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Tertiary conformational transition in sheep hemoglobins induced by reaction with 5,5′-dithiobis(2-nitrobenzoate) and by binding of inositol hexakisphosphate

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ABSTRACT

We have determined the second-order reverse rate constant, k_R, for the reaction of 5,5′-dithiobis(2-nitrobenzoate) – DTNB – with sheep hemoglobins as a function of pH from values of the second-order forward rate constant, k_F, and the equilibrium constant, K_{equ}, at 25 °C: $k_R = \frac{k_F}{K_{equ}}$. We demonstrate that (i) inositol hexakisphosphate (inositol-P_6) decreases k_F and k_R by increasing $K_{\mathbf{rt}}$, the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary conformation transition constant; (ii) the conformation favored for both the forward and reverse reactions is the **r** conformation. For stripped hemoglobin we obtain from the k_F data a **t** isomer population of 34.6% (±14) prior to reaction with DTNB; from the k_R data we calculate a **t** isomer population of 44.8% (±4) following reaction with DTNB. In the presence of inositol-P_6 the latter value is increased to 79.5% (±2). These results demonstrate that an allosteric transition occurs on reaction with DTNB and on inositol-P_6 binding.

1. Introduction

The CysF9[93]β sulphydryl group has served as an indicator for tertiary and quaternary structure changes in hemoglobin [1–9]. Both of these structure changes occur together on ligand binding to ferrous hemoglobin and cannot be distinguished in that system. In an x-ray structure study of oxyhemoglobin at 2.1 Å resolution, Shaanan [8] demonstrated that the CysF9[93]β sulphydryl exists in two conformations, and he suggested that in solution these two conformations might be in dynamic equilibrium. With this suggestion in mind, we performed temperature-jump studies on deoxyhemoglobin and on carbonmonoxyhemoglobin, each mixed with pH indicators [9]. Since deoxyhemoglobin and carbonmonoxyhemoglobin exist in the T and R quaternary conformations, respectively, our temperature-jump data enabled us to characterize the tertiary structure transition in the T and R quaternary states [9]. We refer to this transition as the $\mathbf{r} \rightleftharpoons \mathbf{t}$ transition.

We found that the transition was abolished in both the T and R quaternary states when the CysF9[93]β sulphydryl group of hemoglobin was modified with iodoacetamide [9]. This abolition arises because iodoacetamide binds *irreversibly* to the CysF9[93]β sulphydryl and shifts the $\mathbf{r} \rightleftharpoons \mathbf{t}$ equilibrium completely to the right, in favor of the **t** conformation [9]. By contrast, the reaction of this sulphydryl group with 5,5′-dithiobis(2-nitrobenzoate) – DTNB – is a *reversible* process [10–14] and therefore does not abolish the transition.

The reversible reaction of DTNB with CysF9[93]β may be depicted as:

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

In Eq. (1) PSH is the hemoglobin molecule with its CysF9[93]β sulphydryl in its protonated, unreacting form; DTNB is the sulphydryl reagent 5,5′-dithiobis(2-nitrobenzoate); PS^- is the sulphydryl in its reacting, thiol anion form; k_F and k_R are the apparent forward and reverse rate constants of the DTNB reaction; PS.ST is the mixed disulphide formed; 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 CysF9[93]β and TNBH, respectively.

In the forward reaction of Eq. (1), DTNB forms a mixed disulphide–PS.ST – with the thiol anion form – PS^- – of the hemoglobin sulphydryl group. The formation of such a mixed disulphide gives rise to changes in physiological functions [15–18]. In the reverse reaction the mixed disulphide reacts with 5-thio-2-nitrobenzoate – TNB^- – to reproduce the sulphydryl in its thiol form. This reverse reaction is similar to the protective action of reduced glutathione (GSH) in transforming the cysteine residues of hemoglobin and other red blood cell proteins from the modified form to the reduced form [19]. Therefore, as far as the maintenance of physiological function is concerned, the reverse reaction in Eq. (1) is very important.

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It is currently not feasible to determine directly by experiment the apparent second-order rate constant, k_R, of the reverse reaction (see Eq. (1)) because it is difficult to isolate the two putative reactants: the mixed disulphide, PS.ST, and TNB^-. Nevertheless, k_R can be assessed from knowledge of the forward rate constant, k_F, and the equilibrium constant, K_{equ}, for the DTNB reaction (see Eq. (1)), since $k_R = \frac{k_F}{K_{equ}}$.

We recently reported that the organic phosphate inositol hexakisphosphate – inositol-P_6 – increases the affinity of the CyF9[93]β sulphydryl group of sheep hemoglobins for DTNB by about an order of magnitude in the pH range 5.6 to 9 [14]. Here we report the determination of the apparent forward second-order rate constant, k_F, over the same pH range. Using the K_{equ} values that we recently published [14], together with the k_F values reported here, we have calculated values of k_R as a function of pH for the oxy, carbonmonoxy and aquomet derivatives of the major and minor hemoglobins of the sheep. We find that inositol-P_6 reduces the k_R values of each of the hemoglobin derivatives by about an order of magnitude throughout the pH range 5.6 to 9. Quantitative analyses of the pH dependence of k_R demonstrate that inositol-P_6 reduces k_R by increasing $K_{\mathbf{rt}}$, the equilibrium constant for the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary conformational transition. The increase of $K_{\mathbf{rt}}$ by inositol-P_6 has a remarkably lower effect on the apparent second-order forward rate constant, k_F.

2. Materials and methods

2.1. Separation of hemoglobins

The preparation of sheep hemolysate has been described previously [14].

The separation of the major and minor hemoglobins was achieved as follows in a cold room at 5 °C [14]. A 3 cm (diameter) by 30 cm column of Whatman CMC-52 carboxymethyl cellulose, a microgranular, preswollen cation exchanger, was used. The resin was pre-equilibrated with 10 mmol dm^{-3} phosphate buffer, pH 6.5. The minor hemoglobin was completely eluted with the pH 6.5 buffer, whereas the major component remained bound to the resin. It was eluted with phosphate buffer, pH 8.0, ionic strength 0.2 mol dm^{-3}. Prior to use for experiments, each hemoglobin was passed through a Dintzis ion exchange column to remove endogenous organic phosphates and undesired ions.

2.2. Kinetics measurements

We employed the method previously described [14] to study the kinetics of the reaction of DTNB with hemoglobin derivatives under pseudo-first order conditions. This was achieved by reacting each of the hemoglobin samples with at least a 60-fold excess of DTNB per sulphydryl group. The kinetics of the reaction of DTNB with the sheep hemoglobins were monitored at 412 nm on a Cecil Bioquest UV–visible spectrophotometer coupled to an on-line data acquisition system. The hemoglobin concentration was 10 μmol (heme) dm^{-3} (5 μmol dm^{-3} in reactive sulphydryl groups), while the DTNB concentration varied between 300 and 600 μmol dm^{-3}. The maximum increase in ionic strength arising from the addition of DTNB was less than 4 percent. Each kinetic run was repeated at least three times under the same experimental conditions and was allowed to proceed to near completion. The data were analyzed with a 1990 update of DISCRETE, a computer program for the analysis of multiple exponential signals. The standard error in the determination of the pseudo-first order rate constant was about 5%.

3. Results

As reported previously, the pseudo-first order rate plots for the reaction of stripped hemoglobin with DTNB are linear for at least 2.5 half-lives [14]. We obtained similar results in the presence of inositol-P_6 for the major and minor hemoglobins at all pH values in the range 5.6 to 9. We plotted the pseudo-first order rate constants, k_{obs}, calculated from the least squares slopes of the pseudo-first order plots at a given pH, against the DTNB concentration. Each plot was linear and had a non-zero intercept. These results (not shown) demonstrate that the reaction of DTNB with the sheep hemoglobins in the presence of inositol-P_6 is a reversible process, as it is for stripped hemoglobin [14]. Each linear graph gave a slope, k_F, which is the apparent second-order forward rate constant.

