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Transition of haemoglobin between two tertiary conformations: Inositol hexakisphosphate increases the transition constant and the affinity of sheep haemoglobin for 5,5′-dithiobis(2-nitrobenzoate)

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ABSTRACT

The equilibrium constant (K_{equ}) for the reaction of 5,5′-dithiobis(2-nitrobenzoate) — DTNB — with the CysF9 [93]β sulphydryl group of the haemoglobins of the sheep decreases by about two orders of magnitude between pH ≈ 5.6 and 9.2: from a mean of 7.2 ± 1 to a mean of 0.044 ± 0.01. Calculations from the pH dependence of K_{equ} show that in the **r** ⇌ **t** tertiary conformational transition of haemoglobin the **t** isomer population is 50.7 and 61.8% for the major and minor haemoglobins, respectively. In the presence of inositol hexakisphosphate (inositol-P_6), K_{equ} increases for both haemoglobins by about an order of magnitude through most of the pH range. The **t** isomer population also increases to 82.1 and 79.6% for the major and minor haemoglobins, respectively. These results indicate that inositol-P_6 increases the affinity of the sulphydryl for DTNB by increasing the population of the **t** isomer. It is highly probable that a minimum four-state model that includes the **r** ⇌ **t** transition is required for a full understanding of haemoglobin function.

1. Introduction

The CysF9[93]β sulphydryl group has been employed as an indicator for tertiary and quaternary structure change in haemoglobin [1–9]. In ligand binding studies of ferrous haemoglobin it is usually difficult to separate these two structure changes. A clear separation of the two structure changes became possible from the results of an X-ray structure study of oxyhaemoglobin at 2.1 Å resolution, which showed that the CysF9[93]β sulphydryl exists in two conformations [8]. In a temperature-jump kinetic study of deoxy- and of carbonmonoxyhaemoglobin [9], we demonstrated that the two sulphydryl conformations are coupled to a tertiary structure transition in haemoglobin.

References to the tertiary structure transition in haemoglobin have generally been subsumed under discussions of what, for ligand binding to ferrous haemoglobin, was thought to be the more important structural transition: the R ⇌ T quaternary transition. However, recent molecular dynamics evidence seems to favour the tertiary structure transition as the more important transition in ligand binding to ferrous haemoglobin [10].

The high oxygen affinity quaternary R form of haemoglobin has been associated with a higher reactivity of the CysF9[93]β sulphydryl group compared to the reactivity of this sulphydryl in the low oxygen affinity T quaternary form. Heterotropic effector molecules — such as 2,3-bisphosphoglycerate (2,3-BPG), inositol hexakisphosphate (inositol-P_6) and bezafibrate — lower the oxygen affinity of haemoglobin [11–19] and the reactivity of the CysF9[93]β sulphydryl group. These results have been interpreted in terms of a shift in the equilibrium between the R and T quaternary states in favour of the T state, the shift arising from the preferential binding of effector molecules to the T state. This conclusion has been questioned for a number of reasons: (i) The results of haemoglobin–oxygen equilibrium binding studies have demonstrated that 2,3-BPG not only binds preferentially to the T quaternary state but also lowers the oxygen affinity of the T state [19]. (ii) It has been demonstrated that the oxygen affinity of the R state falls in the presence of bezafibrate without a change in quaternary structure [15,16]. (iii) Kinetic experiments have demonstrated that the oxygen affinity of the R (and also of the T) quaternary state decreases in the presence of allosteric effectors [18]. (iv) Similarly, kinetic experiments have demonstrated that the reactivity of CysF9 [93]β in the R quaternary state decreases in the presence of 2,3-BPG and of inositol-P_6 [20]. These findings indicate that structural changes at the quaternary level are not the only determinants of oxygen affinity or of CysF9[93]β reactivity but that structural changes at the tertiary level must also be considered as important determinants of both parameters.

The CysF9[93]β sulphydryl group of haemoglobin in the R (and also in the T) quaternary state of haemoglobin exists in two conformations relative to the main chain: *cis*-to-amino and *cis*-to-carbonyl [8,9]. These two conformations are coupled to two tertiary isomeric forms of haemoglobin (**r** and **t**, respectively) in dynamic equilibrium [9].

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Evidence for the **r** ⇌ **t** isomerization process comes from temperature-jump studies on carbonmonoxy- and deoxyhaemoglobin: We found that τ^{-1}, the reciprocal relaxation time for the isomerization, varies strongly with pH (circles in Figs. 2 and 4 of [9], respectively). However, the pH dependence of τ^{-1} is abolished when CysF9[93]β is modified with iodoacetamide (triangles in Figs. 2 and 4 of [9]). This arises because iodoacetamide binds irreversibly to this thiol group and tilts the **r** ⇌ **t** equilibrium fully to the right, in favour of the **t** isomer [9]. We demonstrate that the reaction of the CysF9[93]β sulphydryl group of the haemoglobins of the sheep with 5,5′-dithiobis(2-nitrobenzoate) — DTNB — is an equilibrium process. Using this finding, we have determined K_{rt}, the equilibrium constant for the **r** ⇌ **t** tertiary structure transition in sheep haemoglobins. The influence of allosteric effectors on this transition has not yet been tested [21–24]. With this in mind, we have embarked on a pH dependence study of the reaction of DTNB with CysF9[93]β of sheep haemoglobins, stripped as well as in the presence of inositol-P_6. We find that inositol-P_6, in a 4:1 molar ratio with respect to haemoglobin tetramers, increases the relative population of the **t** isomer from 50.7 (±2) to 82.1 (±1)% in the major haemoglobin, and from 61.8 (±4) to 79.6 (±2)% in the minor haemoglobin. We also find that inositol-P_6 *reduces* the reactivity of CysF9[93]β, while *increasing* its affinity for DTNB. These results imply that an increase in the population of the **t** isomer may be associated with a reduction in the reactivity of the CysF9[93]β sulphydryl group of sheep haemoglobins and with an increase in its affinity for DTNB.

2. Materials and methods

Sheep blood was obtained from the local abattoir. The blood was collected in bottles containing freshly prepared acid–citrate–dextrose anticoagulant. Haemoglobin was prepared as described previously [21], except that normal saline (9.5 g NaCl per dm^{-3}) was used to wash the red blood cells. It was stored as the carbonmonoxy derivative. The stock DTNB solution was prepared as previously described [22].

2.1. Separation of haemoglobins

Sheep haemolysate contains two haemoglobins, major and minor. The separation of the haemoglobins was achieved as follows in a cold room at 5 °C. A 3 cm (diameter) by 30 cm column of Whatman CMC-52 carboxymethylcellulose, a microgranular, preswollen cation exchanger, was used. The resin was pre-equilibrated with 10 mmol dm^{-3} phosphate buffer, pH 6.5. The minor haemoglobin was completely eluted with the pH 6.5 buffer, whereas the major component remained bound to the resin. The major haemoglobin was eluted with phosphate buffer, pH 8.0, ionic strength 0.2 mol dm^{-3}. The haemoglobins were stored in the freezer and thawed when required. Prior to use for experiments, each haemoglobin was passed through a Dintzis ion exchange column [25] to remove endogenous organic phosphates and undesired ions.

2.2. Preparation of buffer solutions for equilibrium and kinetics measurements

Phosphate buffers ($5.6 \le pH \le 8.0$) were prepared as follows: 25 cm^3 portions of a stock solution of NaH_2PO_4 (0.4 mol dm^{-3}) were mixed with the appropriate volumes of a stock solution of NaOH (0.4 mol dm^{-3}) in 1 dm^3 standard flasks. The appropriate weight of NaCl was added to each flask so that when the solution was made up to the mark with distilled water the total ionic strength was 50 mmol dm^{-3}. Borate buffers ($8.0 \le pH \le 9.0$) were similarly prepared, except that 250 cm^3 portions of boric acid (0.3 mol dm^{-3}) were mixed in 1 dm^3 standard flasks with the appropriate volumes of 0.3 mol dm^{-3} NaOH. The appropriate amount of NaCl required to make the final ionic strength 50 mmol dm^{-3} was added to each flask and the solution made up to the mark with distilled water. The pH of each prepared buffer was checked for correctness. It should be noted that the pH values reported in this paper are those of experimental solutions.

