10 cm^3 of buffer of known pH. This procedure was repeated for several buffers with pH values in the range $2.2 \le pH \le 10$. After being shaken to ensure good mixing, the tubes containing these TNB^- solutions were placed in a thermostat bath at 25 °C for 1 h to allow equilibrium to be attained. The absorbance of the solution in each tube was determined at 412 nm; the pH of the TNB^- solution in each tube was also determined. pK_{TNB} was calculated for the contents of each tube using the Henderson–Hasselbalch equation.

2.3. Determination of equilibrium constants for the reaction of DTNB with CysF9[93]β

3 cm^3 aliquots of hemoglobin (50 μmol heme dm^{-3}, that is, 25 μmol dm^{-3} in DTNB-reactive sulfhydryl groups) in a buffer at a given pH were measured into several clean, dry tubes. Increasing volumes (2.5 to 35 mm^3) of the stock 29 mmol dm^{-3} DTNB solution were added to the different tubes. The mixtures were stirred and left to equilibrate in a thermostat at 25 °C for about 6 h. The absorbance of each mixture was determined at 412 nm on a Pharmacia Biotech Nova Spec II visible spectrophotometer. Each absorbance was corrected for dilution. The absorbance of the hemoglobin solution to which no DTNB had been added was then subtracted from the absorbance read from each tube. For each tube the concentration of 5-thio-2-nitrobenzoate (TNB^-), the product of the DTNB reaction, was calculated from the change in absorbance, assuming a molar absorption coefficient of 14,000 mol^{-1} dm^3 cm^{-1} for TNB^- at 412 nm. To calculate K_{equ}, the TNB^- concentration, $[TNB^-]$, was then substituted into Eq. (13) (see below), together with values of K_{SH}, K_{TNB}, $[Hb]_{total}$ (the total hemoglobin concentration in terms of reactive sulfhydryl groups), and $[DTNB]_{total}$ (the total DTNB concentration). A computer program was written on a *Micro-Maths Scientist* software to aid these calculations.

3. Results

We define the parameters that appear in Eq. (1) as follows:

$$K_{SH} = \frac{[H^+][HbS^-]_f[DTNB]_f}{[HbSH][DTNB]_f} = \frac{[H^+][HbS^-]_f}{[HbSH]} \tag{2}$$

$$K_{TNB} = \frac{[H^+][HbS\cdot TNB][TNB^-]}{[HbS\cdot TNB][TNBH]} = \frac{[H^+][TNB^-]}{[TNBH]} \tag{3}$$

$$K_{equ} = \frac{k_f}{k_r} = \frac{[H^+][HbS\cdot TNB][TNB^-]}{[H^+][HBS^-]_f[DTNB]_f} = \frac{[HbS\cdot TNB][TNB^-]}{[HbS^-]_f[DTNB]_f} \tag{4}$$