Fig. 1 reports the variation of k_F with pH for the oxy, carbonmonoxy and aquomet derivatives of the (stripped) *major* hemoglobin (open symbols) and for the major hemoglobin in the presence of inositol-P_6 in a 4:1 molar ratio with respect to hemoglobin tetramers (filled symbols). Fig. 2 shows the corresponding data for the *minor* hemoglobin. It is seen that inositol-P_6 decreases k_F over the pH range 5.6 to 9 for the hemoglobin derivatives, except that for three of the derivatives – the major oxy (Fig. 1a); the major carbonmonoxy (Fig. 1b); the minor oxy (Fig. 2a) – k_F is higher for inositol-P_6-bound hemoglobin than for the stripped hemoglobin in the interval pH < 6.5.

Since $k_R = \frac{k_F}{K_{equ}}$, each k_F value at a given pH was divided with the corresponding value of K_{equ} to give k_R, the apparent second-order reverse rate constant. In Fig. 3a we compare the values of $\log_{10} k_R$ as a function of pH for the stripped major oxyhemoglobin (open symbols) with the corresponding values in the presence of inositol-P_6 (filled symbols). It is seen that k_R for stripped hemoglobin is about an order of magnitude higher than in the presence of the organic phosphate. Similar results were obtained for the carbonmonoxy and aquomet derivatives (Fig. 3b and c, respectively), and for the corresponding derivatives of the minor hemoglobin (Fig. 4).

3.1. Analyses of pH dependence profiles

3.1.1. Analyses of k_F versus pH profiles for the forward reaction

Since the CysF9[93]β sulphydryl group exists as a mixture of two tertiary conformations, **r** and **t**, in dynamic equilibrium [8,9] it is necessary to determine with which of the two conformations DTNB preferentially reacts. We first assume that it is the **r** conformation. On the basis of this assumption, and because of the rather complex nature of the pH dependence of k_F, we propose the following reaction scheme (Scheme 1) to explain (i) the relative magnitudes of k_F, with and without inositol-P_6, and (ii) the pH dependence of k_F seen in Figs. 1 and 2.

In Scheme 1, $(H_{n-i+1}PSH)_t$ ($i = 1, 2, \ldots n+1$) are hemoglobin species in which the sulphydryl groups are protonated, and they are in the **t** conformation; $(H_{n-i+1}PS^-)_{\mathbf{t}}$ are the corresponding thiol anion forms; species $(H_{n-i+1}PS^-)_{\mathbf{r}}$ are the thiol anion species in the **r** conformation;

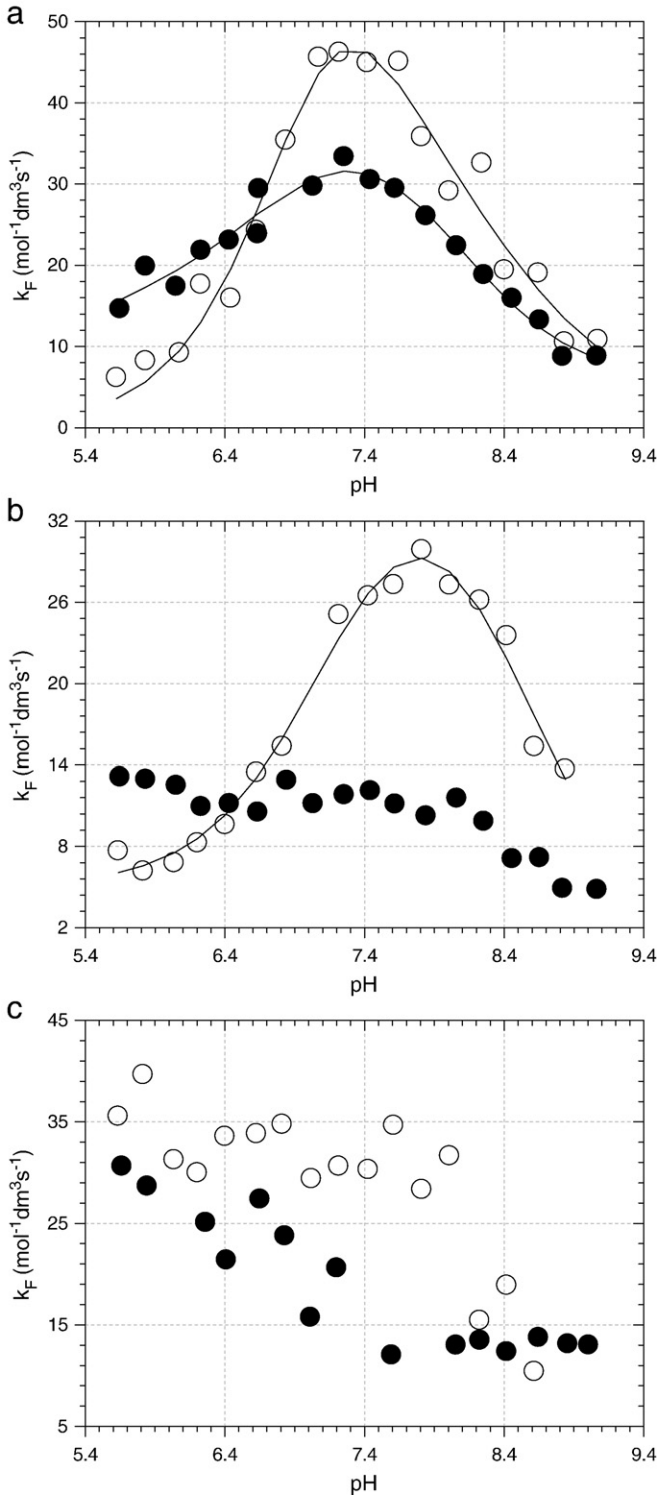


Fig. 1. Reaction of the *major* sheep hemoglobin with DTNB: variation of k_F, the apparent second-order forward rate constant, with pH. (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Open symbols, stripped hemoglobin; filled symbols, hemoglobin + inositol-P_6. Each point is the mean of three determinations and is subject to a standard error of *ca* 20%. Conditions: phosphate buffers (pH 5.6–8.0); borate buffers (pH ≥ 8.0); 25 °C; hemoglobin concentration, 10 μmol (haem) dm^{-3} (5 μmol dm^{-3} in reactive sulphydryl groups); [inositol-P_6] = 10 μmol dm^{-3}. The DTNB concentration was between 300 and 600 μmol dm^{-3}. The total ionic strength of each buffer was 50 mmol dm^{-3}. The lines through the data points are the best-fit lines calculated with the parameters reported in Table 1 (cf. Scheme 1 and Eq. (2)). Data points without lines were not fitted.