2.3. Equilibrium measurements for the reaction of DTNB with haemoglobin

The method [22] employed for the determination of equilibrium constants, K_{equ}, was adopted with some modifications. For measurements involving stripped haemoglobin, 10 cm^3 aliquots of haemoglobin (50 μmol (haem) dm^{-3}) in a buffer at a given pH were measured into several clean, dry tubes. Increasing volumes (between 1 and 120 mm^3) of a stock 29 mmol dm^{-3} DTNB solution, prepared as detailed in [22], were added to the different tubes. The mixtures were stirred and left to equilibrate at 25 °C for about 6 h. The absorbances were determined with a Zeiss PMQ II uv–visible spectrophotometer using a 2 cm light path cuvette. The reference solution was a 50 μmol (haem) dm^{-3} haemoglobin solution to which no DTNB had been added. The transmittance of this reference solution was adjusted to 100% (zero absorbance) by increasing the slit width. Measurements in the presence of inositol-P_6 were similarly carried out, except that 3 cm^3 aliquots of haemoglobin (25 μmol (haem) dm^{-3}) were used. The inositol-P_6 concentration was 25 μmol dm^{-3}, that is, a 4:1 molar ratio with respect to haemoglobin tetramers. The volume of stock DTNB added to the tubes containing haemoglobin was between 1 and 30 mm^3. The absorbances of the mixtures were determined on a Cecil Bioquest instrument. The absorbance of the 25 μmol (haem) dm^{-3} reference solution was adjusted to zero by pressing the 'auto zero' button. The absorbance change, ΔA_{412}, of each haemoglobin/DTNB mixture was then determined at 412 nm relative to this zero absorbance. A molar absorption coefficient of 14,000 mol^{-1} dm^3 cm^{-1} was assumed for 5-thio-2-nitrobenzoate (TNB), the chromophoric product of the reaction. The TNB concentration so determined was substituted into Eq. (3) below to obtain K_{equ}. Tables 1 and 2 show tabulations of the raw data for the oxy derivative of the major

Table 1
Reaction of DTNB with CysF9[93]β of the major sheep oxyhaemoglobin: raw data for the determination of K_{equ}. (a) [Hb] = 50 μmol (haem) dm^{-3}; pH 5.71; 25 °C; (b) [Hb] = 25 μmol (haem) dm^{-3}; pH 5.65; [inositol-P_6]: [Hb_4] = 4:1; 25 °C

DTNB (mm^3)	ΔA_{412}	[TNB] (μmol dm^{-3})	K_{equ}
(a)			
7	0.068	2.428	8.495
15	0.064	2.286	3.141
20	0.082	2.929	4.018
25	0.086	3.071	3.529
30	0.092	3.286	3.392
35	0.09	3.214	2.747
40	0.08	2.857	1.838
45	0.085	3.036	1.863
50	0.09	3.214	1.900
60	0.09	3.214	1.577
70	0.092	3.286	1.416
80	0.088	3.143	1.120
90	0.096	3.429	1.208
Mean			2.788 ± 0.56
(b)			
3	0.062	4.429	87.31
4	0.059	4.214	52.44
6	0.07	5.00	57.13
8	0.057	4.071	21.73
10	0.091	6.50	92.28
12	0.08	5.714	43.33
16	0.079	5.643	30.44
18	0.089	6.357	44.23
20	0.089	6.357	39.61
Mean			52.05 ± 7.3

Errors quoted for K_{equ} are standard errors. Stock [DTNB] = 29 mmol dm^{-3}. ΔA_{412} is the change in absorbance at 412 nm relative to a reference haemoglobin solution instrumentally adjusted to zero absorbance.

Table 2
Reaction of DTNB with CysF9[93]β of the major sheep oxyhaemoglobin: raw data for the determination of K_{equ}. (a) [Hb] = 50 μmol (haem) dm^{-3}; pH 8.59; 25 °C; (b) [Hb] = 25 μmol (haem) dm^{-3}; pH 8.64; 25 °C; [inositol-P_6]: [Hb_4] = 4:1. Stock [DTNB] = 29 mmol dm^{-3}

DTNB (mm^3)	ΔA_{412}	[TNB] (μmol dm^{-3})	K_{equ}
(a) Stripped haemoglobin			
2	0.044	1.571	0.038
5	0.075	2.679	0.041
7	0.090	3.214	0.042
10	0.100	3.571	0.036
15	0.140	5.0	0.049
20	0.160	5.714	0.049
25	0.185	6.607	0.055
30	0.205	7.321	0.058
35	0.230	8.214	0.066
40	0.250	8.929	0.071
45	0.262	9.357	0.070
50	0.280	10.0	0.075
60	0.310	11.071	0.082
70	0.330	11.786	0.084
Mean			0.058 ± 0.003
(b) Haemoglobin + inositol-P_6			
1	0.051	3.643	0.363
2	0.058	4.143	0.197
3	0.042	3.00	0.053
6	0.072	5.143	0.099
8	0.116	8.286	0.345
10	0.113	8.071	0.243
12	0.127	9.071	0.329
14	0.126	9.00	0.269
16	0.114	8.143	0.152
18	0.137	9.786	0.316
30	0.124	8.857	0.113
Mean			0.225 ± 0.02

ΔA_{412} is the change in absorbance at 412 nm relative to a reference haemoglobin solution instrumentally adjusted to zero absorbance. Errors quoted for K_{equ} are standard errors.

haemoglobin. They also display the results of calculations carried out using Eq. (3). The absorbance readings reported in Tables 1 and 2 were those determined directly on the spectrophotometers and were corrected for dilution within the programme written for calculating K_{equ} from Eq. (3).

2.4. Kinetics of the DTNB reaction

The kinetics of the reaction of DTNB with the sheep haemoglobins were monitored at 412 nm on a Cecil Bioquest uv–visible spectrophotometer coupled to an on-line data acquisition system. The reaction was carried out under pseudo-first order conditions, with DTNB in at least 30-fold excess over the concentration of (DTNB-reactive) sulphydryl groups. The haemoglobin concentration was 10 μmol (haem) 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%. 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 programme for the analysis of multiple exponential signals [26,27]. A slight modification of DISCRETE allowed the determination of the absorbance reading at the 'end' of the reaction. The standard error in the determination of the pseudo-first order rate constant was about 5%.

2.5. Determination of dissociation constant of the haemoglobin/inositol-P_6 complex

We determined K_d, the dissociation constant of the haemoglobin/inositol–P_6 complex, essentially as described previously [20], based on the method of Scrutton and Utter [28].

A Shimadzu 1600PC double beam uv–visible spectrophotometer interfaced to a computer was employed for this purpose. 3 cm^3 of a haemoglobin solution, pre-equilibrated with inositol-P_6 in the appropriate buffer, was pipetted into a 1 cm path length cuvette in the sample compartment of the spectrophotometer. The same solution was placed in the reference cell. The haemoglobin concentration was 10 μmol (haem) dm^{-3} (5 μmol dm^{-3} in DTNB reacting sulphydryl groups), and the inositol-P_6 concentration was between 100 and 250 μmol dm^{-3}. An appropriate small volume of a stock (pH 7) solution of DTNB (concentration in the sample cuvette, 215 μmol dm^{-3}) was added with stirring into the sample cell. [The increase in the ionic strength of the buffers, arising from the addition of inositol-P_6 and DTNB, did not exceed 20%.] The kinetics were monitored as described above. The pseudo-first order rate constants, $V_{inositol\text{-}P_6}$, at various inositol-P_6 concentrations were calculated from the least squares slopes of semi-log plots of the data. The same experiment was performed in the absence of inositol-P_6; the slope of the semi-log plot in this case gave V_o. K_d, the dissociation constant of the haemoglobin/inositol-P_6 complex, was calculated by substituting $V_{inositol\text{-}P_6}$ and V_o into the equation [20,28]:

$$\frac{V_{inositol\text{-}P_6}}{V_o} = K_d \cdot \frac{1 - \frac{V_{inositol\text{-}P_6}}{V_o}}{[inositol\text{-}P_6]} + \frac{k_{inositol\text{-}P_6}}{k_o} \ldots \quad (1)$$

In Eq. (1) $\frac{k_{inositol\text{-}P_6}}{k_o}$ is a measure of the maximum protection of the sulphydryl group from reaction with DTNB [20,28].

2.6. Analyses of data

All equilibrium data were analysed with a MicroMaths Scientist software package (Salt Lake City, Utah, USA).

3. Results

In Fig. 1a and b we report the pseudo-first order rate plots for the reaction of DTNB with the carbonmonoxy derivative of the major sheep haemoglobin at pH 6.04 and 8.66. It is seen that the plots are linear for 7 and 3 half-lives, respectively. Similar results were obtained for the minor haemoglobin (Fig. 1c and d) at pH 6.11 and 9.04. Here the plots are linear for 6 and 2.5 half-lives, respectively. These results are in contrast with our findings for cat haemoglobins at pH ≥ 8.6, a range in which we found that the pseudo-first order plots were non-linear [21]. The linearity of the plots in Fig. 1 demonstrates that our results were obtained under true pseudo-first order conditions.

In Fig. 2 we report the plots of k_{obs}, the pseudo-first order rate constant, against the DTNB concentration at low and high pH for the carbonmonoxy derivative of the major and minor sheep haemoglobins. Each plot was fitted by linear least squares and R^2, the square of the correlation coefficient, was at least 0.9. Each plot has a non-zero intercept. The intercepts, together with their calculated standard deviations, are reported in the legend to Fig. 2. The non-zero intercepts demonstrate that the reaction of DTNB with the sheep haemoglobins is a reversible process over the pH range 5.6 to 9 of our study. The reversible reaction between CysF9[93]β and DTNB may be depicted as:

$$PSH + DTNB \underset{}{\overset{Q_{SH}}{\rightleftharpoons}} H^+ + PS^- + DTNB \overset{K_{equ}}{\rightleftharpoons} H^+ + PS.ST + TNB^- \overset{Q_{TNB}}{\rightleftharpoons} PS.ST + TNBH \quad (2)$$

In Eq. (2) PSH is haemoglobin with the CysF9[93]β sulphydryl in its protonated, unreacting (with DTNB) form; PS^- is the corresponding (reacting) anion form in the *cis*-to-amino conformation; PS.ST is the mixed disulfide formed after reaction with DTNB, and it is in the *cis*-to-carbonyl conformation [8,9]; 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; K_{equ} is the equilibrium constant