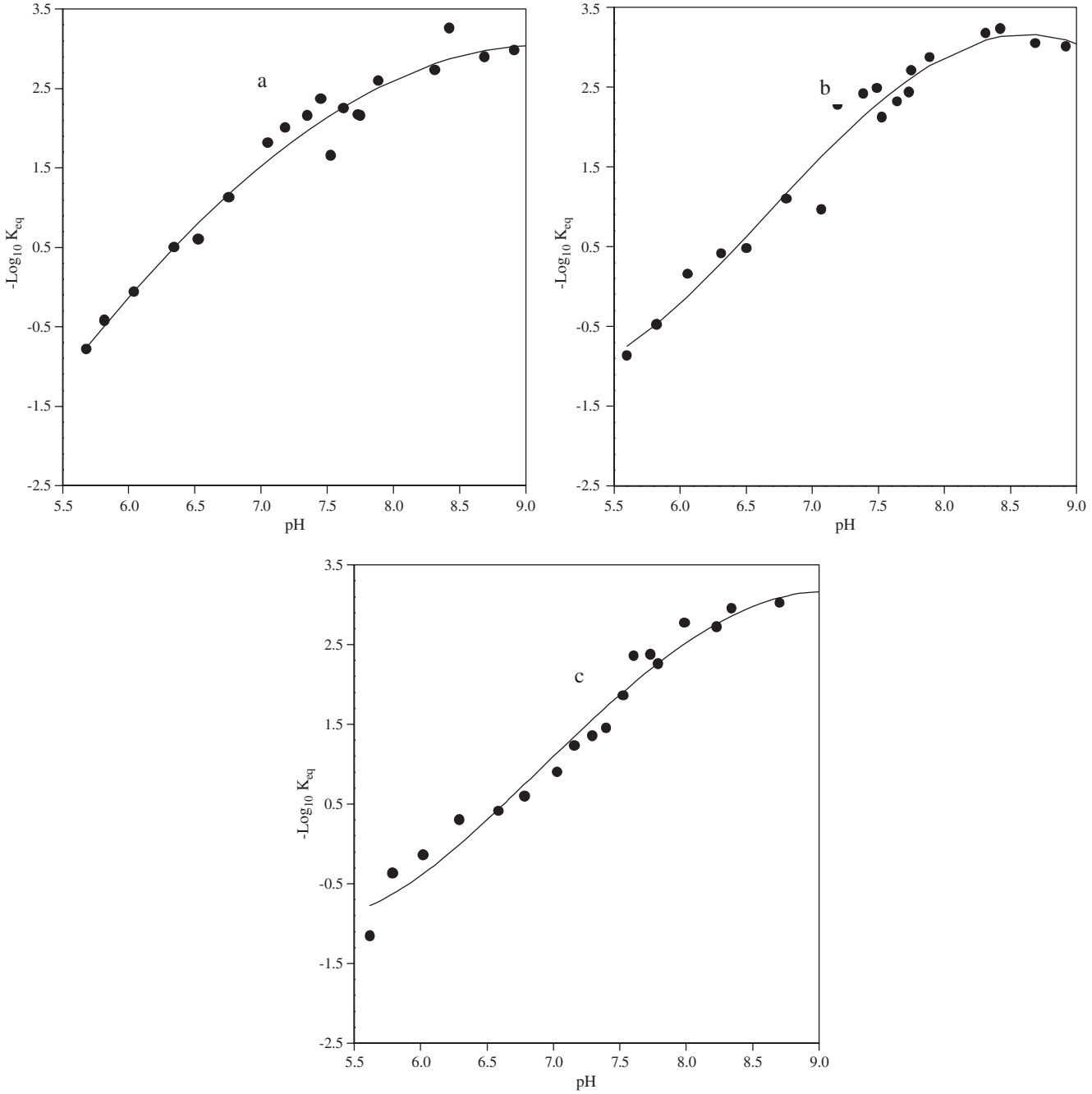


Fig. 1. Reaction of 5,5′-dithiobis(2-nitrobenzoate) — DTNB — with CysF9[93]β of the *major* hemoglobin of the domestic cat. Variation of $-\log_{10}K_{equ}$ with pH: (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Conditions: phosphate buffers, pH 5.6 to 8.0; borate buffers, pH 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 reactive sulfhydryl groups); volume of hemoglobin used, 3 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. The lines through the experimental points are not theoretical.

In Eqs. (2)–(4) the subscript f denotes the unreacted species. From Eq. (3) it is clear that

$$[\mathrm{TNBH}] = \frac{[\mathrm{H^+}][\mathrm{TBN^-}]}{K_{\mathrm{TNB}}} \tag{5}$$

From the stoichiometry of Eq. (1),

$$[\mathrm{HbS\cdot TNB}] = [\mathrm{TNB^-}] + [\mathrm{TNBH}] \tag{6}$$

Substituting for [TNBH] from Eq. (5) into Eq. (6), we obtain

$$[\mathrm{HbS\cdot TNB}] = [\mathrm{TNB^-}]\left\{1 + \frac{[\mathrm{H^+}]}{K_{\mathrm{TNB}}}\right\} \tag{7}$$

The total concentration of hemoglobin is given by

$$[\mathrm{Hb}]_{\mathrm{total}} = [\mathrm{HbSH}] + [\mathrm{HbS^-}]_{\mathrm{f}} + [\mathrm{HbS\cdot TNB}] \tag{8}$$