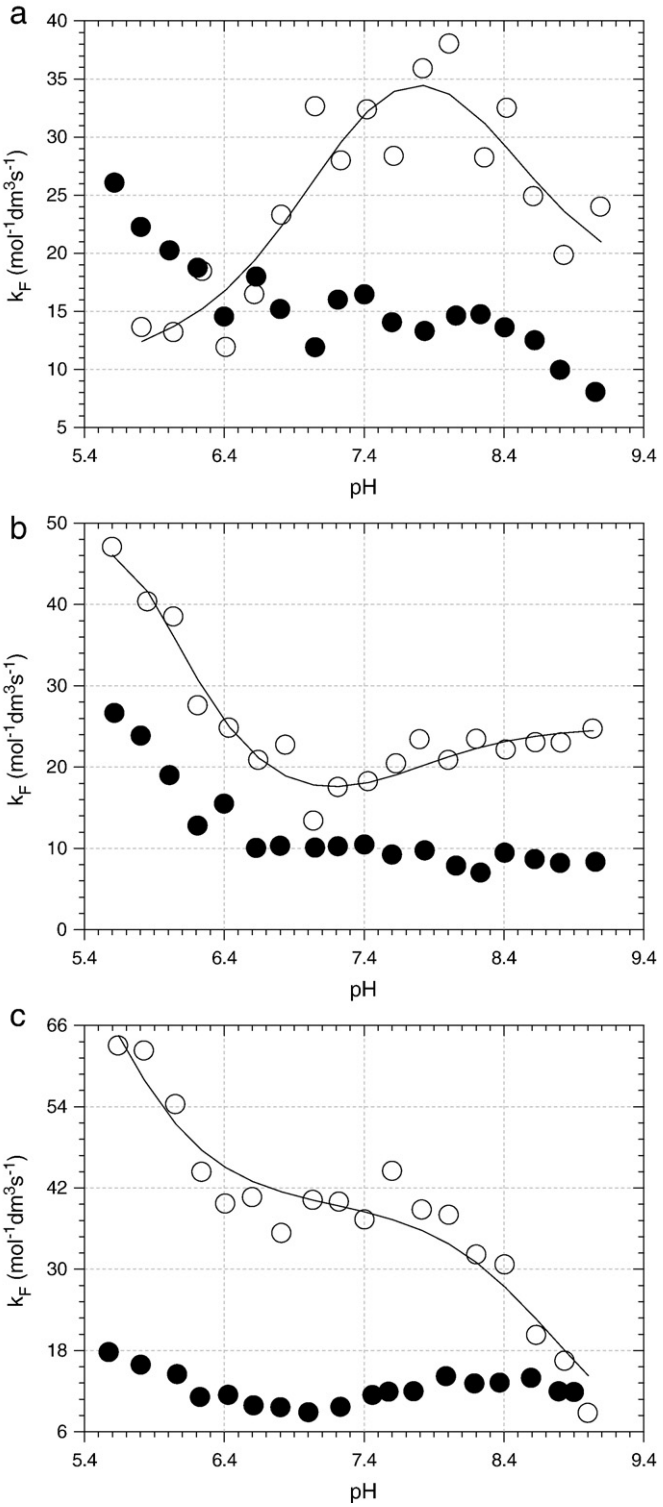


Fig. 2. Reaction of the *minor* sheep hemoglobin with DTNB: variation of k_F, the apparent second-order forward rate constant, with pH. (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Open symbols, stripped hemoglobin; filled symbols, hemoglobin + inositol-P_6. Conditions as in Fig. 1. The lines through the data points are the best-fit lines calculated with the parameters reported in Table 1 (cf. Scheme 1 and Eq. (2)). Data points without lines were not fitted.

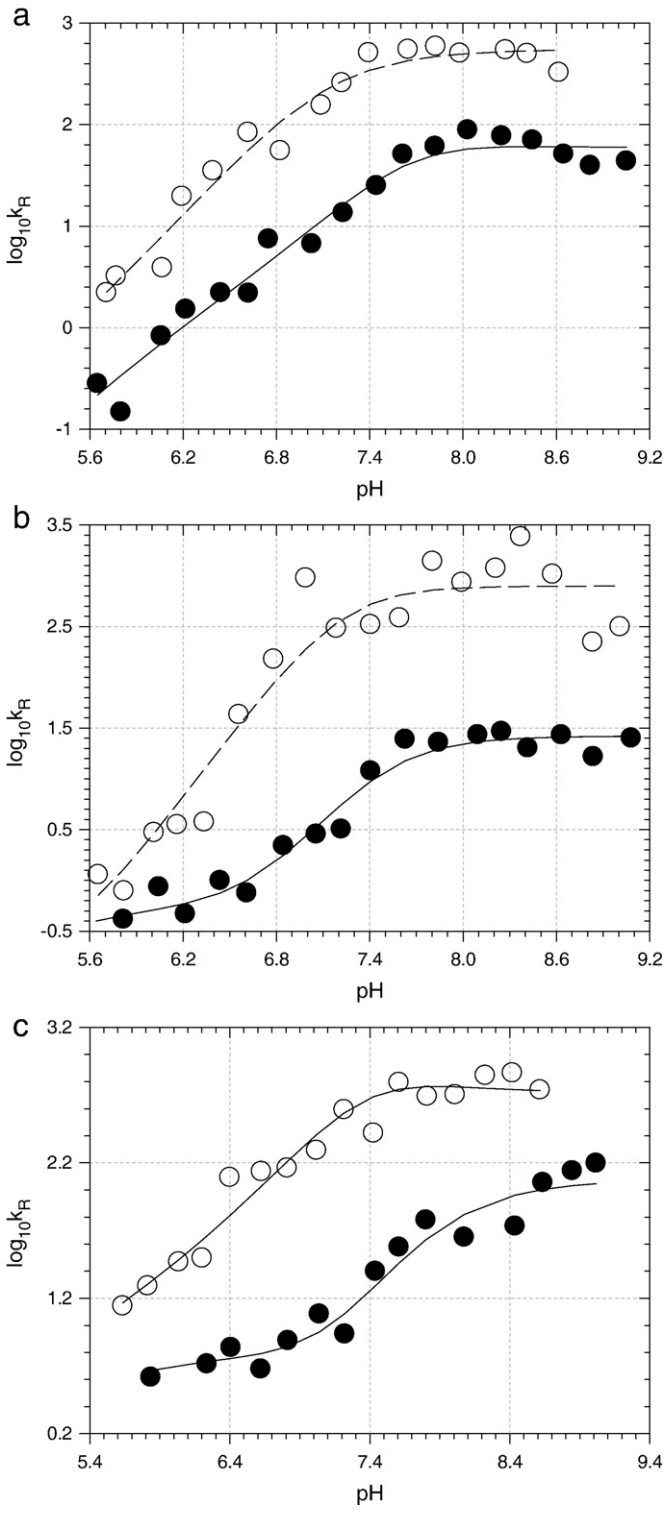


Fig. 3. Reaction of the major sheep hemoglobin with DTNB: variation of k_R, the apparent second-order reverse rate constant, with pH. (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Open symbols, stripped hemoglobin; filled symbols, hemoglobin + inositol-P_6. Conditions as in Fig. 1. The lines through the data points are the best-fit lines calculated with the parameters reported in Tables 2 and 3 (cf. Scheme 2 and Eq. (4)).

