

# Role of the Relaxation of the Iron(III) Ion Spin States Equilibrium in the Kinetics of Ligand Binding to Methaemoglobin

Emmanuel I. Iwuoha† and Kehinde O. Okonjo

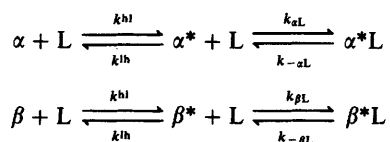
Biophysical Chemistry Laboratory, Department of Chemistry, University of Ibadan, Ibadan, Nigeria

Temperature-jump experiments of the reaction of the thiocyanate ion with human aquomethaemoglobin have been performed in the presence of a 10-fold excess of inositol hexakisphosphate (inositol- $P_6$ ). Two kinetic phases corresponding to the  $\alpha$  and  $\beta$  subunits were observed. Kinetic parameters of the reaction were evaluated from the reciprocal relaxation times on the basis of a fast relaxation of the iron(III) ion spin states equilibrium before binding of the ligand. The association,  $k_{iL}$ , and dissociation,  $k_{-iL}$ , rate constants determined were:  $k_{\alpha L} = 225 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ ,  $k_{-\alpha L} = 1.52 \text{ s}^{-1}$ ,  $k_{\beta L} = 2430 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ ,  $k_{-\beta L} = 6.51 \text{ s}^{-1}$  at 27 °C, pH 6.44. There was good agreement between the equilibrium constant of the ligand binding step determined by static methods ( $K_{\text{equ}} = 204 \pm 11 \text{ dm}^3 \text{ mol}^{-1}$ ) and that evaluated from kinetic data [ $(K_{\alpha L} K_{\beta L})^{1/2} = 235 \pm 12 \text{ dm}^3 \text{ mol}^{-1}$ ]. The value  $k_{\beta L}/k_{\alpha L} = 11$  obtained ensured proper separation of the two kinetic phases. Analyses of the subunit relaxation amplitudes,  $\delta E_{iL}$ , showed that inositol- $P_6$  perturbed the absorption spectrum of the  $\beta$  subunits. This suggests that in the presence of the organic phosphate, methaemoglobin behaves as a protein with independent binding sites rather than as an allosteric molecule. The kinetic and relaxation amplitude spectral characteristics of the subunits, in the presence of inositol- $P_6$  have demonstrated that the kinetic dynamics are effectively decoupled in a stable tetramer.

Methaemoglobin can form high-spin or low-spin compounds, depending on the nature of the ligand. There are high-spin and low-spin ligands corresponding to the formation of methaemoglobin and ligand complexes with a final high-spin or low-spin state. Fluoride, formate and thiocyanate are high-spin ligands, whereas azide, cyanide and imadazole are low-spin ligands.<sup>1–3</sup> In aqueous solution, methaemoglobin forms a complex with one water molecule. This complex is referred to as aquomethaemoglobin. Aquomethaemoglobin is a mixture of high- and low-spin species in thermal equilibrium.<sup>1</sup> This means that two types of haem exist in methaemoglobin: one type in which the iron atom is out of plane with respect to the porphyrin ring and another type in which the iron atom is in the porphyrin plane. The ligand-binding dynamics of each haem type would be determined by the associated stereochemical characteristics.

Two-phase kinetics have been observed for the binding of a number of ligands<sup>4–6</sup> to methaemoglobin. Each kinetic phase consists of a bimolecular reaction. Gibson *et al.*<sup>5</sup> attributed the biphasic kinetics to the heterogeneity of the  $\alpha$  and  $\beta$  subunits of methaemoglobin. On the basis of spectroscopic evidence and temperature-jump studies the fast and slow phases were attributed to the  $\beta$  and  $\alpha$  subunits, respectively.<sup>5–10</sup>

Experimental evidence is available that demonstrates that a high-spin to low-spin transition occurs in aquomethaemoglobin.<sup>1,6,9,10</sup> Interpretations of kinetic data on the reaction of ligands with aquomethaemoglobin which are based on a simple differential bimolecular scheme preclude any involvement of the transition between iron spin states.<sup>4–6</sup> A scheme has been proposed for ligand binding<sup>10</sup> which involves a fast iron spin state transition that precedes the ligand-binding step:



Scheme 1

In Scheme 1,  $\alpha$  and  $\beta$  refer to the subunits in the high-spin state;  $\alpha^*$  and  $\beta^*$  are the corresponding low-spin species; L is the ligand;  $\alpha^*L$  and  $\beta^*L$  are the liganded  $\alpha$  and  $\beta$  subunits, respectively;  $k^{hl}$  and  $k^{lh}$  are the rate constants for the high-to-low and low-to-high spin transitions, respectively; and  $k_{iL}$  and  $k_{-iL}$  are the association and dissociation rate constants for the ligand-binding step of each subunit.