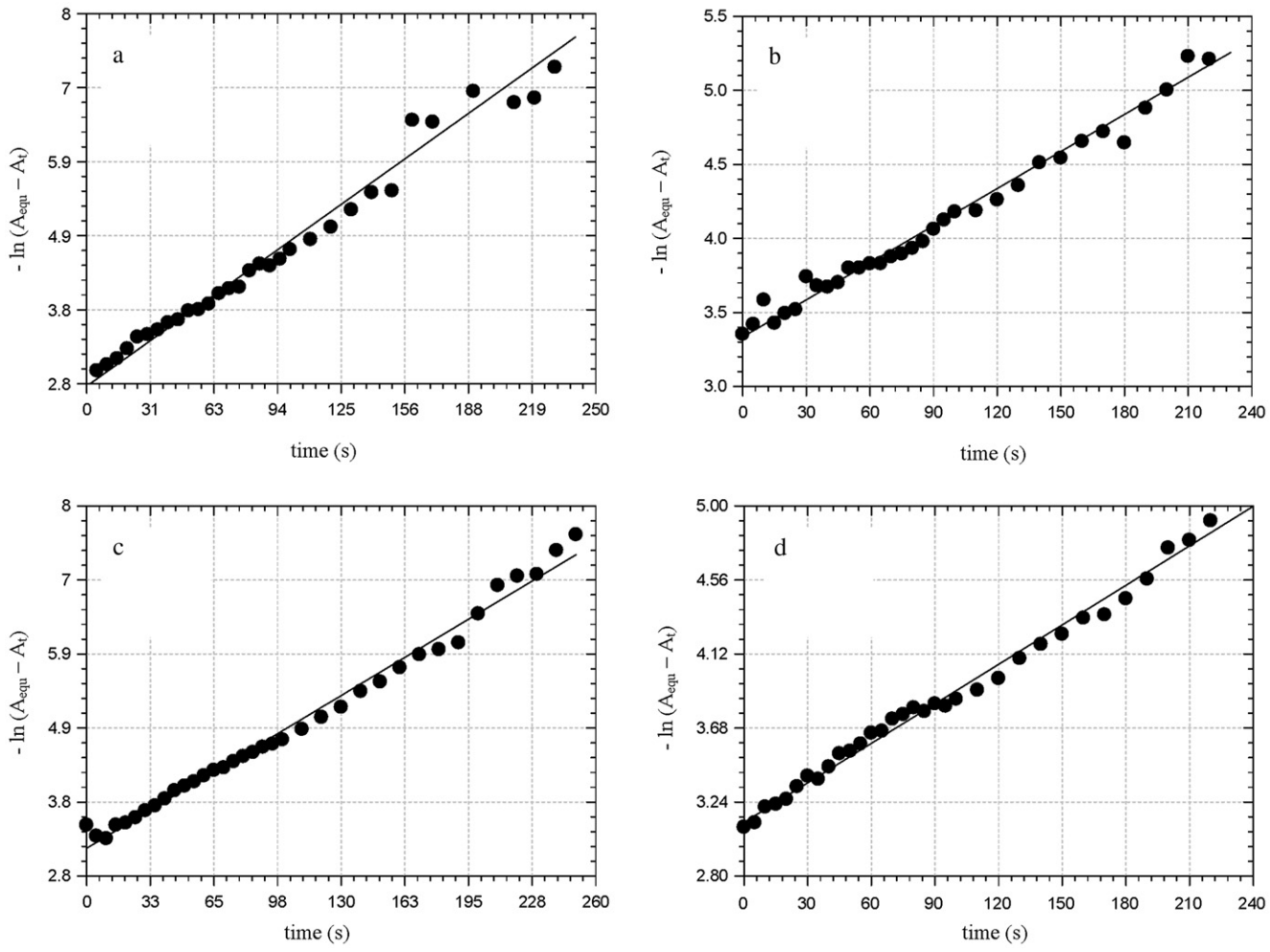


Fig. 1. Semi-logarithmic plots of the time courses for the reaction of DTNB with the carbonmonoxy derivatives of the sheep haemoglobins at 25 °C. The least squares slopes give k_{obs}, the pseudo-first order rate constants. (a) Major haemoglobin at pH 6.04; k_{obs} = 0.020 (±0.006) s^{-1}; (b) major haemoglobin at pH 8.66; k_{obs} = 0.008 (±0.002) s^{-1}; (c) minor haemoglobin at pH 6.11; k_{obs} = 0.016 (±0.001) s^{-1}; (d) minor haemoglobin at pH 9.04; k_{obs} = 0.008 (±0.001) s^{-1}. The errors quoted are standard deviations. Conditions: haemoglobin concentration, 10 μmol (haem) dm^{-3} (5 μmol dm^{-3} in reactive sulphydryl groups); DTNB concentration, 300 μmol dm^{-3}; phosphate buffers, 5.6<pH<8.0; borate buffers, pH≥8. Each buffer had a total ionic strength of 50 mmol dm^{-3}.

for the formation of the mixed disulfide, that is, the DTNB reaction step. The equation used to calculate K_{equ} from Eq. (2) is

$$K_{equ} = \frac{[TNB^-]^2\left\{1+\frac{[H^+]}{Q_{TNB}}\right\}\left\{1+\frac{[H^+]}{Q_{SH}}\right\}}{\left\{[P]_{total}-[TNB^-]\left(1+\frac{[H^+]}{Q_{TNB}}\right)\right\}\left\{[DTNB]_{total}-[TNB^-]\left(1+\frac{[H^+]}{Q_{TNB}}\right)\right\}} \quad (3)$$

In Eq. (3) $[P]_{total}$ refers to the haemoglobin concentration in terms of reacting sulphydryl groups. A full derivation of Eq. (3) has been reported elsewhere [22]. A computer programme aided the calculation of K_{equ} from the experimental data. The standard error in the determination of K_{equ} was about 20%. The values of pQ_{SH} and pQ_{TNB} used in these calculations were 8.30 and 5.27, respectively, and the extinction coefficient of TNB was assumed to be 14,000 mol^{-1} dm^3 cm^{-1} [22]. Tables 1 and 2 report typical raw data and the K_{equ} values calculated from them.

It has been established that inositol-P_6 binds to liganded haemoglobin at the allosteric site, with a 1:1 stoichiometry with respect to haemoglobin tetramers [29–31]. A 2:1 stoichiometry is only approached when the ionic strength goes as low as 0.01 mol dm^{-3} [31]. Since we performed our experiments at an ionic strength of 0.05 mol dm^{-3}, we worked under conditions of 1:1 stoichiometry. In Fig. 3a we compare the pH dependence of K_{equ} for the reaction of CysF9[93]β of the major oxyhaemoglobin with DTNB in the absence (open circles) and presence (filled circles) of inositol-P_6 in a 4:1 molar ratio with respect to haemoglobin tetramers. The corresponding data for carbonmonoxy- and aquomethaemoglobin are reported in Fig. 3b and c, respectively. It is very striking that in each case the organic phosphate *increases* K_{equ} by about an order of magnitude for most of the pH range ~5.6 to ~9. This result is interesting because inositol-P_6 is a heterotropic allosteric effector, and such effectors are known to *reduce* the oxygen affinity of haemoglobin [11–19].

Similarly striking results are seen in Fig. 4 for the corresponding derivatives of the minor haemoglobin. As we demonstrate below, inositol-P_6 also increases the value of K_{rt} of haemoglobin. The effect of inositol-P_6 in increasing the DTNB affinity (Figs. 3 and 4) may be compared with the well-known effect of heterotropic organic phosphates in lowering the oxygen affinity of R state and of T state haemoglobin [18,19]. A higher affinity of sheep haemoglobins for DTNB may therefore imply a lower affinity for oxygen.

As may be seen in Figs. 3 and 4, the increases in K_{equ} produced by inositol-P_6 at alkaline pH are about as large as, or even larger than, those produced in the neutral and acid pH ranges. Given the fact that the affinity of haemoglobin for inositol-P_6 increases as the pH decreases, these results are somewhat surprising.

It is a well characterized phenomenon that decreasing pH promotes the dissociation of haemoglobin tetramers to dimers [32–35]. For