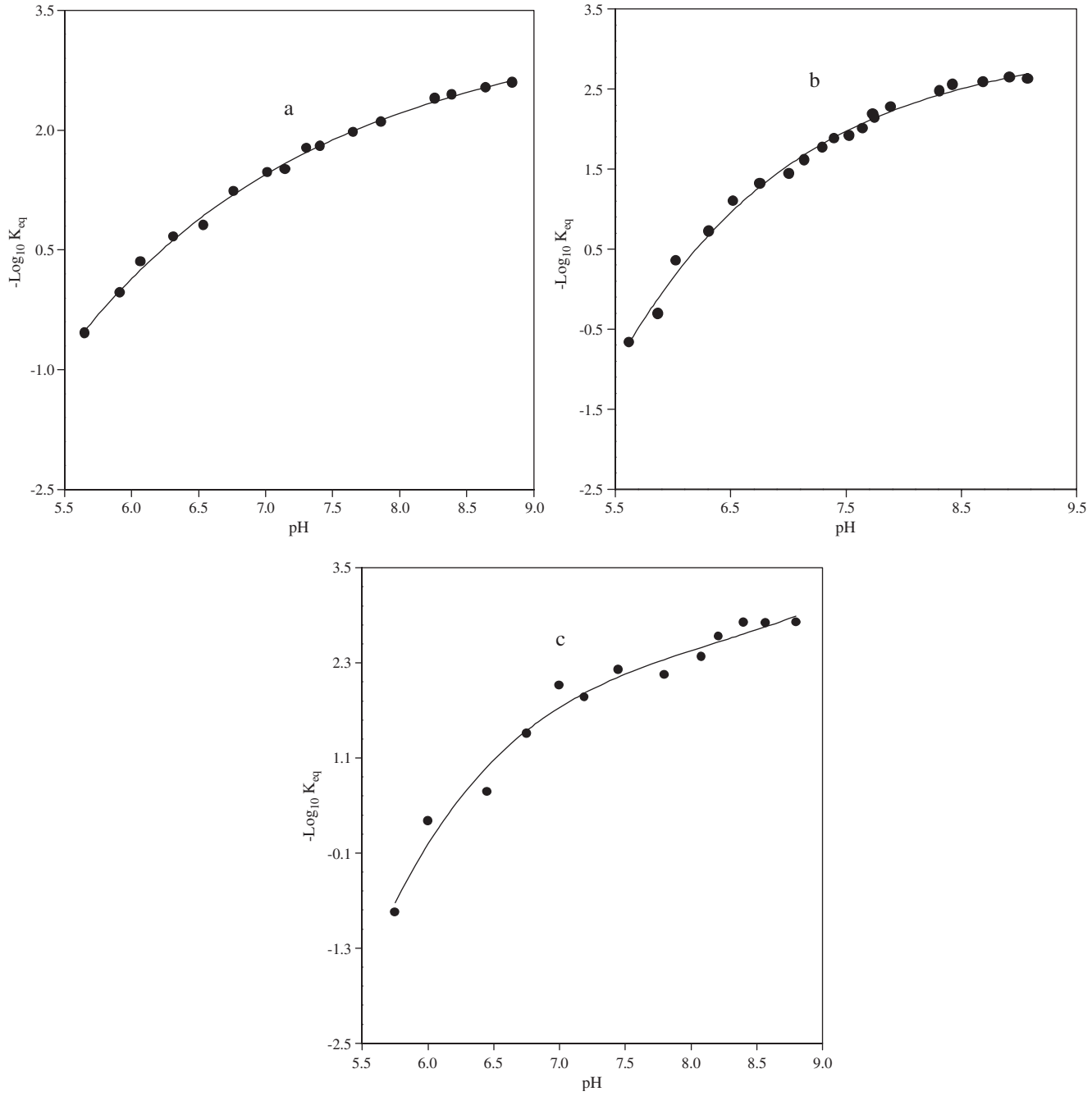


Fig. 2. Reaction of 5,5′-dithiobis(2-nitrobenzoate) — DTNB — with CysF9[93]β of the *minor* hemoglobin of the domestic cat. Variation of $-\log_{10}K_{equ}$ with pH: (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Conditions as in Fig. 1.

Substituting from Eq. (2) for [HbSH] and from Eq. (7) for [HbS·TNB] into Eq. (8) we obtain

$$[\mathrm{Hb}]_{\mathrm{total}} = [\mathrm{HbS}^-]_{\mathrm{f}}\left\{1+\frac{[\mathrm{H}^+]}{K_{\mathrm{SH}}}\right\} + [\mathrm{TNB}^-]\left\{1+\frac{\mathrm{H}^+}{K_{\mathrm{TNB}}}\right\} \qquad (9)$$

Consequently,

$$[\mathrm{HbS}^-]_{\mathrm{f}} = \frac{[\mathrm{Hb}]_{\mathrm{total}} - [\mathrm{TNB}^-]\left\{1+\frac{[\mathrm{H}^+]}{K_{\mathrm{TNB}}}\right\}}{\left\{1+\frac{[\mathrm{H}^+]}{K_{\mathrm{SH}}}\right\}} \qquad (10)$$

The total concentration of DTNB, $[\mathrm{DTNB}]_{\mathrm{total}}$, is given by

$$[\mathrm{DTNB}]_{\mathrm{total}} = [\mathrm{DTNB}]_{\mathrm{f}} + \frac{1}{2}\{[\mathrm{HbS}\cdot\mathrm{TNB}] + [\mathrm{TNB}^-] + [\mathrm{TNBH}]\} \qquad (11)$$

Therefore,

$$[\mathrm{DTNB}]_{\mathrm{f}} = [\mathrm{DTNB}]_{\mathrm{total}} - \frac{1}{2}\{[\mathrm{HbS}\cdot\mathrm{TNB}] + [\mathrm{TNB}^-] + [\mathrm{TNBH}]\} = [\mathrm{DTNB}]_{\mathrm{total}} - [\mathrm{TNB}^-]\left\{1+\frac{[\mathrm{H}^+]}{K_{\mathrm{TNB}}}\right\} \qquad (12)$$

Substituting for [HbS·TNB] from Eq. (7), for $[HbS^-]_f$ from Eq. (10), and for $[DTNB]_f$ from Eq. (12) into Eq. (4), one obtains the final expression for K_{equ},

$$K_{equ} = \frac{[TNB^-]^2\left\{1+\frac{[H^+]}{K_{TNB}}\right\}\left\{1+\frac{[H^+]}{K_{SH}}\right\}}{\left\{[Hb]_{total}-[TNB^-]\left(1+\frac{[H^+]}{K_{TNB}}\right)\right\}\left\{[DTNB]_{total}-[TNB^-]\left(1+\frac{[H^+]}{K_{TNB}}\right)\right\}} \quad (13)$$