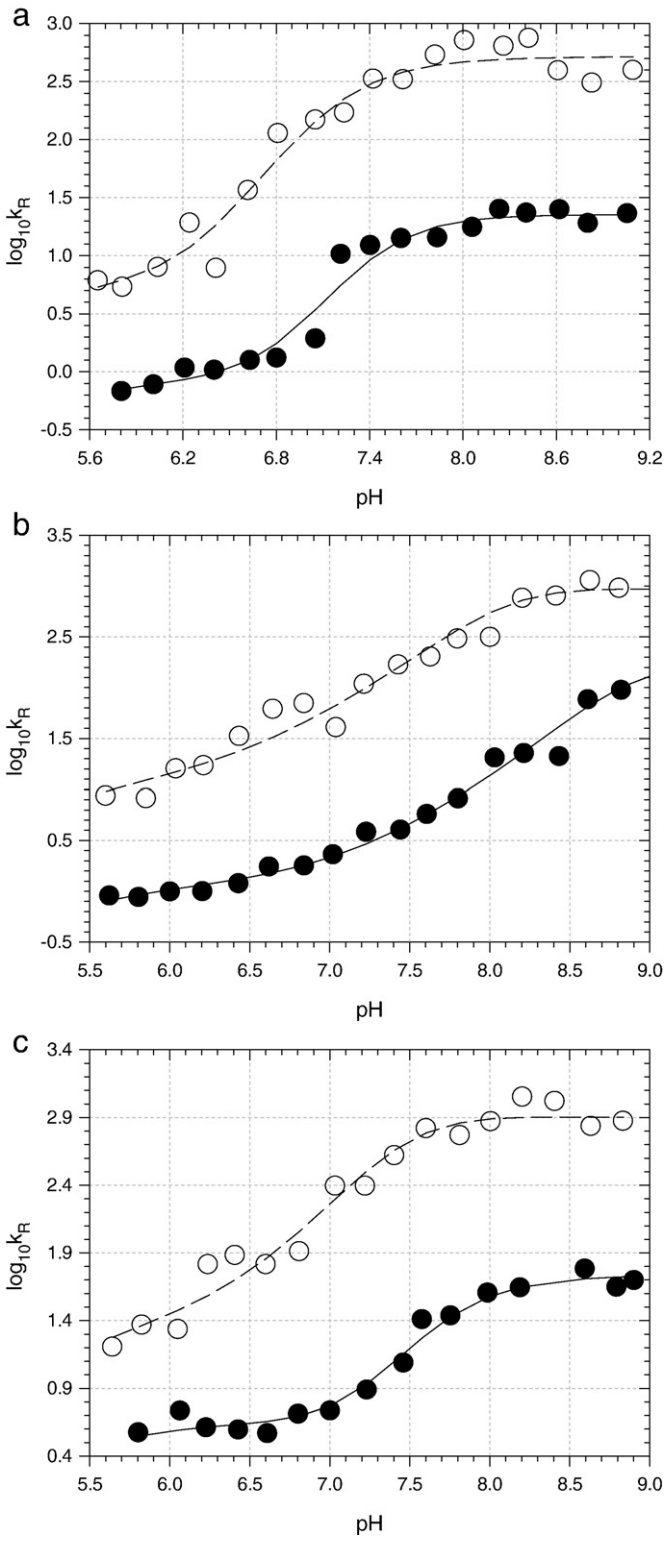
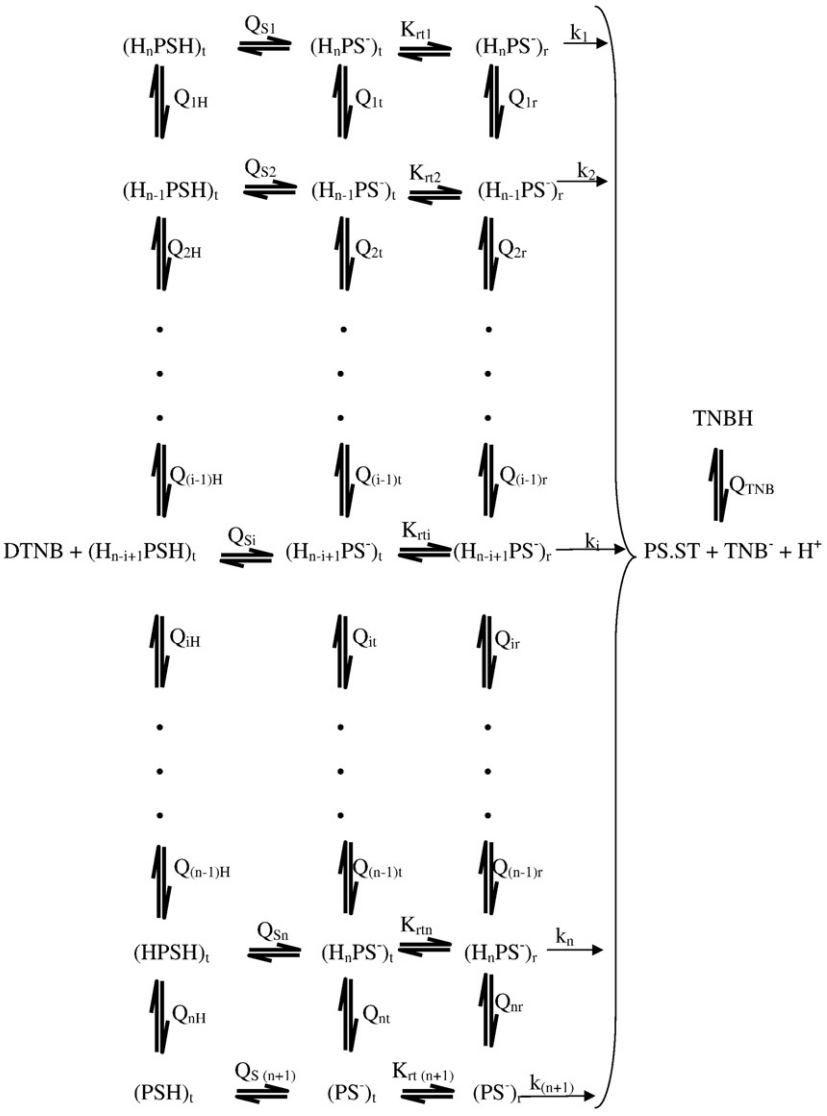


Fig. 4. Reaction of the minor sheep hemoglobin with DTNB: variation of k_R, the apparent second-order reverse rate constant, with pH. (a) oxyhemoglobin; (b) carbonmonoxyhemoglobin; (c) aquomethemoglobin. Open symbols, stripped hemoglobin; filled symbols, hemoglobin + inositol-P_6. Conditions as in Fig. 1. The lines through the data points are the best-fit lines calculated with the parameters reported in Tables 2 and 3 (cf. Scheme 2 and Eq. (4)).



Scheme 1.

Q_{si} are the dissociation constants of the sulphydryl group; $K_{\mathbf{rt}i}$ are the tertiary conformation transition constants; and k_i are the second-order forward rate constants for the reaction of DTNB with the various $(H_{n-i+1}PS^-)_{\mathbf{r}}$ species in the **r** tertiary conformation. The equation relating k_F to the parameters of Scheme 1 is:

$$k_F = \frac{k_{n+1} + \sum_{i=1}^{n} k_i [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jr} \right)^{-1}}{1 + \sum_{i=1}^{n} [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jr} \right)^{-1} + K_{\mathbf{rt}(n+1)} \left[1 + [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jt} \right)^{-1} + \frac{[H^+]}{Q_{s(n+1)}} \left\{ 1 + \sum_{i=1}^{n} [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jH} \right)^{-1} \right\} \right]} \quad (2)$$

It is seen that the transition constant for the thiol anion form of hemoglobin, $K_{\mathbf{rt}(n+1)}$, appears in the denominator of Eq. (2). Therefore, assuming the values of all the other parameters in Eq. (2) to be fixed, any factor that causes $K_{\mathbf{rt}(n+1)}$ to increase, will give rise to an increase in the denominator and, consequently, to a decrease in k_F.

We have already established [14] that inositol-P_6 increases the magnitude of $K_{\mathbf{rt}(n+1)}$. Therefore, from Eq. (2) we can predict that the magnitude of the second-order forward rate constant, k_F, will be lower for inositol-P_6-bound hemoglobin compared to the stripped hemoglobin. Our previous kinetic data on the reaction of DTNB with various hemoglobins, with and without inositol-P_6, support this prediction [20–23]. Although the inositol-P_6 effect is much greater in our previous reports [20–23] than for the sheep hemoglobin data presented here, the prediction contained in Eq. (2) is also justified by most of the data in Figs. 1 and 2 (see especially Fig. 2b and c).

Had we assumed that DTNB reacts preferentially with the **t** tertiary conformation, the scheme that would arise from such an assumption would give Eq. (3) in place of Eq. (2):

$$k_F = \frac{k_{n+1} + \sum_{i=1}^{n} k_i [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jt} \right)^{-1}}{1 + \sum_{i=1}^{n} [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jt} \right)^{-1} + \frac{1}{K_{\mathbf{rt}(n+1)}} \left[1 + [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{j\mathbf{r}} \right)^{-1} + \frac{[H^+]}{Q_{s(n+1)}} \left\{ 1 + \sum_{i=1}^{n} [H^+]^{n-i+1} \left(\prod_{j=i}^{n} Q_{jH} \right)^{-1} \right\} \right]} \quad (3)$$

Table 1
Reaction of DTNB with hemoglobin: Best-fit parameters used to fit the k_F data in Figs. 1 and 2 using Scheme 1 and Eq. (2) of the text, with $n=2$. Columns 2–7, stripped hemoglobin.