The two reactions in Scheme 1 are coupled *via* the common ligand, L. For the two kinetic phases to be effectively decoupled, three conditions must be satisfied: (i) the relaxation times of the fast ( $\tau_\beta$ ) and slow ( $\tau_\alpha$ ) kinetic phases have to be related by the expression,  $\tau_\alpha/\tau_\beta \geq 10$ ; (ii) there has to be quasi-ligand-buffering; and (iii) the methaemoglobin tetramer has to be devoid of cooperativity. In previous studies<sup>10,11</sup> where Scheme 1 was tested, only the second condition can be said to have been adequately fulfilled. This is because the reactions were carried out in the presence of excess ligand concentration compared with methaemoglobin. Inositol- $P_6$  binds to the  $\beta$  subunit of methaemoglobin (not at the haem iron)<sup>7,12</sup> and brings about an up to 10-fold increase in the reaction rate of the subunit, while that of the  $\alpha$  subunit is unaffected. In addition, inositol- $P_6$  stabilizes methaemoglobin in the tetrameric form (even at low methaemoglobin concentrations when the protein is usually dimeric<sup>9</sup>) and at the same time removes whatever cooperativity exists in the tetramer.<sup>13,14</sup> Schwartz and Schimmel have demonstrated<sup>9</sup> that the binding of inositol- $P_6$  does not alter the haem environment, instead the flexibility of the iron atom is maintained. It is, therefore, possible to ensure complete separation of the kinetics of the  $\alpha$  and  $\beta$  subunits, while having the methaemoglobin tetramer intact, by carrying out ligand binding in the presence of a saturating amount of inositol- $P_6$ . In this work we test the validity of Scheme 1 by carrying out temperature-jump studies of the reaction of methaemoglobin with thiocyanate ion in the presence of excess of inositol- $P_6$ .

## Experimental

### Materials

Haemoglobin was prepared from adult human blood samples containing haemoglobin A. The procedures were those of

† Present address: School of Chemical Sciences, Dublin City University, Dublin 9, Ireland.

Beetlestone and Irvine.<sup>15</sup> The erythrocytes in the blood were collected by centrifuging (MSE Hi-spin 21 centrifuge) the blood sample at 18 000 r.p.m. for 20 min, at 5 °C. The packed cells were washed three times with isotonic saline [9.5 g(NaCl) dm<sup>-3</sup>], lysed with an equal volume of ice-cold distilled water. The haemolysate was centrifuged at 10 000 r.p.m. to remove the cell debris. Organic phosphates were removed from the resulting haemoglobin solution by dialysing at 5 °C against phosphate buffer, pH 7.5, made up to 1 mmol dm<sup>-3</sup> with NaCl. Three changes of the dialysing solution were made at 3 h intervals. The concentration of the haemoglobin solution was determined by measuring the absorbance of the cyanomethaemoglobin complex at 540 nm, using an absorption coefficient (per haem) of 0.109 dm<sup>3</sup> mol<sup>-1</sup> cm<sup>-1</sup>. Haemoglobin was stored at 4 °C and used within 10 days of preparation. Methaemoglobin was prepared using the procedures previously reported,<sup>14</sup> stored at 4 °C and used within three days of preparation.

Inositol hexakis(dihydrogen phosphate), *i.e.* inositol-*P*<sub>6</sub>, was obtained from Aldrich as the dodecasodium hydrate salt.

### Apparatus and Procedures

Temperature-jump and equilibrium binding studies were undertaken on mixtures of methaemoglobin, thiocyanate ion and inositol-*P*<sub>6</sub> as described before,<sup>14</sup> with either the thiocyanate or inositol-*P*<sub>6</sub> concentration being varied. Unless, in the case where its concentration was varied, inositol-*P*<sub>6</sub> was in 10-fold molar excess over methaemoglobin tetramers, the final pH, ionic strength and temperature of the mixtures were 6.44, 0.25 mol dm<sup>-3</sup> and 27 °C, respectively.