The pK_{SH} of CysF9[93]β lies between ca 8.0 and 8.6 [7–9]. In calculating K_{equ} from Eq. (13) we therefore assumed a pK_{SH} of 8.3. The pK_{TNB} determined above (see Experimental) was 5.27±0.1 The K_{SH} and K_{TNB} values calculated from these pK values, together with the TNB^- concentration determined at known pH values, were substituted into Eq. (13) to obtain K_{equ}. The standard error involved in the determination of K_{equ} was about 10%. In doing these calculations it was assumed that the absorbance of TNBH is negligible. This is readily demonstrated at low pH values, ca pH 2.2.

We found that K_{equ} decreased rapidly as the pH increased. For this reason we have reported the pH dependence of K_{equ} in logarithmic form. Fig. 1(a–c) shows the variation of

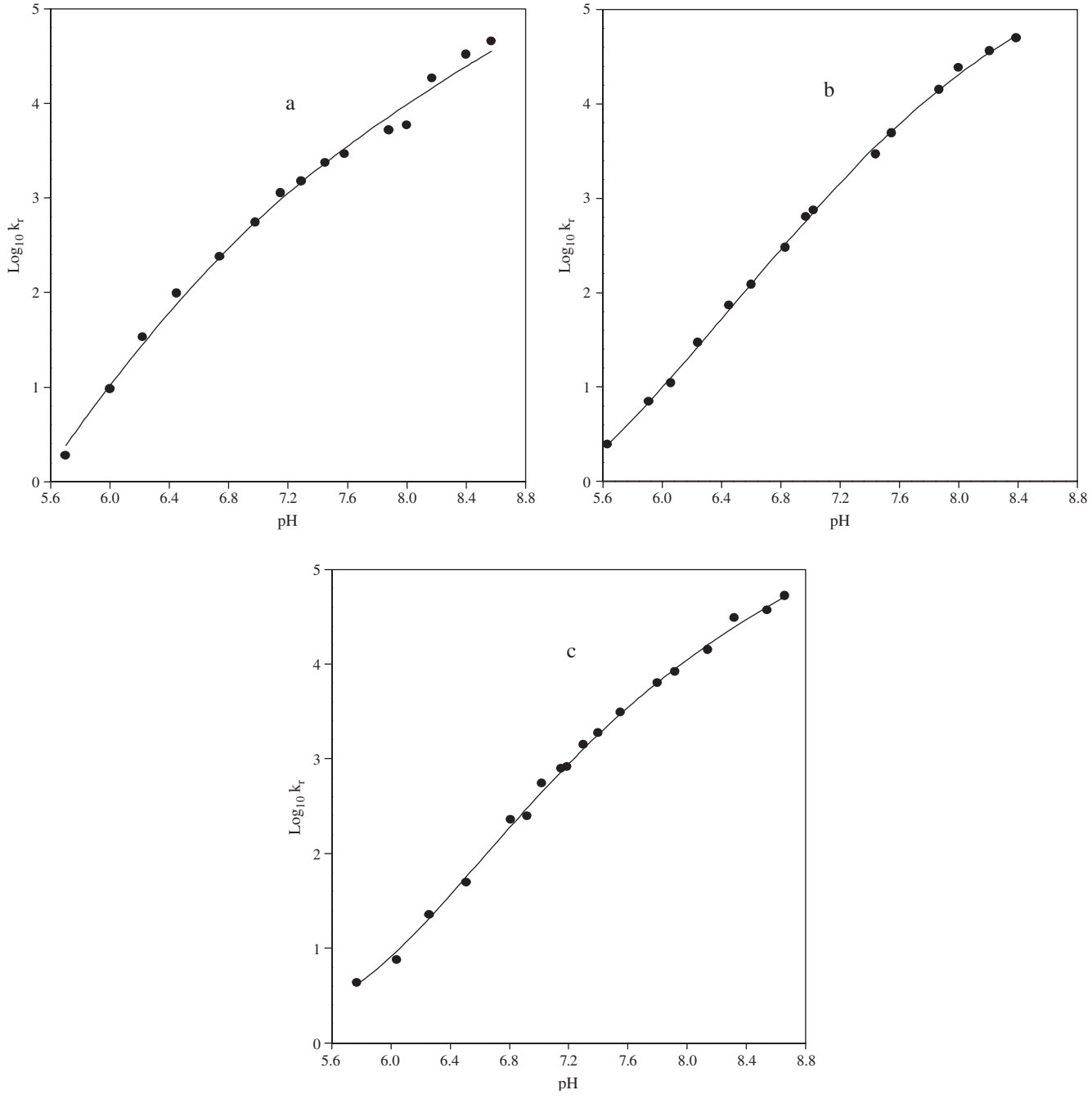


Fig. 3. Reaction of 5,5′-dithiobis(2-nitrobenzoate) — DTNB — with CysF9[93]β of the *major* hemoglobin of the domestic cat. Variation of $\log_{10} k_r$, the logarithm of the apparent second order *reverse* rate constant, with pH: (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Conditions as in Fig. 1. The lines through the data points are theoretical lines drawn with Eq. (14), with $n=2$ (compare with Scheme 1).