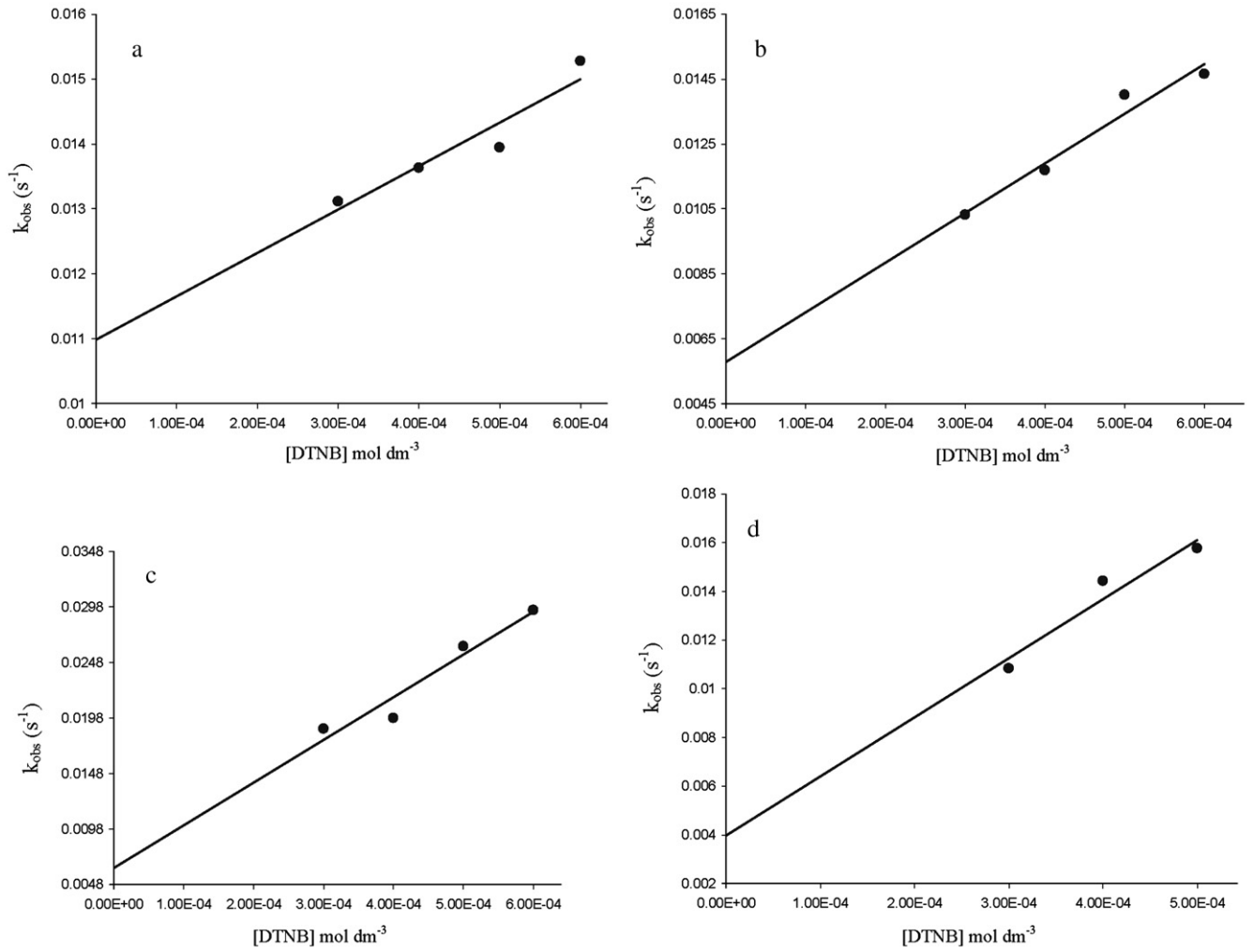


Fig. 2. Dependence of the pseudo-first order rate constant, k_{obs}, on the DTNB concentration for the reaction of DTNB with CysF9[93]β of the carbonmonoxy derivative of the sheep haemoglobins. (a) major haemoglobin at pH 6.04; slope, 6.80 (±1.5) mol^{-1} dm^3s^{-1}; intercept, 0.011 (±0.001) s^{-1}; (b) major haemoglobin at pH 8.66; slope, 15.34 (±2.2) mol^{-1} dm^3s^{-1}; intercept, 0.0058 (±0.001) s^{-1}; (c) minor haemoglobin at pH 6.11; slope, 38.51 (±7.2) mol^{-1} dm^3s^{-1}; intercept, 0.0063 (±0.003) s^{-1}; (d) minor haemoglobin at pH 9.04; slope, 24.66 (±6.5) mol^{-1} dm^3s^{-1}; intercept, 0.004 (±0.003) s^{-1}. Conditions as in Fig. 1. Errors quoted are standard deviations.

example, at pH 6, 7.2, and 8.7 the values of $K_{4,2}$, the tetramer–dimer dissociation constant of human haemoglobin, are 3.16, 0.2, and 0.032 μmol dm^{-3}, respectively [32]. At our experimental haemoglobin concentration — 25 μmol (haem) dm^{-3} — these $K_{4,2}$ values give percentage tetramer contents of 70.2, 91.5 and 96.5, respectively, at pH 6, 7.2 and 8.7. If these values are roughly valid for sheep haemoglobins, then our experimental haemoglobin samples are likely to be fully tetrameric at pH 9, even when stripped of organic phosphates. Indeed it has been found that at pH>8 stripped human haemoglobin exists exclusively as tetramers [36]. As the pH decreases, the tetramer content of our samples also decreases.

It has been established that inositol-P_6 binds about 30 times more tightly to tetramers than to dimers [36,37]. The considerable increase in K_{equ} (Figs. 3 and 4) caused by inositol-P_6 at pH 9, where stripped haemoglobin exists exclusively as the tetramer [36], suggests that inositol-P_6 acts almost exclusively on the tetramer.

In sheep haemoglobins there is a deletion of one amino acid at the beginning of the β chain [38,39]. In what follows we retain the usual system of numbering the sequence where there is no deletion. Of the basic groups at the organic phosphate binding site, only LysEF6[82]β (pK_a ~ 10.5) retains its charge and binds inositol-P_6 at pH 9. As the pH decreases, the tetramer content becomes reduced. The increase in K_{equ} caused by inositol-P_6 would be considerably reduced in magnitude but for the fact that the organic phosphate binds more tightly to haemoglobin as the remaining organic phosphate binding groups — HisH21[143]β and the terminal amino group of MetNA2[2]β — regain their charges. Tighter inositol-P_6 binding with decreasing pH favours an increase in the tetramer content, thus balancing out the effect of decreasing pH. It is therefore not surprising that the changes in K_{equ} reported in Figs. 3 and 4 are almost the same through our experimental pH range, 5.6 to ~9. The actual increase in K_{equ} will depend on the extent to which these two effects balance out. In the case of the minor aquomethaemoglobin (Fig. 4c), the decrease in the tetramer population with decreasing pH appears to predominate.

3.1 Analyses of pH dependence of K_{equ} data

The fairly strong pH dependences seen in Figs. 3 and 4 imply that the DTNB reaction (Eq. (2)) is coupled to the ionizations of groups on the haemoglobin molecule. To gain an understanding of the nature and number of these groups, and of the possible effect of inositol-P_6 on these groups and on the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary conformational equilibrium, we have analysed the data in Figs. 3 and 4 quantitatively. We previously analysed curves like those in Figs. 3 and 4 on the basis of a scheme and equation that were strictly not thermodynamically correct [23,24]. We employed this scheme and equation because they enabled us to fit the equilibrium data, whereas the thermodynamically correct scheme and equation (Scheme 1 and Eq. (4) below) did not. In Scheme 1 the protons arising

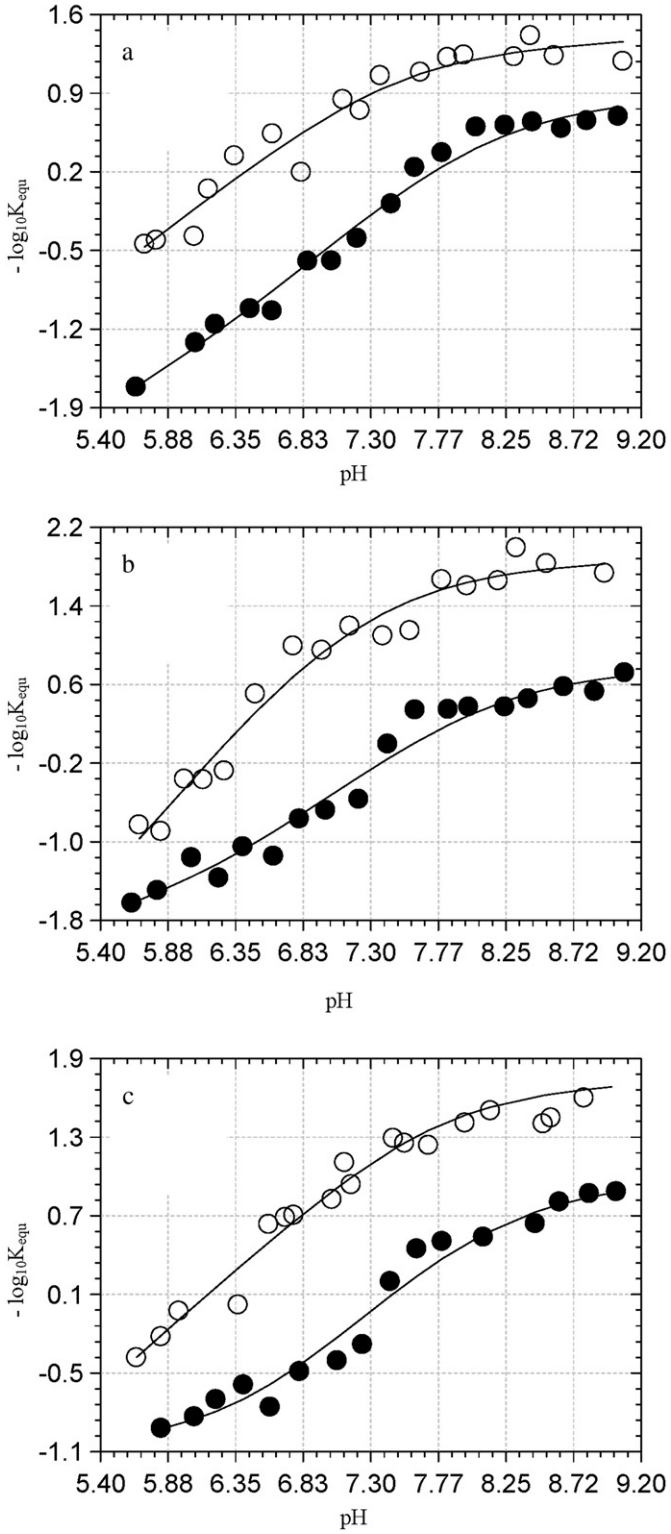


Fig. 3. Variation of $-\log_{10}K_{equ}$ with pH for the reaction of 5,5′-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulphydryl group of various derivatives of the major haemoglobin of the sheep in the absence (open symbols) and presence (filled symbols) of inositol-P_6. (a) Oxyhaemoglobin; (b) carbonmonoxyhaemoglobin; (c) aquomethaemoglobin. Conditions: stripped haemoglobin concentration, 50 μmol (haem) dm^{-3}; haemoglobin + inositol-P_6, 25 μmol (haem) dm^{-3} and 25 μmol dm^{-3}; 25 °C; phosphate buffers, pH 5.6–8.0; borate buffers, pH 8.0–9.2, each of final ionic strength 50 mmol dm^{-3} (added salt, NaCl). The change in ionic strength due to added inositol-P_6 was less than 2%. Each experimental point is subject to a standard error of about 20%. The lines through the data points are the theoretical best-fit lines drawn with the parameters reported in Table 3 (*cf.* Scheme 1 and Eq. (5) for $n=2$).