The magnetic susceptibility of methaemoglobin was determined by the method of Evans<sup>16</sup> as applied to methaemoglobin by Anusiem.<sup>17</sup> The high concentration of methaemoglobin, which is required for the experiment, was obtained by vacuum filtration. From this an 8 mmol dm<sup>-3</sup> (haem) methaemoglobin solution was prepared in phosphate buffer (pH 6.44, ionic strength 0.1 mol dm<sup>-3</sup>) containing cyanomethane (acetonitrile, BDH). The acetonitrile served as an inert reference.<sup>17</sup> The methaemoglobin solution was saturated with a 10-fold excess of inositol-*P*<sub>6</sub>. NMR measurements were carried out with a 60 MHz Varian T-60 NMR spectrometer. Magnetic susceptibilities and  $k^h/k^{hl}$  were calculated from the relevant resonance lines as described in ref. 17. In order to correct for possible diamagnetic contributions, the experiment was also performed on oxyhaemoglobin, a diamagnetic substance. All experiments were carried out at 27 °C.

## Results

### Kinetic Parameters

From Scheme 1, the rate equation for the reaction of subunit *i*, with ligand is given by

$$-dC_i^*/dt = k_{iL} C_i^* C_L - k_{iL} C_{iL}^* \quad (1)$$

In eqn. (1), *C* denotes concentration. If we assume a small perturbation of the equilibria by a temperature pulse, and a relaxation of the iron spin states equilibrium before that of the ligand binding, then it can be shown that, in the presence of a large excess of ligand compared with methaemoglobin, the reciprocal relaxation time,  $1/\tau_i$  is given by

$$1/\tau_i = k_{iL} C_L + k_{-iL}(1 + k^h/k^{hl}) \quad (2)$$

In the presence of inositol-*P*<sub>6</sub> the reaction of thiocyanate ion with methaemoglobin is characterized by two relaxation times. The faster relaxation phase is assigned to the  $\beta$  sub-

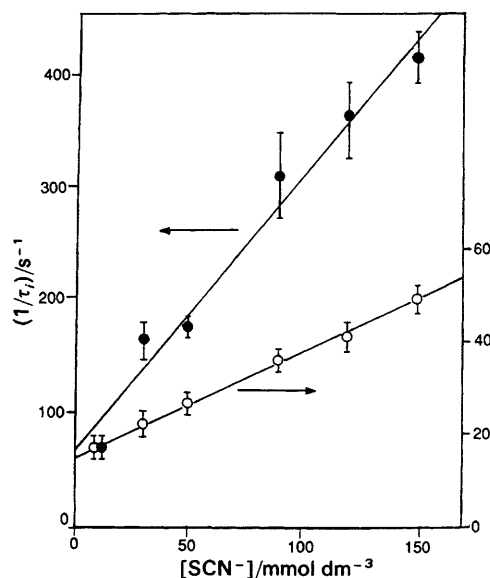


Fig. 1 Reaction of thiocyanate ion with methaemoglobin in the presence of excess of inositol-*P*<sub>6</sub>. Dependence of reciprocal relaxation time on thiocyanate concentration at 27 °C. Conditions: 20 mmol dm<sup>-3</sup> tris-maleate buffer, pH 6.44; ionic strength, 0.25 mol dm<sup>-3</sup> (added salt, NaCl); methaemoglobin concentration, 58  $\mu$ mol dm<sup>-3</sup>; inositol-*P*<sub>6</sub> concentration, 145  $\mu$ mol dm<sup>-3</sup>; observation wavelength, 427 nm. Error bars indicate standard errors from at least three temperature jumps. Fast (or  $\beta$ ) subunit (●). Right-hand ordinate: slow (or  $\alpha$ ) subunit (○).

units.<sup>4,5</sup> In Fig. 1 we present the dependence of the reciprocal relaxation times on thiocyanate concentration,  $C_L$ . The slopes of these plots give the association rate constants,  $k_{iL}$ ; the intercepts,  $k_D^i$ , are only apparent dissociation rate constants defined in eqn. (2) as

$$k_D^i = k_{-iL}(1 + k^h/k^{hl}) \quad (3)$$

The dissociation rate constants  $k_{-iL}$  can be evaluated from eqn. (3) provided we know the value of the iron spin state equilibrium constant,  $k^h/k^{hl}$ . The  $k^h/k^{hl}$  value obtained for inositol-*P*<sub>6</sub>-saturated aquomethaemoglobin at pH 6.44 and 27 °C by magnetic susceptibility measurements is 9.2. The  $k_{iL}$  and  $k_{-iL}$  values are collected in Table 1. These rate constants are related to the thermodynamically determined equilibrium constant  $K_{eq}$  by<sup>4</sup>