$-\log_{10}K_{equ}$ (pK_{equ}) with pH for the oxy, carbonmonoxy and aquomet derivatives of the major hemoglobin. In Fig. 2 we report the corresponding data for the minor hemoglobin. It is seen that in each case K_{equ} decreases by about 3 to 4 orders of magnitude between pH 5.6 and 9.

Since $k_r = \frac{k_f}{K_{equ}}$, it is possible to determine k_r from knowledge of k_f and K_{equ}. k_r values were determined from the ratio of the k_f values of Ref. [1] and the K_{equ} values reported in Figs. 1 and 2 of the present paper. Fig. 3 reports the pH dependences of $\log_{10} k_r$ for the oxy, carbonmonoxy and aquomet derivatives of the major hemoglobin, and Fig. 4 reports those for the corresponding derivatives of the minor hemoglobin.

3.1. Analyses of the pH dependence of log_{10} k_r

The strong pH dependence of $\log_{10}$ k_r (Figs. 3 and 4) indicates that the reverse of the DTNB reaction, $TNB^- + HbS \cdot TNB \xrightarrow{k_r} HbS^- + DNTB$, is coupled to the ionization of groups on the protein. To determine the nature and the number of these groups we propose the following reaction scheme.

In Scheme 1 $H_{n-i+1}HbS \cdot TNB$ ($i = 1, 2, \ldots n$) is the mixed disulfide species in solution; k_{-i} is the second order *reverse* rate constant for the reaction of this species with TNB^-; Q_{jT} is the dissociation constant for the release of the jth proton