	Major oxy	Major carbon-monoxy	Minor oxy	Minor carbon-monoxy	Minor aquomet	Mean ± S.E.	Major oxy + inositol-P_6
pQ_{1H}	7.06	5.09	4.93	5.30	5.75	5.63 ± 0.4	5.25
pQ_{2H}	7.85	4.96	6.90	5.82	5.46	6.20 ± 0.6	6.42
$pQ_{1\mathbf{r}}$	6.00	6.01	6.91	5.15	4.89	5.79 ± 0.4	7.01
$pQ_{2\mathbf{r}}$	8.66	8.59	7.46	6.85	8.71	8.05 ± 0.4	6.49
$pQ_{1\mathbf{t}}$	7.17	8.98	9.04	5.32	4.89	7.08 ± 0.8	7.42
$pQ_{2\mathbf{t}}$	7.77	7.09	6.42	7.64	8.75	7.53 ± 0.5	7.34
pQ_{s3}	8.31	8.25	8.71	8.17	8.25	8.34 ± 0.1	8.19
$K_{\mathbf{rt}3}$	0.76	0.57	0.74	0.26	0.33	0.53 ± 0.1	1.58
k_1 mol^{-1} dm^3 s^{-1}	2404	91.2	259.8	2713	360.5		811.1
k_2 mol^{-1} dm^3 s^{-1}	44	52.1	616.2	100.9	58.6		3706.2
k_3 mol^{-1} dm^3 s^{-1}	5	0.7	29	31.5	0		13.9

Eq. (3) is similar to Eq. (2), except that all the $Q_{j\mathbf{r}}$ terms in the latter are replaced by $Q_{j\mathbf{t}}$ terms , and vice versa; and $\frac{1}{K_{\mathbf{rt}(n+1)}}$ appears in the denominator of Eq. (3) in place of $K_{\mathbf{rt}(n+1)}$ in Eq. (2). Assuming the values of the other parameters in Eq. (3) to be fixed, the equation predicts that in the presence of inositol-P_6 – with the consequent increase in $K_{\mathbf{rt}(n+1)}$ [14] – k_F should increase. This is contrary to previous DTNB kinetic data [20–23] and to most of the data presented in Figs. 1 and 2. We are led to conclude that DTNB reacts preferentially with the **r** tertiary conformation, at least in the range pH > 6.5. Therefore, in what follows we shall employ Scheme 1 and Eq. (2) to analyze the data in Figs. 1 and 2.

Scheme 1 is similar to the scheme that we employed previously to fit complex, bell-shaped pH dependence profiles for the reaction of DTNB with other hemoglobins [10,20,21], except that we have now included the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary transition in the present scheme. We employed Eq. (2) to fit the complex, bell/bowl-shaped profiles reported in Figs. 1 and 2, namely, Fig. 1a (open and filled symbols); 1b (open symbols); 2a (open symbols); 2b (open symbols); and 2c (open symbols). (We could not use this scheme to obtain sensible fits to the other data in Figs. 1 and 2 partly because some of the rate constants k_i became negative.) We fitted the bell/bowl-shaped data using an n value of 2. The best-fit lines are drawn through the data points, and the fitting parameters are reported in Table 1.

Some results in Figs. 1 and 2 remain to be explained, namely, the finding in Figs. 1a, b and 2a that at pH < 6.5 k_F is higher for inositol-P_6-bound compared to the stripped hemoglobin. These results can be rationalized on the basis of Eq. (3). This is the equation that arises if we assume that DTNB reacts preferentially with the **t** tertiary isomer. Thus, while DTNB may react with both the **r** and **t** isomers, for the sheep major and minor oxyhemoglobins, and the minor carbonmonoxyhemoglobin, reaction with the **r** isomer predominates at pH > 6.5 whereas reaction with the **t** isomer seems to predominate below pH 6.5.

3.1.2. Analyses of k_R profiles

As may be seen in Figs. 3 and 4, between pH 5.6 and 9.2 k_R varies by about two orders of magnitude for each hemoglobin derivative. These strong pH dependences of k_R suggest that the reverse reaction, like the forward reaction (see Eq. (1)), is linked to the ionization of certain groups on the protein. Moreover, k_R for stripped hemoglobin is greater than for inositol-P_6-bound hemoglobin by about an order of magnitude. To understand (i) the nature of the linked ionizable groups and (ii) the relative magnitudes of k_R, with and without inositol-P_6, we propose the following scheme (Scheme 2).

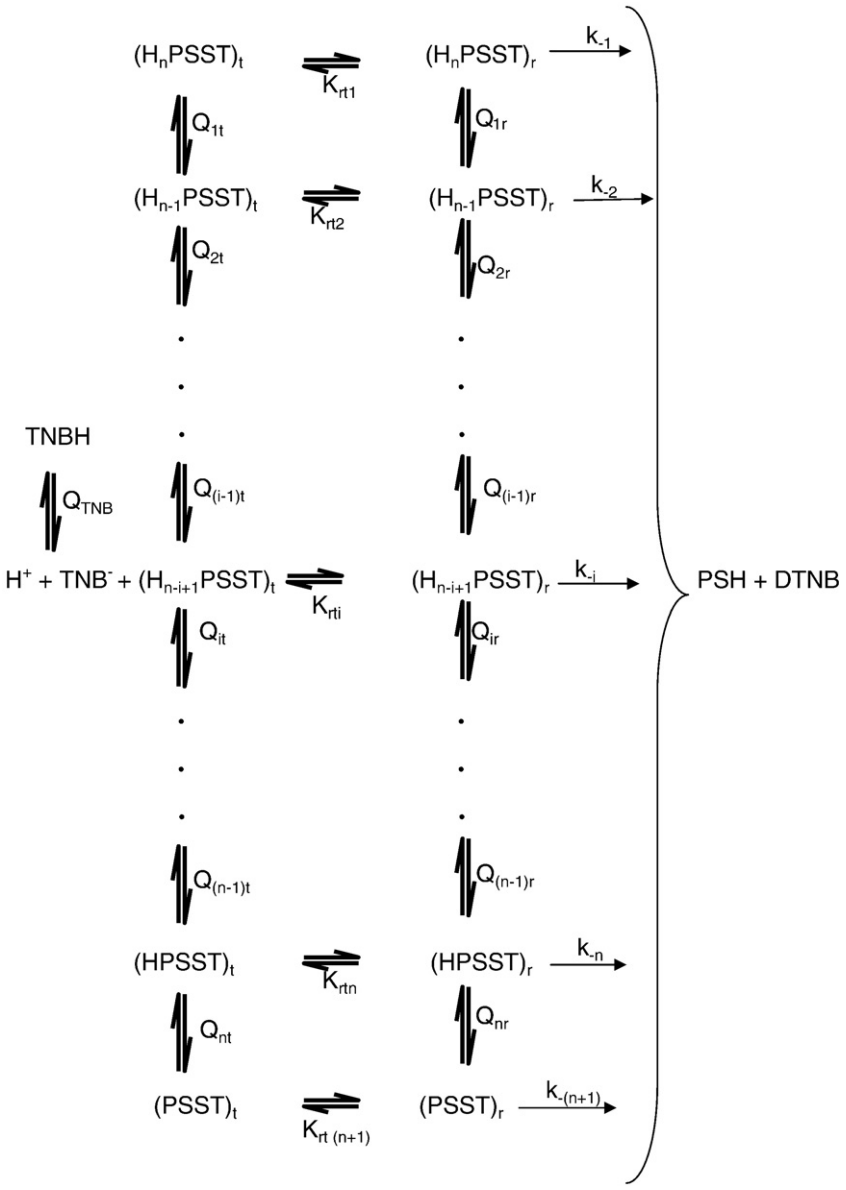
In Scheme 2, H_{n-i+1}PS.ST ($i=1, 2,\ldots, n+1$) are the mixed disulphide species in solution; k_{-i} ($i=1, 2,\ldots, n+1$) are the second-order reverse rate constants for the reactions of these species with TNB^-; $Q_{j\mathbf{r}}$ ($j=1, 2,\ldots, n$) are the dissociation constants for the release of the jth proton from H_{n-i+1}PS.ST in the **r** tertiary conformation; $Q_{j\mathbf{t}}$ ($j=1, 2,\ldots, n$) are the corresponding dissociation constants for the release of the jth proton from H_{n-i+1}PS.ST in the **t** tertiary conformation. The apparent second-order reverse rate constant, k_R, is related to the various parameters in Scheme 2 by Eq. (4).