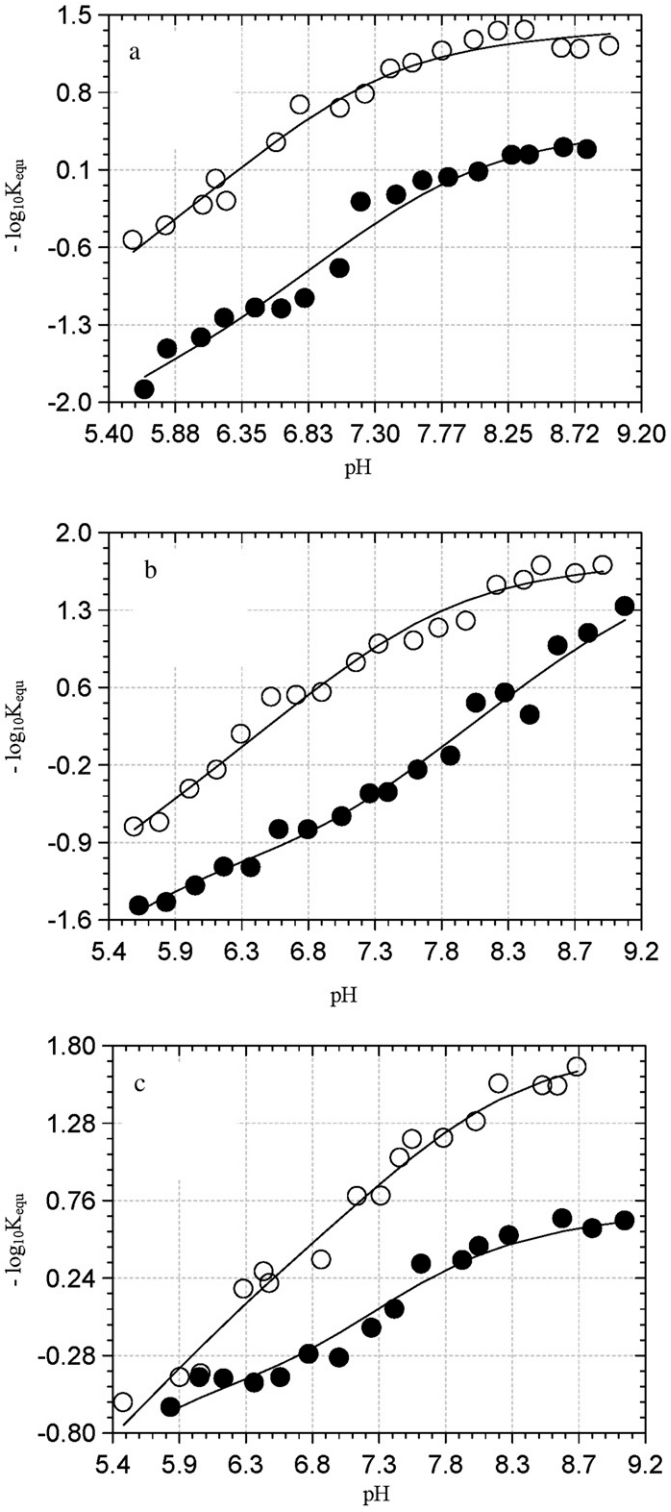
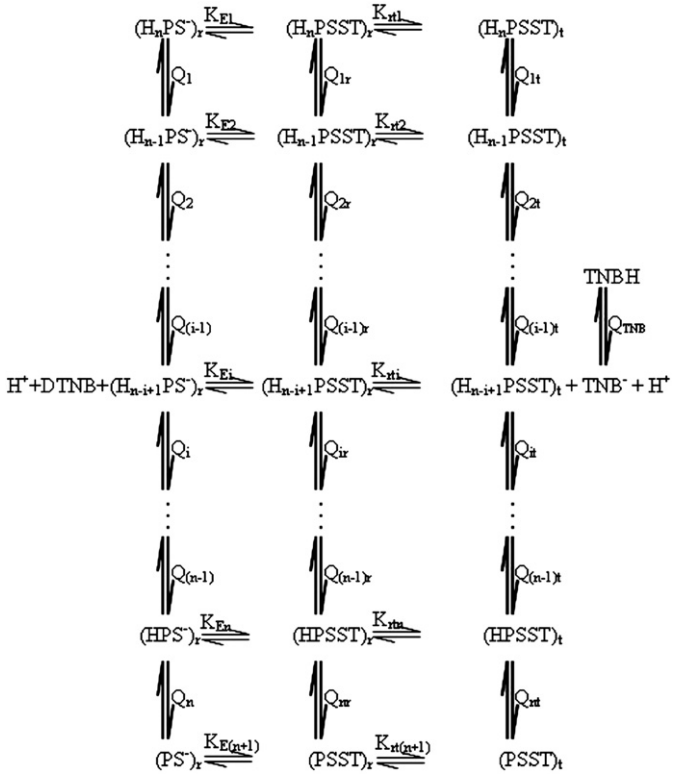


Fig. 4. Variation of $-\log_{10}K_{equ}$ with pH for the reaction of 5,5′-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulphydryl group of various derivatives of the minor haemoglobin of the sheep in the absence (open symbols) and presence (filled symbols) of inositol-P_6. (a) Oxyhaemoglobin; (b) carbonmonoxyhaemoglobin; (c) aquomethaemoglobin. Conditions as in Fig. 3. Each experimental point is subject to a standard error of about 20%. The lines through the data points are the theoretical best-fit lines drawn with the parameters reported in Table 4 (*cf.* Scheme 1 and Eq. (5) for $n=2$).

from the various ionization steps have been omitted for clarity. The species $H_{n-i+1}PSH$ ($i=1, 2,\ldots, n$) are hemoglobin species in which the sulphydryl group is in its protonated form, which does not react with DTNB. These species are therefore omitted from Scheme 1. $H_{n-i+1}PS^-$



Scheme 1.

($i=1, 2,\ldots, n$) are species in which the sulphydryl is in its thiolate anion form, the form that reacts with DTNB [21–24]; $H_{n-i+1}PS.ST$ ($i=1, 2,\ldots, n$) are the mixed disulfide species formed after the reaction of the sulphydryl with DTNB. Species marked with subscripts **r** and **t** are those in which the sulphydryl is in the **r** and **t** tertiary isomeric forms of haemoglobin, respectively. The various proton ionization constants are represented as Q_i, 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; and $K_{rt(n+1)}$ is the equilibrium constant at high pH for the $\mathbf{r} \rightleftharpoons \mathbf{t}$ isomerization. A relationship between K_{equ} and the parameters of Scheme 1 is given by the equation:

$$K_{equ} = \frac{K_{E(n+1)}\left\{1 + \sum_{i=1}^{n} (H^+)^{n-i+1}\left(\prod_{j=i}^{n} Q_{jr}\right)^{-1} + K_{rt(n+1)}\left(1 + \sum_{i=1}^{n} (H^+)^{n-i+1}\left(\prod_{j=i}^{n} Q_{jt}\right)^{-1}\right)\right\}}{1 + \sum_{i=1}^{n} (H^+)^{n-i+1}\left(\prod_{j=i}^{n} Q_j\right)^{-1}} \quad (4)$$

We attempted to use Eq. (4) to analyse the data presented in Figs. 3 and 4, but we had considerable difficulty fitting the data with this equation. In particular, for $n=2$, the best value required to fit K_{equ} data [23,24], the pQ_1 value fluctuated between the lower (5) and upper (9) limits placed on it and failed to converge near the value 6.5 initially estimated for it. For this reason we have derived Eq. (5), a relationship between K_{equ} and the parameters of Scheme 1 that is equivalent to Eq. (4), except that it does not involve Q_i values. With the relationship in Eq. (5) we were able to analyse the data in Figs. 3 and 4 successfully with $n=2$. This was possible because we could set good initial estimates of

$$K_{equ} = \frac{K_{E(n+1)}\left\{1 + \sum_{i=1}^{n} (H^+)^{n-i+1}\left(\prod_{j=i}^{n} Q_{jr}\right)^{-1} + K_{rt(n+1)}\left(1 + \sum_{i=1}^{n} (H^+)^{n-i+1}\left(\prod_{j=i}^{n} Q_{jt}\right)^{-1}\right)\right\}}{1 + K_{E(n+1)}\left\{\sum_{i=1}^{n} (H^+)^{n-i+1}\left(\prod_{j=i}^{n} Q_{jr}\right)^{-1} K_{Ei}^{-1}\right\}} \quad (5)$$

K_{E3}/K_{E1} and K_{E3}/K_{E2}, the parameters that appear in the denominator of Eq. (5) for $n=2$. For each haemoglobin derivative we employed its K_{equ} value at the highest experimental pH as an initial estimate for K_{E3} and the K_{equ} value at the lowest experimental pH as an initial estimate for K_{E1}. In this way we obtained a good initial estimate for K_{E3}/K_{E1}. Since K_{equ} decreases as the pH increases, we set $0<K_{E3}/K_{E2}<1$. With these good initial estimates, we successfully fitted the data in Figs. 3 and 4. The results of the analyses for the major haemoglobin (Fig. 3) are presented in Table 3; those for the minor haemoglobin (Fig. 4) are reported in Table 4. With the mean values of pQ_{1r}, pQ_{2r} and K_{E3}/K_{E1}, K_{E3}/K_{E2} in Table 3, microscopic reversibility enabled us to obtain for the (stripped) major haemoglobin $pQ_1=4.09$; $pQ_2=6.37$. In the presence of inositol-P_6, $pQ_1=4.37$; $pQ_2=6.79$. From the corresponding data in Table 4 we obtained for the (stripped) minor haemoglobin $pQ_1=4.42$; $pQ_2=6.97$. In the presence of inositol-P_6, $pQ_1=4.78$; $pQ_2=7.30$. The mean of the pQ_1 values, 4.41, is so low that pQ_1 cannot make any significant contribution in the pH range 5.6–9 of our experiments. The pQ_2 values are similar to the pQ_{2r} values in Tables 3 and 4 and, in the following discussion, are treated as the same.