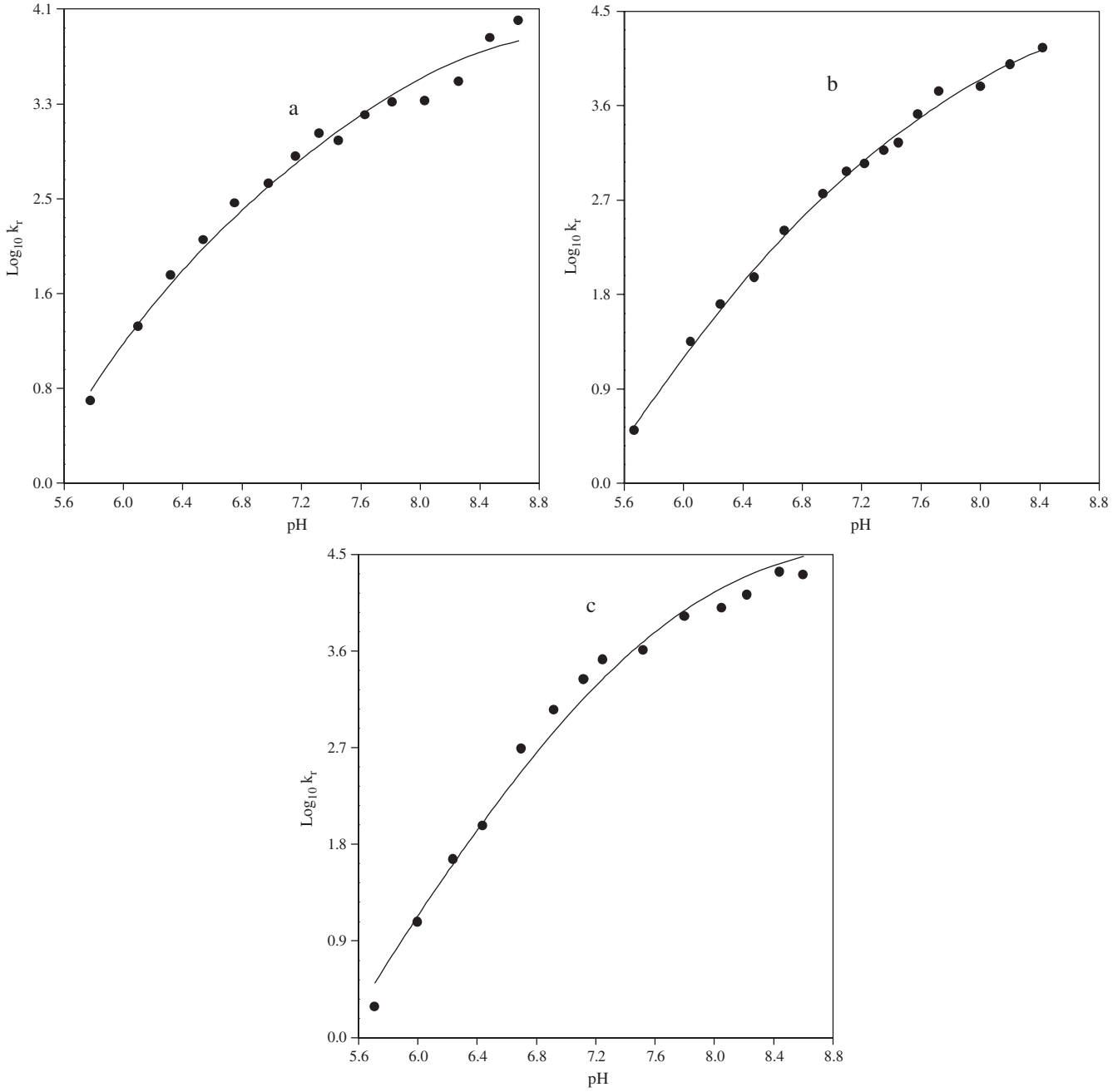
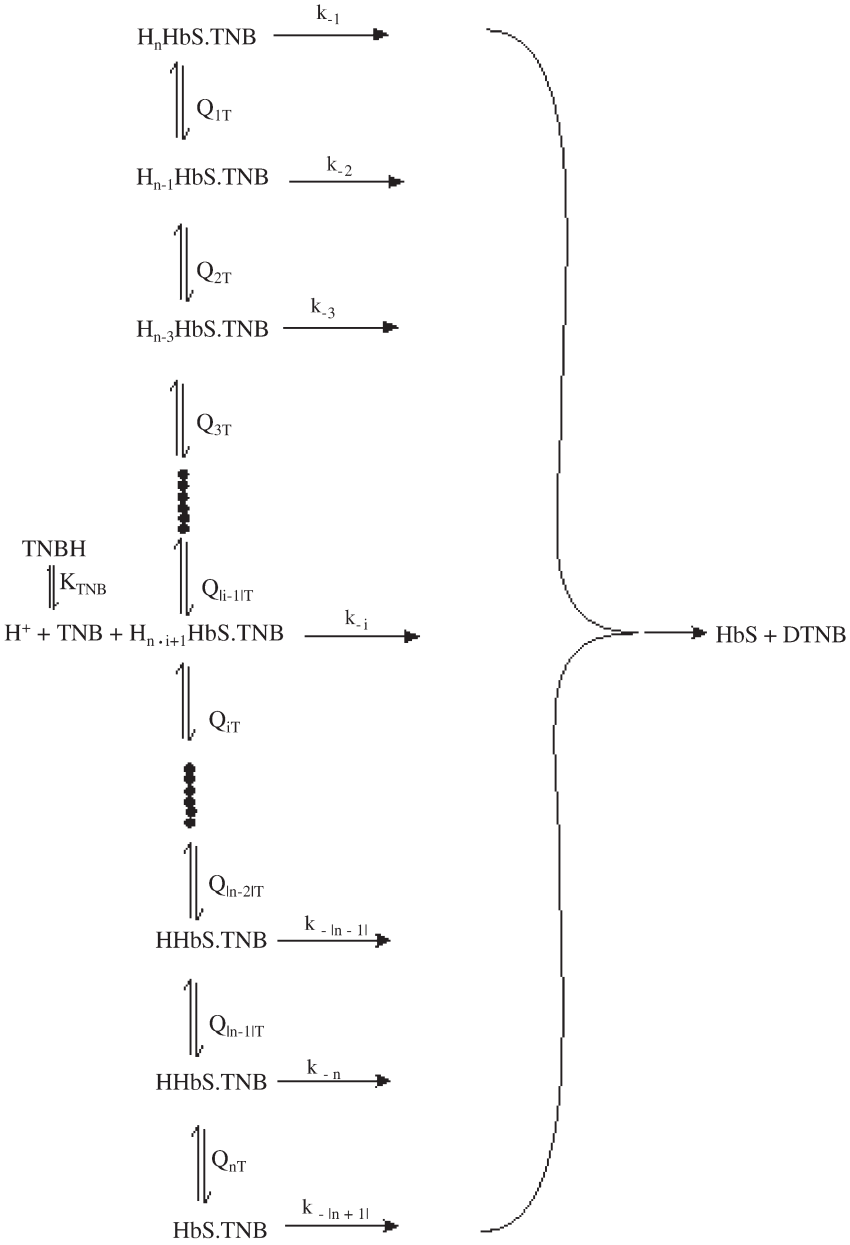


Fig. 4. Reaction of 5,5′-dithiobis(2-nitrobenzoate) — DTNB — with CysF9[93]β of the *minor* hemoglobin of the domestic cat. Variation of $\log_{10} k_r$, the logarithm of the apparent second order *reverse* rate constant, with pH: (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Conditions as in Fig. 1. The lines through the data points are theoretical lines drawn with Eq. (14), with $n = 2$ (compare with Scheme 1).