$$k_R = \frac{\left[\frac{Q_{TNB}}{Q_{TNB}+[H^+]}\right]\left(k_{-(n+1)} + \sum_{i=1}^{n} k_{-i}[H^+]^{n-i+1}\prod_{j=i}^{n} Q_{j\mathbf{r}}{}^{-1}\right)}{\left(1+\sum_{i=1}^{n}[H^+]^{n-i+1}\prod_{j=i}^{n} Q_{j\mathbf{r}}{}^{-1}\right) + K_{\mathbf{rt}(n+1)}\left(1+\sum_{i=1}^{n}[H^+]^{n-i+1}\prod_{j=i}^{n} Q_{j\mathbf{t}}{}^{-1}\right)} \tag{4}$$

It is clear from Eq. (4) that the magnitude of k_R will, among other factors, be determined by the magnitude of $K_{\mathbf{rt}(n+1)}$, the constant for the tertiary structure transition *within the mixed disulphide species*. As reported previously [14], inositol-P_6 increases $K_{\mathbf{rt}(n+1)}$. Consequently, if one assumes that the values of all the other parameters in Eq. (4) are fixed, an increase in the value of $K_{\mathbf{rt}(n+1)}$ should cause the reverse rate constant, k_R, to be lowered in the presence of the effector. The results in Figs. 3 and 4 confirm this expectation: inositol-P_6 lowers the values of k_R by about an order of magnitude throughout the pH range 5.6 to 9. Using a pQ_{TNB} value of 5.27 [11], we quantitatively analyzed the data presented in these figures with Eq. (4). We obtained the best fits to the data with an n value of 2. The lines drawn through the *major* hemoglobin data in Fig. 3 are the best-fit lines; those in Fig. 4 are for the *minor* hemoglobin data. The fitting parameters for the stripped hemoglobin data in both figures are reported in Table 2; those for data obtained in the presence of inositol-P_6 are presented in Table 3.

It should be noted that in arriving at Eq. (4) from Scheme 2 we tacitly assumed that the **r** conformation of the mixed disulphide species, PS.ST, is the conformation with which TNB^- preferentially reacts. Had we assumed that reaction with the **t** conformation was preferred, we would have obtained Eq. (5) from the relevant alternative to Scheme 2:

$$k_R = \frac{\left[\frac{Q_{TNB}}{Q_{TNB}+[H^+]}\right]\left(k_{-(n+1)} + \sum_{i=1}^{n} k_{-i}[H^+]^{n-i+1}\prod_{j=i}^{n} Q_{j\mathbf{t}}{}^{-1}\right)}{\left(1+\sum_{i=1}^{n}[H^+]^{n-i+1}\prod_{j=i}^{n} Q_{j\mathbf{t}}{}^{-1}\right) + \frac{1}{K_{\mathbf{rt}(n+1)}}\left(1+\sum_{i=1}^{n}[H^+]^{n-i+1}\prod_{j=i}^{n} Q_{j\mathbf{r}}{}^{-1}\right)} \tag{5}$$



Scheme 2.

Eq. (5) predicts that in the presence of inositol-P_6 – with the consequent increase in $K_{\mathbf{rt}(n+1)}$ [14] – k_R should increase. Since this is contrary to the evidence in Figs. 3 and 4, we are led to conclude that in the reverse reaction with TNB^-, $PS.ST + TNB^- \xrightarrow{k_R} PS^- + DTNB$, (cf Eq. (1)), the preferred tertiary conformation of PS.ST is the **r** conformation. This conclusion is identical with the conclusion we reached above for the forward reaction of the thiol anion species, PS^-, with DTNB.

3.2. Assignment of pQ_i values

3.2.1. Assignment of pQ_i values for forward reaction

We demonstrated previously that at an ionic strength of 50 mmol dm^{-3} the reaction of the negatively charged DTNB at the F9[93]β site of hemoglobin (see Eq. (1)) is subject to the electrostatic effects of the positively charged groups at the organic phosphate binding site [10,20,21]. In human hemoglobin these groups are ValNA1[1]β, HisNA2[2]β, HisH21[143]β and LysEF6[82]β [24,25]. With the exception of LysEF6[82]β, which has $pK_a \approx 10.5$, these groups are ionizable in the pH range 5.6 to 9 of our experiments. In sheep hemoglobins there is a deletion of one amino acid at the beginning of the β chain, and HisNA2[2]β is mutated to a methionine residue, which now becomes the NH_3^+-terminal residue [26,27]. (In what follows we retain the usual system of numbering the sequence were there no deletion.) With these changes, the only ionizable organic phosphate binding groups left in the sheep hemoglobins are the NH_3^+-terminal MetNA2[2]β and HisH21[143]β. Thus we expect two ionizable groups to be linked to the reaction of DTNB with CysF9[93]β, in agreement with the value $n = 2$ required to fit Eq. (2) to our data in Figs. 1 and 2.

For the stripped hemoglobins (see Table 1), we assign the mean $pQ_{1\mathbf{r}}$ value of 5.79 ± 0.4 to HisH21[143]β in the **r** tertiary conformation and the mean $pQ_{2\mathbf{r}}$ value of 8.05 ± 0.4 to the β-chain terminal NH_3^+ group; we assign the mean $pQ_{1\mathbf{t}}$ value of 7.08 ± 0.8 to HisH21[143]β in the **t** conformation, and the mean $pQ_{2\mathbf{t}}$ value of 7.53 ± 0.5 to the β-chain terminal NH_3^+ group. Thus $pQ_{1\mathbf{t}} > pQ_{1\mathbf{r}}$ by 1 pQ unit, but $pQ_{2\mathbf{t}} < pQ_{2\mathbf{r}}$ by 0.5 of a pQ unit.

Table 2
Reaction of DTNB with stripped hemoglobin: Best-fit parameters employed to fit the reverse kinetics data reported in Figs. 3 and 4 (open symbols) using Scheme 2 and Eq. (4) of the text, with $n=2$.

	Major oxy	Major carbon-monoxy	Major aquomet	Minor oxy	Minor carbon-monoxy	Minor aquomet	Mean ± S.E.
$pQ_{1\mathbf{r}}$	6.94	6.59	6.63	6.29	6.83	6.61	6.65 ± 0.1
$pQ_{2\mathbf{r}}$	7.09	6.33	7.10	7.33	7.92	6.90	7.11 ± 0.3
$pQ_{1\mathbf{t}}$	7.34	6.61	7.15	6.84	7.71	7.21	7.14 ± 0.2
$pQ_{2\mathbf{t}}$	7.03	8.48	7.88	7.74	8.91	8.26	8.05 ± 0.3
$K_{\mathbf{rt}3}$	0.77	0.54	1.05	1.33	0.74	0.40	0.81 ± 0.2
k_{-1} mol^{-1} dm^3 s^{-1}	811	40.7	253.5	93.5	674	839.7	
k_{-2} mol^{-1} dm^3 s^{-1}	3706	≈0	2057.4	≈0	1795	1618.7	
k_{-3} mol^{-1} dm^3 s^{-1}	13.9	1763	1072.2	1212	1580	1110.4	

It is rather unfortunate that we were able to fit only one of the k_F data sets for inositol-P_6-bound hemoglobin: that of the oxy derivate of the major hemoglobin. This allows only a limited comparison of the data, with and without inositol-P_6 (columns 2 and 8 of Table 1). It is seen that $pQ_{1\mathbf{r}}$ and $pQ_{1\mathbf{t}}$ are lower for stripped compared to inositol-P_6-bound oxyhemoglobin, but $pQ_{2\mathbf{r}}$ and $pQ_{2\mathbf{t}}$ are higher; the pQ_{s3} values for the sulphydryl ionization are in reasonable agreement. More interestingly, $K_{\mathbf{rt}3}$ for the major oxyhemoglobin is increased 2-fold by the organic phosphate, in line with our previous result [14]. From these $K_{\mathbf{rt}3}$ values we calculate, for the major oxyhemoglobin, **t** conformation populations of 43.2% and 61.2%, with and without inositol-P_6. It is difficult to rationalize the values of the rate constants k_i: although the k_1 value is decreased by inositol-P_6, as expected, the k_2 and k_3 values are increased.