3.2 Effect of inositol-P_6 on K_{rt3}, K_{equ} and pQs of coupled ionizable groups

The most striking results seen in Tables 3 and 4 are the effect of inositol-P_6 on the values of K_{rt3}, K_{equ} and the p*Q*s of the ionizable groups linked to the reaction of DTNB with CysF9[93]β. We now consider these results in detail.

3.2.1. Effect of inositol-P_6 on K_{rt3}

It is seen in Tables 3 and 4 that inositol-P_6 increases K_{rt3} 4.5-fold in the major haemoglobin and 2.4-fold in the minor haemoglobin. From the mean value of K_{rt3} for the (stripped) major haemoglobin, 1.03±0.4 (see Table 3), we obtain a value of 50.7 (±6)% as the relative population of the **t** isomer. The value of K_{rt3} in the presence of inositol-P_6 is 4.59±0.4; this gives a value of 82.1 (±1)% for the relative population of the **t** isomer. From the corresponding data for the minor haemoglobin, 1.62±0.4 and 3.90±0.4 (Table 4), we obtain relative **t** isomer populations of 61.8 (±4) and 79.6 (±2)%, respectively, for stripped haemoglobin and for

Table 3
Reaction of 5,5′-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulphydryl group of various derivatives of the major haemoglobin of the sheep in the absence and presence of inositol hexakisphosphate (inositol-P_6): best-fit parameters employed to fit the equilibrium data reported in Fig. 3 using Scheme 1 and Eq. (5) of the text

Derivative	pQ_{1r}	pQ_{1t}	pQ_{2r}	pQ_{2t}	K_{E3}/K_{E2}	K_{E3}/K_{E1}	K_{E3}	K_{rt3}
Stripped haemoglobin								
Oxy	5.95	7.43	7.66	7.11	0.51	0.021	0.098	0.88
Carbonmonoxy	5.52	6.54	6.41	8.03	0.18	0.002	0.030	0.45
Aquomet	5.43	6.73	7.34	7.84	0.64	0.012	0.018	1.77
Mean	5.63±0.2	6.92±0.3	6.75±0.4	7.66±0.3	0.42 ±0.14	0.012 ±0.006	0.048 ±0.03	1.03 ±0.4
Haemoglobin +inositol-P_6								
Oxy	5.96	5.74	6.94	8.17	0.63	0.0089	0.233	4.54
Carbonmonoxy	6.49	4.55	7.59	8.17	0.085	0.0063	0.190	4.01
Aquomet	5.90	4.72	7.14	8.14	0.95	0.016	0.170	5.21
Mean	6.12±0.2	5.00±0.4	7.22±0.2	8.16±.01	0.56 ±0.3	0.010 ±0.003	0.198±0.02	4.59±0.4

Table 4
Reaction of 5,5′-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulphydryl group of various derivatives of the minor haemoglobin of the sheep in the absence and presence of inositol hexakisphosphate (inositol-P_6): best-fit parameters employed to fit the equilibrium data reported in Fig. 4 using Scheme 1 and Eq. (5) of the text.

Derivative	pQ_{1r}	pQ_{1t}	pQ_{2r}	pQ_{2t}	K_{E3}/K_{E2}	K_{E3}/K_{E1}	K_{E3}	K_{rt3}
Stripped haemoglobin								
Oxy	5.92	6.47	7.23	7.37	0.37	0.0091	0.087	1.49
Carbonmonoxy	6.32	5.84	6.81	8.02	0.65	0.0032	0.097	1.04
Aquomet	6.73	6.73	7.91	8.07	0.34	0.0058	0.024	2.28
Mean	6.32 ±0.3	6.35 ±0.3	7.32 ±0.4	7.82 ±0.2	0.45 ±0.1	0.0057 ±0.002	0.069 ±.02	1.62 ±0.4
Haemoglobin +inositol-P_6								
Oxy	5.67	5.62	6.77	7.93	0.83	0.0094	0.52	4.34
Carbonmonoxy	6.99	5.98	7.72	9.37	0.88	0.0056	0.06	3.15
Aquomet	5.98	5.29	7.92	7.64	0.31	0.059	0.28	4.21
Mean	6.21 ±0.4	5.63 ±0.2	7.47 ±0.4	8.31 ±0.6	0.67 ±0.2	0.025 ±0.02	0.29 ±0.2	3.90 ±0.4

haemoglobin in the presence of inositol-P_6. Consequently, the organic phosphate appears to have a greater effect on K_{rt3} for the major compared to the minor haemoglobin. This is not because the major haemoglobin has a higher net positive charge and therefore binds the organic phosphate more strongly, resulting in a greater effect on K_{rt3}. We demonstrate below (see Table 6) that K_d, the dissociation constant of the haemoglobin/inositol-P_6 complex, is approximately the same for the two haemoglobins.

3.2.2. Effect of inositol-P_6 on the affinity for DTNB

From Figs. 3 and 4 it is seen that inositol-P_6 increases the affinity of CysF9[93]β of each haemoglobin for DTNB by about an order of magnitude throughout the pH range 5.6 to 9.2. From Table 3 it is seen that, for the major haemoglobin, inositol-P_6 increases 4-fold the mean value of K_{E3}, the limiting value of K_{equ} at high pH: from a value of 0.069 (±0.02) for stripped haemoglobin to a value of 0.290 (±0.2). Inositol-P_6 also increases 4-fold the value of K_{E3} for the minor haemoglobin. The increase (Table 4) is from 0.048 (±0.03) to 0.198 (±0.02). These increases are less than the *ca* 10-fold increases observed at lower pH values (Figs. 3 and 4). This is because inositol-P_6 binds less strongly to haemoglobin as the pH increases [40,41].

3.2.3. Effect of inositol-P_6 on the ionization constants of groups coupled to the DTNB reaction

The results of a molecular dynamics study indicate that the binding site of inositol-P_6 in R-state haemoglobin is the same as in T-state haemoglobin, namely, at the molecular dyad axis [42]. Inositol-P_6 binds to R-state haemoglobin at three sites: one site whose identity is not certain and two sites at the dyad axis, identified as ValNA1[1]β and HisH21[143]β [42,43]. According to our analyses, two sites are detectable (Tables 3 and 4). These sites may be associated with pQ_{1r}/pQ_{1t} and pQ_{2r}/pQ_{2t}. On the basis of the above assignments [42,43], we assign the mean pQ_{1r} and pQ_{1t} values (see Tables 3 and 4) to HisH21[143]β of sheep haemoglobin in the **r** and **t** tertiary conformations, respectively. The deletion of an amino acid at the NH_3^+-terminals of the β chains of the sheep haemoglobins [38,39] makes it difficult for us to assign the pQ_{2r} and pQ_{2t} values.

Examination of the mean pQ values of the ionizable groups linked to the DTNB reaction (Tables 3 and 4) shows that inositol-P_6 increases pQ_{1r}, pQ_{2r} and pQ_{2t} by a mean value of about 0.5 pK_a unit, but decreases pQ_{1t} by between 0.7 and 2.1 pK_a units. HisH21[143]β is the only histidine group whose pK_a is known to decrease on going from the R to the T quaternary structure [44,45]. The decrease in the pK_a of this histidine in the sheep haemoglobins caused by inositol-P_6 (Tables 3 and 4) occurs, however, within the **t** tertiary structure of the major and minor haemoglobins.

3.3 Effect of inositol-P_6 on the kinetics of the DTNB reaction

To gain an insight into the basis for the increases caused by inositol-P_6 in the values of K_{rt} and, consequently, of the **t** isomer population, we determined k_f, the apparent forward second order rate constant for the DTNB reaction (Eq. (3)). Table 5 reports the data obtained at pH 7.0 for the various derivatives of the major and minor haemoglobins in the absence and in the presence of inositol-P_6. For the (stripped) major haemoglobin the mean value of k_f is 50.6±16 mol^{-1} dm^3 s^{-1}. This value is decreased 2.8-fold to 18.1±5 mol^{-1} dm^3 s^{-1} in the presence of inositol-P_6. For the (stripped) minor haemoglobin k_f is 28.7±9 mol^{-1} dm^3 s^{-1}. This is again decreased 2.8-fold to 10.2±1 mol^{-1} dm^3 s^{-1} in the presence of inositol-P_6. These results are in line with previous observations on the effect of organic phosphates on the reactivity of the CysF9[93]β sulphydryl group of haemoglobin [20]. It is remarkable that the major haemoglobin is 1.8-fold faster reacting than the minor haemoglobin in the absence of inositol-P_6. It is highly likely that the higher reactivity of the major haemoglobin is not unconnected with its lower tertiary **t** isomer population. Thus, the lowering of the CysF9[93]β reactivity by allosteric effectors, which had in the past been associated solely with a transition from the R to the T quaternary state, is at least in part due to the transition from the **r** to the **t** tertiary isomeric form which takes place during the overall R to T quaternary transition.