Scheme 1.

from H_{n-i+1} HbS·TNB. For the sake of clarity the protons involved in the various protolytic steps of Scheme 1 have been omitted. Assuming that the rates of the protolytic steps are much faster than the rate of the reverse of the DTNB reaction [7–9], it can be readily shown that the apparent second order *reverse* rate constant, k_r, is related to the various k_{-i} of Scheme 1 by Eq. (14):

$$k_r = \frac{K_{TNB}}{K_{TNB} + [H^+]} \cdot \frac{k_{-(n+1)} + \sum_{i=1}^{n} k_{-i}[H^+]^{n-i+1}\left(\prod_{j=i}^{n} Q_{jT}\right)^{-1}}{1 + \sum_{i=1}^{n} [H^+]^{n-i+1}\left(\prod_{j=i}^{n} Q_{jT}\right)^{-1}}. \quad (14)$$

Using a pK_{TNB} value of 5.27 (see Experimental section), we have employed Eq. (14) to fit the data in Figs. 3 and 4. (We used a computer program written on the MicroMaths Scientist curve fitting software.) We fit the data to $\log_{10} k_r$ rather than to k_r because the goodness of fit to the data in the low pH range (pH <7.5) is more easily visualized with the data expressed in logarithmic form.

Numerous attempts to fit the $\log_{10} k_r$ data with an n value of 1 failed: the resulting theoretical curves did not even come close to fitting the data. By contrast, an n value of 2 gave excellent fits to the data. The lines through the data points in Fig. 3 are the best-fit lines calculated with the parameters reported in Table 1 for the major hemoglobin. The mean pQ_{1T} and pQ_{2T} values are 7.2±0.2 and 9.4±0.1, respectively. The lines through the data points in Fig. 4 are the best-fit lines calculated with the

Table 1
Reverse of the reaction of DTNB with the *major* cat hemoglobin

Hemoglobin derivative	k_{-1} mol^{-1} dm^3 s^{-1}	k_{-2} mol^{-1} dm^3 s^{-1}	k_{-3} mol^{-1} dm^3 s^{-1}	pQ_{1T}	pQ_{2T}
Oxy	9.4×10^{-4}	0.54	4.1×10^{5}	6.89	9.58
Carbonmonoxy	2.5	47.9	2.8×10^{5}	7.41	9.29
Aquomet	2.3	45.6	2.8×10^{5}	7.41	9.29

Parameters employed to fit the $\log_{10} k_r$ versus pH data (Fig. 3). Compare with Eq. (14) and Scheme 1 of the text, with $n=2$.

parameters reported in Table 2 for the minor hemoglobin. The mean pQ_{1T} and pQ_{2T} values are 6.7±0.3 and 8.4±0.1, respectively.

4. Discussion

4.1. Equilibrium constants

In a previous report [1] we concluded from kinetic data that the reaction of DTNB with cat hemoglobins must be a reversible process. This conclusion has now been justified by the equilibrium constants reported in this paper (Figs. 1 and 2). In particular, the conclusion [1] that the non-linear pseudo-first order plots at pH >8.6 (Fig. 2d of Ref. [1]) arise because K_{equ} must be very low in this pH range has been fully borne out by the present experimental results (see Figs. 1 and 2).

4.2. Fitting parameters to log_{10} k_r data

The best fits to the $\log_{10} k_r$ data (Figs. 3 and 4) were obtained with an n value of 2. This means that the reverse of the DTNB reaction, $TNB^- + HbS\cdot TNB \xrightarrow{k_r} HbS^- + DTNB$, is coupled to the ionization of two groups on the protein. The pQ_{1T} values of 7.2±0.2 and 6.7±0.3 for the major and minor hemoglobins (see Tables 1 and 2) are similar to the values (6.9 and 6.5, respectively) assigned to histidines for the forward reaction [1]. We therefore assign the pQ_{1T} values to histidines. The pQ_{2T} values of 9.4±0.1 and 8.4±0.1 are difficult to assign at present. Although the latter figure is similar to the pK_a of ionization of sulfhydryl groups in hemoglobins, the sulfhydryl groups in the various mixed disulfide species (Scheme 1) are not free and therefore cannot ionize. Consequently, the pQ_{2T} values cannot be assigned to sulfhydryl groups.

The pQ_{2T} value of the major hemoglobin (9.4) is one pK_a unit higher than that of the minor hemoglobin (8.4). This is an extreme example of a general observation on the pQ differences between identical ionizable groups in the major and minor hemoglobins: for both the forward and reverse reactions (Tables 1 and 2 of [1] and Tables 1 and 2 of this paper, respectively) the pQ values obtained for the major hemoglobin are generally higher than those obtained for the minor hemoglobin. The finding that the pQ values of the groups on the major hemoglobin linked to the DTNB reaction are higher than those on the minor hemoglobin suggests that the former groups contribute to the higher net positive charge of the major compared to the minor hemoglobin. This is demonstrated by the tighter binding of the major hemoglobin to carboxymethylcellulose (see Experimental section of Ref. [1]): while the minor hemoglobin easily comes off the resin, the binding of the major hemoglobin is so tight that a higher salt concentration and a higher pH are required for its release from the resin [1]. There are, of course, the extra positive charges on the β chain amino terminal residues of the major hemoglobin; the same residues on the minor hemoglobin carry no charge [10].