3.2.2. Assignment of pQ_i values for reverse reaction

From the parameters calculated from Scheme 2 and Eq. (4) for stripped hemoglobin (see Table 2), the mean value of $pQ_{1\mathbf{r}}$ is 6.65 ± 0.1 and the mean $pQ_{2\mathbf{r}}$ value is 7.11 ± 0.3. We assign these values respectively to HisH21[143]β and the NH_3^+ terminal group of the β chain in the **r** tertiary conformation. The corresponding pQ values in the **t** conformation are $pQ_{1\mathbf{t}} = 7.14 \pm 0.2$ and $pQ_{2\mathbf{t}} = 8.05 \pm 0.3$. In the presence of inositol-P_6 (see Table 3) the values are: $pQ_{1\mathbf{r}} = 7.12 \pm 0.4$; $pQ_{2\mathbf{r}} = 8.11 \pm 0.2$; $pQ_{1\mathbf{t}} = 7.04 \pm 0.5$; and $pQ_{2\mathbf{t}} = 8.41 \pm 0.3$. Comparison of these later values with the corresponding values for stripped hemoglobin shows that, with the exception of $pQ_{1\mathbf{t}}$, all the pQ values are increased in the presence of inositol-P_6. A similar result was obtained from the equilibrium data (Tables 3 and 4 of [14]). Increases in pQs of the organic phosphate binding groups are expected as the negatively charged effector approaches the positively charged basic groups at the binding site.

3.3. Effect of inositol-P_6 on k_{-i} and $K_{\mathbf{rt}3}$ values

Comparison of the last three rows of Tables 2 and 3 reveals that inositol-P_6 decreases the values of the reverse rate constants — in some cases by more than an order of magnitude. These decreases in the k_{-i} values of the various species (H_2PS.ST, HPS.ST and PS.ST) contribute to the overall decrease of k_R by inositol-P_6 (Figs. 3 and 4).

Further examination of Tables 2 and 3 reveals that inositol-P_6 increases the mean values of $K_{\mathbf{rt}3}$, the constant for the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary structure transition, from 0.81 ± 0.2 to 3.87 ± 0.5, that is 4.8-fold. This increase translates into a change in the relative **t** isomer population from 44% to 79.6%. These results, which have been obtained from analyses of reverse kinetics data, are in reasonably good agreement with the corresponding values obtained from analyses of equilibrium constant data for the reaction of DTNB with the sheep hemoglobins [14].

4. Discussion

4.1. Origin of the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary structure transition

Since we have recently reported on the $\mathbf{r} \rightleftharpoons \mathbf{t}$ equilibrium from studies of the reaction of CysF9[93]β with DTNB, it might *erroneously* be construed that the tertiary **t** isomer is distinguishable from the tertiary **r** isomer only by reason of this reaction. In other words, the $\mathbf{r} \rightleftharpoons \mathbf{t}$ equilibrium might be *erroneously* construed as having no independent existence of its own, apart from CysF9[93]β or its reaction with sulphydryl reagents.

We successfully employed the temperature-jump technique to study the kinetics of the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary structure transition in stripped deoxyhemoglobin and carbonmonoxyhemoglobin, under conditions *in which CysF9[93]β had not reacted, and was not reacting,* with any sulphydryl reagent [9]. In other words, CysF9[93]β had *nothing* to do with the transition as studied by temperature-jump.

We also reported that the $\mathbf{r} \rightleftharpoons \mathbf{t}$ transition was completely abolished in hemoglobin after the CysF9[93]β sulphydryl was modified with iodoacetamide (compare circles and triangles in Figs. 2 and 4 of [9]). This abolition arose because iodoacetamide bound to the sulphydryl *irreversibly*, thereby shifting the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary equilibrium entirely to

Table 3
Reaction of DTNB with hemoglobin plus inositol-P_6: Best-fit parameters employed to fit the reverse kinetics data reported in Figs. 3 and 4 (filled symbols) using Scheme 2 and Eq. (4) of the text, with $n=2$.

	Major oxy	Major carbon-monoxy	Major aquomet	Minor oxy	Minor carbon-monoxy	Minor aquomet	Mean ± S.E.
$pQ_{1\mathbf{r}}$	8.14	5.99	6.68	6.37	7.35	8.17	7.12 ± 0.4
$pQ_{2\mathbf{r}}$	7.66	7.98	8.53	7.50	9.00	7.98	8.11 ± 0.2
$pQ_{1\mathbf{t}}$	6.33	6.46	6.80	6.34	9.29	7.02	7.04 ± 0.5
$pQ_{2\mathbf{t}}$	8.73	9.01	8.80	8.68	7.98	7.27	8.41 ± 0.3
$K_{\mathbf{rt}3}$	5.32	3.67	3.33	3.91	2.30	4.68	3.87 ± 0.5
k_{-1} mol^{-1} dm^3 s^{-1}	≈0	61.0	53.5	50.2	23.2	4.8	
k_{-2} mol^{-1} dm^3 s^{-1}	183.2	2.4	6.3	≈0	39.8	≈0	
k_{-3} mol^{-1} dm^3 s^{-1}	374.4	125	516.9	111.5	448.2	306.5	

the right in favor of the **t** conformation. Thus although the **r** ⇌ **t** tertiary equilibrium exists as an independent process *when no reaction has taken place, or is taking place, at the CysF9[93]β site,* it is affected when a reaction has taken place, or is taking place, at this site. It is only in this way that it is linked to the CysF9[93]β site.

We subsequently discovered that DTNB reacts *reversibly* with the CysF9[93]β sulphydryl [10–14]. If iodoacetamide binds *irreversibly* to the CysF9[93]β site, thereby shifting the **r** ⇌ **t** equilibrium completely to the right and making it impossible to detect [9], it follows that, since DTNB reacts with CysF9[93]β *reversibly*, it cannot shift the **r** ⇌ **t** equilibrium completely to the right but can only influence its relative position. This is the justification for including the **r** ⇌ **t** tertiary equilibrium in Schemes 1 and 2. Nevertheless, it must continue to be borne in mind that the **r** ⇌ **t** tertiary equilibrium can exist, and can be studied and defined, independently of the CysF9[93]β sulphydryl [9], just as oxygen binding to hemoglobin can be studied independently of CysF9[93]β, even though the oxygen affinity of hemoglobin is affected when the sulphydryl is chemically modified [15–18].

We conclude that the **r** ⇌ **t** equilibrium is an intrinsic property of the hemoglobin molecule and that it is highly probable that it exists in hemoglobins that do not have CysF9[93]β, such as those of fish and amphibians, which have a serine residue at the F9[93]β site.

4.2. Forward kinetics

The data on the forward kinetics of stripped hemoglobin are limited (see Table 1, columns 2–7) because we could not fit all the pH dependence profiles in Figs. 1 and 2. The data are even more limited for inositol-P_6-bound hemoglobin: only the data on the major oxy derivative could be fitted (Table 1, column 8). In spite of this limitation, one can tentatively conclude from Eq. (2) that inositol-P_6 reduces the forward DTNB reaction rate constant (Figs. 1 and 2) by increasing K_{rt3}, and hence the population of the **t** tertiary isomer (compare the major oxy data, columns 2 and 8 of Table 1, row 4 from the bottom).