At first sight it may seem contradictory that inositol-P_6 increases the affinity of haemoglobin for DTNB, $\Delta G = -RTlnK_{equ}$ (see Figs. 3 and 4), but decreases the forward second order rate constant (k_f) for the same reaction (Table 5). A reasonable check on this apparent contradiction would be to independently determine k_r, the apparent second order reverse rate constant. Unfortunately, it is not feasible to determine k_r experimentally for this system since PS.ST and TNB^- (see Eq. (2)) are produced *in situ* and cannot both be isolated [22]. We have

Table 5
Reaction of DTNB with CysF9[93]β of various derivatives of the major and minor haemoglobins of the sheep in the absence and presence of inositol-P_6: apparent second order forward rate constants, k_f (mol^{-1} dm^3 s^{-1}), and apparent second order reverse rate constants, k_r (mol^{-1} dm^3 s^{-1})

	Stripped haemoglobin		Haemoglobin plus inositol-P_6	
	k_f	k_r	k_f	k_r
Major haemoglobin derivative				
Oxy	45.3	155.1	26.2	6.7
Carbonmonoxy	76.8	952.1	11.1	3.9
Aquomet	29.6	197.7	16.9	12.1
Mean	50.6±16	434±300	18.1±5	9.6±3
Minor haemoglobin derivative				
Oxy	32.6	147.4	11.8	1.9
Carbonmonoxy	13.3	40.4	10.0	2.3
Aquomet	40.1	246.1	8.8	5.4
Mean	28.7±9	144.6±69	10.2±1	3.2±1

The k_r values were obtained from $k_r = \frac{k_f}{K_{equ}}$. Conditions: haemoglobin concentration, 10 μmol (haem) dm^{-3}; [DTNB] = 300–600 μmol dm^{-3}; phosphate buffer, pH 7.0, final ionic strength 50 mmol dm^{-3} (added salt, NaCl); 25 °C. Where present, inositol-P_6 was in a 4:1 ratio with respect to haemoglobin tetramers.

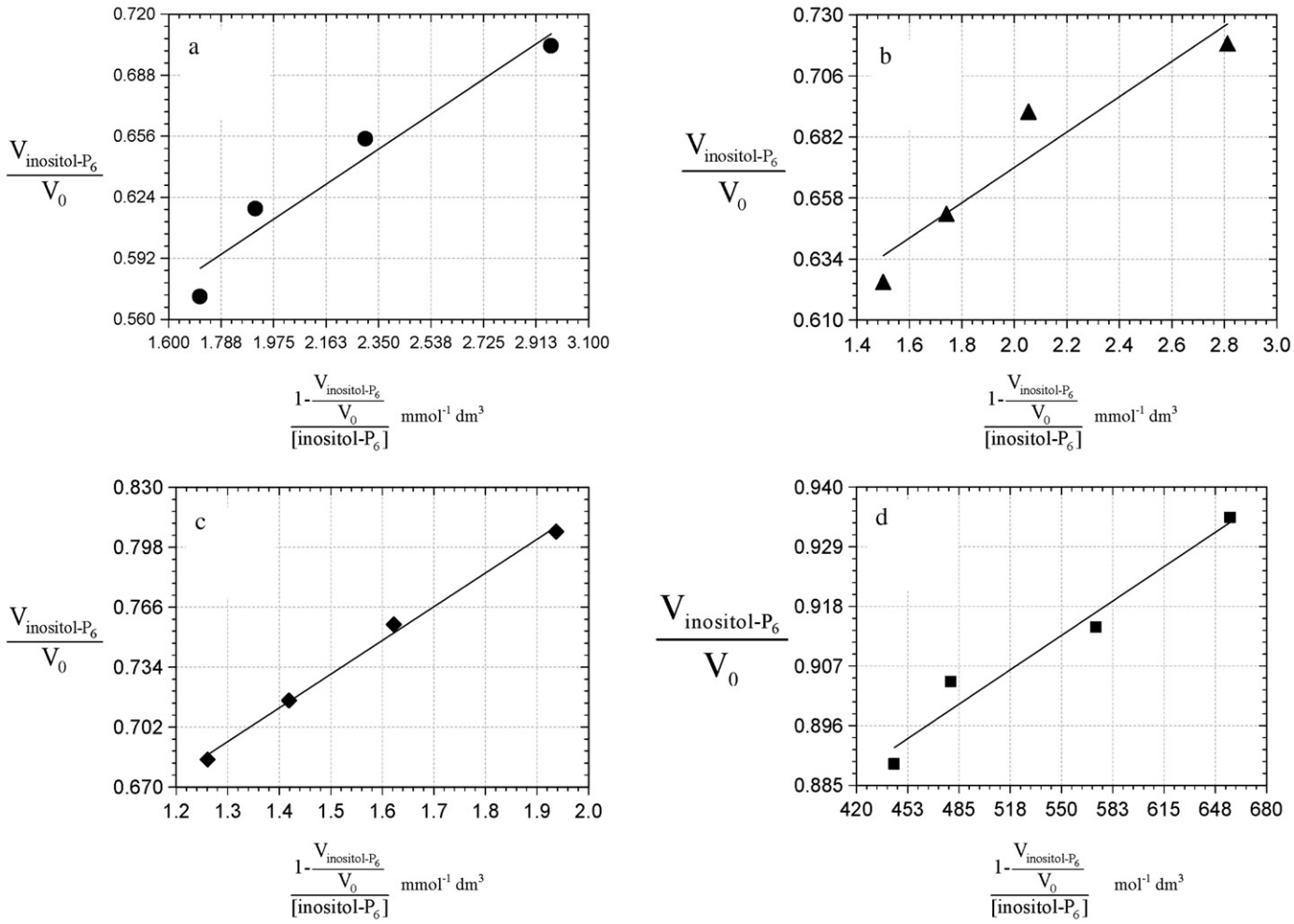


Fig. 5. Determination of K_d, the dissociation constant of the complex formed between the minor sheep carbonmonoxyhaemoglobin and inositol-P_6, at various pH values: Scrutton–Utter plots [20,28], according to Eq. (1), for the reaction of DTNB at various inositol-P_6 concentrations. (a) pH 6.15; (b) pH 7.32; (c) pH 8.13; (d) pH 9.04. Conditions: 25 °C; phosphate buffers, pH<8; borate buffers, pH>8; ionic strength, 50 mmol dm^{-3} (added salt, NaCl); [haemoglobin], 10 μmol (haem) dm^{-3} (5 μmol dm^{-3} in reactive sulphydryl groups); [DTNB], 215 μmol dm^{-3}; [inositol-P_6], 100-250 μmol dm^{-3}. The maximum increase in ionic strength due to added DTNB and inositol-P_6 is *ca* 20%. The K_d values are reported in Table 6.

instead calculated k_r (at pH 7) from the relation $k_r = \frac{k_f}{K_{equ}}$. As the mean values of k_f and the calculated k_r in Table 5 indicate, the 2.8-fold lowering of the value of k_f by inositol-P_6 is more than compensated for by an even greater (46-fold) lowering of k_r. This is the reason for the rise in the affinity for DTNB despite a decrease in the forward rate constant.

To determine the extent of inositol-P_6 binding to liganded sheep haemoglobins at various pH values, we measured K_d, the dissociation constant of the inositol-P_6/haemoglobin complex [20,28]. Fig. 5 reports plots according to Eq. (1) for the carbonmonoxy derivative of the minor haemoglobin. The presence of inositol-P_6 (100–250 μmol dm^{-3}) together with DTNB (215 μmol dm^{-3}) gives rise to an increase in the buffer ionic strength of between 8 and 20%. Nevertheless, as expected from Eq. (1), linear plots are obtained for the data. Similar plots (not shown) were obtained for the aquomet derivative, and for the carbonmonoxy and aquomet derivatives of the major haemoglobin. In Table 6 we report the K_d values, together with the percentage saturations of the samples we employed for determining K_{equ}. These percentages were calculated from the inositol-P_6 concentration of 25 μmol dm^{-3} and haemoglobin tetramer concentration of 6.25 μmol dm^{-3} employed in our equilibrium studies (Figs. 3 and 4). It is seen that the K_d values do not vary much with pH. This finding is similar to that reported for liganded human haemoglobin [40], despite the absence of HisNA2[2]β in the sheep haemoglobins

Table 6
Dissociation constant (K_d) and percentage saturation (in brackets) as a function of pH for the equilibrium reaction between inositol-P_6 and the sheep carbonmonoxy and aquomet derivatives

pH	Major HbCO K_d (μmol dm^{-3})	Minor HbCO K_d (μmol dm^{-3})	Major met K_d (μmol dm^{-3})	Minor met K_d (μmol dm^{-3})	Mean % saturation
6.15	47.7 (32.5)	97.9 (19.5)	89.8 (20.9)	39.0 (36.8)	27.4±4
7.32	53.7 (30.0)	69.4 (25.2)	95.0 (20.0)	78.2 (23.2)	24.6±3
8.13	81.5 (22.4)	180.0 (11.8)	161.3 (13.0)	125.5 (16.0)	15.8±3
9.04	122.6 (16.4)	195.4 (11.1)	167.3 (12.7)	130.0 (15.6)	14±1

The K_d values were determined from the least squares slopes of plots such as those in Fig. 5 (*cf.* Eq. (1)). Conditions: phosphate buffers (pH<8); borate buffers (pH>8); ionic strength, 50 mmol dm^{-3} (added salt, NaCl); 25 °C.