4.3. Value of n required to fit the $log_{10}k_r$ data

Two points need to be clarified regarding the value $n=2$ required to fit the $\log_{10} k_r$ versus pH data for both the major and minor hemoglobins (Figs. 3 and 4). (i) For the forward reaction [1,11,12], higher n values were required to best fit the k_f versus pH data for aquomethemoglobin compared to the oxy and carbonmonoxy derivatives. This is because the ionization of the water molecule at the 6th coordination position of the heme iron (III) ion in aquomethemoglobin is electrostatically linked to the CysF9[93]β sulfhydryl group. (ii) Again, for the forward reaction the values of n required to fit the k_f versus pH data for the major cat hemoglobin are 1 higher than those required to fit the corresponding minor hemoglobin data [1]. This is because the positively charged organic phosphate binding groups are electrostatically linked to CysF9[93]β, and the major hemoglobin has a terminal, positively charged NH_3^+ group on each of its β chains, whereas in the minor hemoglobin the terminal amino group has its charge neutralized [10]. In contrast to the forward reaction [1,11,12], for the reverse reaction the value of n required to best fit the $\log_{10} k_r$ versus pH data (Figs. 3 and 4) is the same for the aquomet and other derivatives. It is also the same for the major and minor cat hemoglobins. How could this discrepancy in the values of n required to fit the data for the forward and reverse reactions arise?

In Shanaan's X-ray crystallographic structure of oxyhemoglobin [13], CysF9[93]β can exist in either of two conformations, *cis* to the main chain carbonyl group or *cis* to the main chain amino group. In a temperature-jump kinetic study [14] we demonstrated that in solution the CysF9[93]β groups of deoxy- and carbonmonoxyhemoglobin exist in two conformations (*cis*-to-carbonyl and *cis*-to-amino) in dynamic equilibrium, with the *cis*-to-amino conformation dominating. We also demonstrated that chemical modification of this sulfhydryl group fixes it in the *cis*-to-carbonyl conformation and completely eliminates the dynamic equilibrium [14].

In the forward DTNB reaction, $HbS^- + DTNB \xrightarrow{k_f} HbS\cdot TNB + TNB^-$, the sulfhydryl group is unmodified and exists mainly in the *cis*-to-amino conformation. By contrast, in the

Table 2
Reverse of the reaction of DTNB with the *minor* cat hemoglobin

Hemoglobin derivative	k_{-1} mol^{-1} dm^3 s^{-1}	k_{-2} mol^{-1} dm^3 s^{-1}	k_{-3} mol^{-1} dm^3 s^{-1}	pQ_{1T}	pQ_{2T}
Oxy	1×10^{-4}	1×10^{-3}	9.7×10^{3}	6.17	8.31
Carbonmonoxy	4.6×10^{-3}	8.6	3.0×10^{4}	6.67	8.49
Aquomet	$\leq1\times10^{-4}$	$\leq1\times10^{-3}$	4.4×10^{4}	7.11	8.29

Parameters employed to fit the $\log_{10} k_r$ versus pH data (Fig. 4). Compare with Eq. (14) and Scheme 1 of the text, with $n=2$.

reverse of the DTNB reaction, $TNB^- + HbS{\cdot}TNB \xrightarrow{k_r} HbS^- +$ DTNB, the mixed disulfide species, HbS·TNB, with which TNB^- reacts has a chemically modified CysF9[93]β group. Consequently, the sulfhydryl is *fixed* in the *cis*-to-carbonyl conformation. The single $n=2$ value required to fit *all* the $\log_{10} k_r$ versus pH data (Figs. 3 and 4) implies that the water molecule at the 6th coordination position of the heme iron in aquomethemoglobin is not electrostatically linked to the F9 [93]β position in this fixed conformation. Were it so linked, the value of n we would have required to fit the aquomet data would be 1 higher than the n value required to fit the oxy and carbonmonoxy data [1]. The single $n=2$ value required to fit the data − major hemoglobin (Fig. 3); minor hemoglobin (Fig. 4) − also implies that the positively charged groups at the organic phosphate binding site are not linked to the F9 [93]β site in this fixed conformation. Were they so linked, the values of n required to fit the major hemoglobin data would have been 1 more than those required to fit the minor hemoglobin data [1]. This suggests that the linked ionizable groups for the forward [1,11,12] and reverse (this paper) portions of the DTNB reaction are not identical.

5. Conclusion

It is clear from the results presented in this paper that the reaction of DTNB with the CysF9[93]β sulfhydryl groups of the hemoglobins of the domestic cat is indeed reversible. Whether the reaction of DTNB with other hemoglobins is reversible is a moot point that may be worth investigating. It is highly unlikely that the ionisable groups linked to the forward and reverse portions of the DTNB reaction are identical.

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