It should be noted that the mean parameters reported for the forward reaction, $PS^- + DTNB \xrightarrow{k_F} PS.ST + TNB^-$, (column 7 of Table 1) refer to the stripped hemoglobin in which CysF9[93]β *has not reacted with DTNB* (see Scheme 1). For this the mean value of $K_{\mathbf{rt3}}$ is 0.53 ± 0.1. This value gives a **t** isomer population of 34.6% (±4). This system is similar to the system that we studied by temperature-jump [9], which involves hemoglobin in which the sulphydryl has not reacted with any reagent.

4.3. Reverse kinetics

The most interesting result obtained in this study is the *ca* one order of magnitude reduction – by inositol-P_6 – of the apparent second-order reverse rate constant, k_R (Figs. 3 and 4). This reduction arises (see Eq. (4)) from the fact that inositol-P_6 increases the transition constant, K_{rt3}, for the **r** ⇌ **t** tertiary conformational transition (compare Tables 2 and 3). The reduction of k_R was expected theoretically, since K_{rt3} appears in the denominator of Eq. (4). It is gratifying that the theoretical prediction has been confirmed by the experimental results, even though the k_R values were determined indirectly from the forward rate constant (k_F) and the equilibrium constant (K_{equ}) data. Previous reports on the effect of inositol-P_6 on the kinetics of reaction of sulphydryl reagents with CysF9[93]β were confined to the forward reaction. This report is the *first* on the effect of inositol-P_6 on both the forward and reverse kinetics of reaction of a sulphydryl reagent.

4.4. Ligand- and effector-induced shifts in the **r** ⇌ **t** equilibrium

It should be noted that the mean parameters reported for the forward reaction, $PS^- + DTNB \xrightarrow{k_F} PS.ST + TNB^-$, (column 7 of Table 1) refer to stripped hemoglobin *in which CysF9[93]β has not reacted with DTNB* (see Scheme 1). For this the mean value of K_{rt3} is 0.53 ± 0.1. This value gives a **t** isomer population of 34.6% (±4). On the other hand, the mean parameters in Table 2 (column 8) refer to stripped hemoglobin in which CysF9[93]β is in the mixed disulphide form, that is, *CysF9[93]β has reacted with DTNB.* For this, the mean K_{rt3} value is 0.81 ± 0.2, giving a **t** isomer population of 44.7% (± 5). Thus reaction with DTNB, leading to the formation of the mixed disulphide, gives rise to a 10% increase in the **t** isomer population, *even in the absence of inositol-P_6.* This is a ligand-induced shift in the allosteric equilibrium between the tertiary **r** and **t** states.

Moreover, a comparison of the mean value of K_{rt3} in column 8 of Table 2 (stripped hemoglobin; **t** isomer population, $44.7 \pm 5\%$) with the mean K_{rt3} value in column 8 of Table 3 (hemoglobin + inositol-P_6; **t** isomer population, $79.5 \pm 4\%$) shows that inositol-P_6 increases the **t** isomer population by 34.8%. This is an effector-induced shift in the allosteric equilibrium between the tertiary **r** and **t** states.

In summary, we have in this study been able to capture both a ligand- and an effector-induced allosteric transition *at the level of the tertiary structure*.

4.5. Interpretation of changes induced by allosteric effectors in terms of tertiary **r** ⇌ **t** or quaternary *R* ⇌ *T* transitions

Heterotropic effector molecules — 2,3-bisphosphoglycerate (2,3-BPG), inositol-P_6 and bezafibrate — lower the oxygen affinity of hemoglobin [28–36] and the reactivity of the CysF9[93]β sulphydryl group [22]. The interpretation of these results in terms of a shift in the equilibrium between the R and T quaternary states in favor of the T state has been questioned with respect to O_2 binding [32,33,35,36] and the reactivity of CysF9[93]β [14]. The current wisdom is that these results are more accurately interpreted in terms of the effects of tertiary, rather than quaternary, structure transitions [37,38].

With particular reference to CysF9[93]β, the greatest challenge is to provide an explanation for the observation that 2,3-BPG and inositol-P_6 lower its reactivity in human oxyhemoglobins A and S, which have the R quaternary structure [22]. Our theoretical considerations (Scheme 1; Eq. (2)) lead to the conclusion that the rates of the forward and reverse reactions of DTNB with CysF9[93]β depend on the position of the tertiary **r** ⇌ **t** equilibrium: a shift in favor of the **t** isomer decreases the rates of both the forward and reverse reactions. A shift in the position of the **r** ⇌ **t** equilibrium in favor of the **t** isomer is the only plausible explanation for the observed decrease in sulphydryl reactivity of human oxyhemoglobins A and S induced by effectors in the *absence of a change in quaternary structure* [22].

4.6. Possible implications for physiological function

The result that inositol-P_6 reduces the rate of the reverse reaction has possible implications for the preservation of the physiological function of hemoglobin. Reduced glutathione (GSH) protects hemoglobin and other red blood cell protein sulphydryl groups from oxidation to the mixed disulphide form [19]. The reverse reaction of Eq. (1) is comparable to the action of GSH in protecting protein sulphydryl groups. A hemoglobin with its CysF9[93]β sulphydryl group in the mixed disulphide, or in the modified, form loses its normal physiological function [15–18]. Consequently, any agent that keeps the protein in this form would tend to make it dysfunctional. It is therefore a bit puzzling that inositol-P_6 does exactly that in sheep hemoglobins by lowering – by one order of magnitude – the rate of the conversion of the mixed disulphide of CysF9[93]β to the thiol anion form. This is the major reason for the inositol-P_6-induced increased affinity of sheep hemoglobins for DTNB [14].

It is known that the concentration of the natural allosteric effector, 2,3-BPG, is low in sheep erythrocytes [39]. This may not be unconnected with the finding that inositol-P_6 reduces the reverse rate constant of the DTNB reaction. If, as is highly likely, 2,3-BPG, like inositol-P_6, reduces the reverse rate constant for the DTNB reaction, then it is physiologically advantageous that its concentration in sheep erythrocytes be as low as possible in order to preserve normal physiological function.

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Appendix A

In analyzing the data in Figs. 1 and 2 with Eq. (2) and those in Figs. 3 and 4 with Eq. (4) we tacitly assumed that the rates of the protolytic steps and of the tertiary conformational transition in Schemes 1 and 2 are much faster than the forward and reverse reactions, respectively. Justification for these assumptions is provided below.

In deriving Eqs. (2) and (4) we tacitly assumed that the protolytic steps and the tertiary transition steps in Schemes 1 and 2 are much faster than the forward and reverse reactions of Eq. (1). We have already justified these assumptions for the forward reaction [10]. These assumptions may be justified for the reverse reaction as follows. From Figs. 3 and 4 the maximum value of k_R is about $1000\ mol^{-1}\ dm3\ s^{-1}$. The highest concentration of DTNB employed in our experiments for the forward reaction was $600\ \mu mol\ dm^{-3}$. Although the concentration of TNB^- involved in the reverse reaction is nowhere near this value, for the purpose of argument we assume that we had $[TNB^-] = 600\ \mu mol\ dm^{-3}$. The maximum value of the product $k_R \times [TNB^-]$ would then be $1000\ mol^{-1}\ dm^3\ s^{-1} \times 600 \times 10^{-6}\ mol\ dm^{-3} = 0.6\ s^{-1}$. To compare this rate with the rate of the protolytic steps, we note that the lowest protolytic rate will occur where the H^+ concentration is least. Under our experimental conditions this will be at $pH \approx 9$. Assuming $[H^+] \approx 10^{-9}\ mol\ dm^{-3}$, and using the second-order rate constant of $10^{10}\ mol^{-1} dm^3\ s^{-1}$ observed for protolytic processes [40–42], we obtain a velocity of $10\ s^{-1}$. Thus the lowest protolytic rate is at least an order of magnitude higher than $0.6\ s^{-1}$, the fastest rate of the reverse reaction of Eq. (1). Furthermore, the rate $0.6\ s^{-1}$ is much slower than the rate of about $10^4 s^{-1}$ observed for the tertiary structure transition [9]. Thus the assumptions of fast protolytic and tertiary transition steps are correct.

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