[38,39]. This is most likely because we determined K_d at a lower ionic strength, 50 mmol dm^{-3}, compared to the 100 mmol dm^{-3} employed in [40]. It is clear from Table 6 that the sheep haemoglobins are sufficiently saturated with inositol-P_6 to produce the effects seen in Figs. 3 and 4, even at pH 9 where the mean saturation is 14 (±1)%.

From Table 6 the mean saturation of the two derivatives of the major haemoglobin with inositol-P_6 in the pH range 6.15–9.04 is 21 (±2) %; the corresponding value for the minor haemoglobin is 19 (±3) %. These results are similar. Since the major haemoglobin has a higher net charge than the minor, this demonstrates that the extent of binding of inositol-P_6 is not a function of net charge. This is similar to our previous conclusion: Human haemoglobin A, which has a lower net charge than haemoglobin S, binds inositol-P_6 more tightly [20].

3.4. Effect of DTNB on the **r** ⇌ **t** equilibrium

We showed previously that when the CysF9[93]β sulphydryl group is modified with iodoacetamide the **r** ⇌ **t** equilibrium is shifted entirely to the right in favour of the **t** isomer [9]. This is because iodoacetamide binds irreversibly to haemoglobin. As we have demonstrated in the present report (Fig. 2), DTNB reacts reversibly with the CysF9[93]β sulphydryl group. It should be expected, therefore, that the position of the **r** ⇌ **t** equilibrium will depend on the degree of saturation of haemoglobin with DTNB: A high saturation should shift the equilibrium to the right in favour of the **t** conformation, and a low saturation should shift the equilibrium to the left in favour of the **r** conformation.

The results presented in Figs. 3 and 4 demonstrate that the affinity of haemoglobin for DTNB increases with decreasing pH. Consequently, as one moves from the high to the low pH region one expects the **r** ⇌ **t** equilibrium to shift increasingly from the **r** to the **t** conformation. Thus one expects the following order for the transition constants: $K_{rt1} > K_{rt2} > K_{rt3}$. From Scheme 1, with $n=2$, and the mean fitting parameters K_{rt3}, pQ_{1r}, pQ_{2r}, pQ_{1t} and pQ_{2t} reported in Tables 3 and 4, microscopic reversibility enables us to calculate K_{rt1} and K_{rt2}. For the stripped major haemoglobin the values of K_{rt1}, K_{rt2} and K_{rt3} are 270.9, 8.37, and 1.03, respectively. The relative **t** populations calculated from these values are 99.6, 89.3 and 50.7%, respectively. The corresponding K_{rt1}, K_{rt2} and K_{rt3} values for the stripped minor haemoglobin are 5.49, 5.12 and 1.62, respectively. The percentage **t** populations are 84.6, 83.7 and 61.8, respectively. So, in addition to the effect of inositol-P_6 in favouring the **t** conformation, increasing saturation of haemoglobin with DTNB in the absence of inositol-P_6 also favours the **t** conformation. At very low saturations with DTNB, such as one encounters at very high pH (see Figs. 3 and 4), the **r** conformation predominates. It should be noted that K_{rt3} is the transition constant closest to the value in haemoglobin to which no sulphydryl reagent has been added.

4. Discussion

We attribute the increase caused by inositol-P_6 in the affinity of sheep haemoglobins for DTNB (Figs. 3 and 4) to two interrelated factors: (i) a shift in the tertiary **r** ⇌ **t** conformational equilibrium in favour of the **t** isomer, arising from the preferential binding of inositol-P_6 to the **t** isomer; (ii) a shift in the tetramer–dimer equilibrium in favour of tetramers. This latter shift also favours an increase in the **t** isomer population, as seen on going from liganded to deoxyhaemoglobin: At 11 °C carbonmonoxyhaemoglobin has a **t** population of 12%, whereas deoxyhaemoglobin, which is more tetrameric, has a **t** population of 24% [9,23]. Our conclusions on the effects seen in Figs. 3 and 4 must remain semi-quantitative until more is known about the tetramer–dimer equilibrium of the liganded sheep haemoglobins.

The question that now arises is whether the **r** ⇌ **t** tertiary equilibrium ought to be taken into consideration in analysing the oxygen binding data of haemoglobins. The Monod–Wyman–Changeux (MWC) model of protein cooperativity [46] has been employed for four decades for interpreting haemoglobin–oxygen equilibrium binding data and appeared to be mostly satisfactory. Deoxyhaemoglobin was identified with the tense (T) quaternary state and liganded haemoglobin with the relaxed (R) quaternary state. The MWC model is a two-state model that takes only quaternary structure transitions into consideration but does not consider tertiary structure transitions. As has been pointed out [47,48], the model does not give a satisfactory representation of the equilibrium or kinetics of the reaction of haemoglobin with oxygen. The inadequacy of the two-state MWC model becomes even more obvious in the presence of allosteric effectors [15,16,18,19]. This is because the effectors do not merely bind preferentially to the T state; they also bind to the R state and lower the oxygen affinities of both T-state and R-state haemoglobin [15,16,18,19], probably by shifting the **r** ⇌ **t** tertiary equilibrium in favour of the **t** isomer.

We now consider the relationship of the tertiary **r** ⇌ **t** transition to the quaternary R ⇌ T transition. The first indication of the existence of a tertiary conformational equilibrium in haemoglobin came from the 2.1 Å crystal data on oxyhaemoglobin [8], which exists in the R quaternary structure. We subsequently carried out a temperature-jump study on carbonmonoxyhaemoglobin which, like oxyhaemoglobin, has the R quaternary structure [9]. We also reported on the T-jump study of deoxyhaemoglobin, which exists in the T quaternary conformation [9]. In each case we found that there is a non-quaternary (i.e., a tertiary) conformational equilibrium in haemoglobin, which we subsequently referred to as the **r** ⇌ **t** tertiary transition [23,24]. Thus the tertiary **r** ⇌ **t** transition occurs within the quaternary R and also within the quaternary T structure and, therefore, cannot have the same identity as the R ⇌ T quaternary transition [9].

We now consider the relationship of the **r** ⇌ **t** transition to the tertiary transitions defined by Laberge and Yonetani for oxygen binding to haemoglobin [49]. These authors state *inter alia*, "We propose that effectors act by fine-tuning the distribution of tertiary conformations within HbA quaternary states … ". This is exactly what our present experimental results have demonstrated: Inositol-P_6 causes a redistribution of a pre-existing tertiary conformational equilibrium in the R quaternary state by increasing the **t** isomer population of the major sheep haemoglobin by 31.4% and that of the minor haemoglobin by 17.8%. These results are remarkable, considering that the mean saturation of our samples with inositol-P_6 is about 20% (see Table 6). Thus the percentage increases in the **t** isomer population are very similar to the percentage saturation of haemoglobin with inositol-P_6.

Inositol-P_6 diminished the relaxation signals when we added it to the unbuffered solutions we used for the temperature-jump study of the **r** ⇌ **t** transition [9]. For this reason we were not able to test the effect of this allosteric effector on the **r** ⇌ **t** transition directly [9]. In the present report we have successfully tested the effect of inositol-P_6 on the **r** ⇌ **t** transition indirectly from measurements of the equilibrium constant for the binding of DTNB to haemoglobin. We emphasize that the **r** ⇌ **t** transition is not defined in terms of the reaction of the CysF9 [93]β sulphydryl group. The existence of the transition is entirely independent of the sulphydryl and is an intrinsic property of the haemoglobin molecule. Consequently, haemoglobins that do not have Cys at the F9[93]β site — such as fish and amphibian haemoglobins, which have Ser at that site — can have their pre-existing **r** ⇌ **t** equilibria perturbed by inositol-P_6, provided the organic phosphate binds at the allosteric site.

The **r** ⇌ **t** transition satisfies the requirements of an allosteric transition: (i) redistribution of the relative populations of protein conformations by an allosteric effector, in this case inositol-P_6 (see Tables 3 and 4); (ii) changes in ligand affinity, in this case the binding of DTNB, arising from a redistribution in the relative populations of protein conformations (see Figs. 3 and 4); and (iii) sensitivity to chemical modification [9].

All the haemoglobin derivatives employed in this study are in the R quaternary state in the absence or presence of inositol-P_6. The only possible exception is methaemoglobin at low pH in the presence of the effector. Considerable controversy surrounds the exact effect of inositol-P_6 on high spin ligated methaemoglobin at acid pH. Perutz and his associates propose that these complexes assume the T quaternary conformation [50–52]. On the other hand, Gibson and his associates have provided evidence to demonstrate that the conformation induced by inositol-P_6 is not identical with a normal T state [5]. We have also demonstrated (Figs. 3 and 4 of [53]) that the inositol-P_6 induced spectral changes in high spin methaemoglobins noted by Perutz et al. [52] are confined to the β chains.

5. Conclusion

The data presented in this and in our previous reports [9,23,24] have thrown more light on the nature of the tertiary structure transition in both T and R state haemoglobin.

The present results indicate that the structural transition induced by inositol-P_6 is not simply quaternary. It also involves the tertiary $\mathbf{r} \rightleftharpoons \mathbf{t}$ transition. It is highly probable that a full understanding of oxygen binding to haemoglobin requires, as a minimum, a four-state model that takes the $\mathbf{r} \rightleftharpoons \mathbf{t}$ tertiary structure transition into consideration.

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