$$(K_{\alpha L} K_{\beta L})^{1/2} = K_{eq} \quad (4)$$

where  $K_{iL} = k_{iL}/k_{-iL}$ . From Table 1,  $(K_{\alpha L} K_{\beta L})^{1/2}$  is  $235 \pm 12$  dm<sup>3</sup> mol<sup>-1</sup> which is in reasonable agreement with a  $K_{eq}$  value of  $204 \pm 11$  dm<sup>3</sup> mol<sup>-1</sup>. However, if a simple bimolecular binding scheme, which precludes a spin transition step, is assumed,  $(K_{\alpha L} K_{\beta L})^{1/2}$  would be 23.2 mol dm<sup>-3</sup>, which substantially disagrees with the  $K_{eq}$  value. Therefore, Scheme 1 adequately accounts for thiocyanate binding to methaemoglobin in the presence of inositol-*P*<sub>6</sub>.

### Effect of Inositol-*P*<sub>6</sub> on Subunit Kinetics

A comparison of the  $k_{iL}$  values obtained from the slopes of the plots in Fig. 1 shows that  $k_{\beta L}/k_{\alpha L} = 11$ . This means that in the presence of inositol-*P*<sub>6</sub>, thiocyanate binds 11 times faster to the  $\beta$  than the  $\alpha$  subunits. Fig. 2 shows the effect of varying the concentration of inositol-*P*<sub>6</sub> on the kinetics of thiocyanate binding. Here the thiocyanate concentration was kept constant at 11 mmol dm<sup>-3</sup> while a very low concentration range of inositol-*P*<sub>6</sub> was chosen to avoid saturating the methaemoglobin. To ensure that methaemoglobin does not

**Table 1** Comparison of kinetic and equilibrium parameters for thiocyanate binding to subunits of methaemoglobin within the tetramer in the presence of inositol hexakisphosphate at 27 °C

methaemoglobin subunit	$k_{iL}/\text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$	$k_{-iL}/\text{s}^{-1}$	$(k_{iL}/k_{-iL})/\text{dm}^3 \text{ mol}^{-1}$	$[(K_{\alpha L} K_{\beta L})^{1/2}]/\text{dm}^3 \text{ mol}^{-1}$
$\alpha$	$225 \pm 6$	$1.52 \pm 0.05$	$148 \pm 4$	$235 \pm 12$
$\beta$	$2430 \pm 189$	$6.51 \pm 1.67$	$374 \pm 29$	

The kinetic parameters shown in this table were determined with eqn. (2) and (3).  $k^h/k^{hl} = 9.2$  at pH 6.44, 27 °C. The binding constant determined from equilibrium titration is  $K_{eq} = 204 \pm 11 \text{ dm}^3 \text{ mol}^{-1}$ . Conditions: 20 mmol  $\text{dm}^{-3}$  tris-maleate buffer, pH 6.44; ionic strength 0.25 mol  $\text{dm}^{-3}$  (added salt NaCl); methaemoglobin concentration, 58  $\mu\text{mol dm}^{-3}$  (haem). Errors quoted are standard errors.

disproportionate and form dimers,<sup>9</sup> the concentration was kept high (58  $\mu\text{mol dm}^{-3}$ ). Under these conditions, the reaction of thiocyanate ion with the subunits would be sensitive to changes in inositol- $P_6$  concentration. The graphs show that the kinetics of the  $\beta$  subunits are affected, while the  $\alpha$  subunits are not affected.

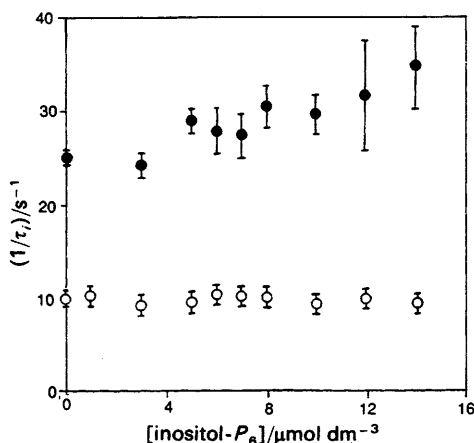
### Spectral Heterogeneity of $\alpha$ and $\beta$ Subunits

In Fig. 3 the relaxation amplitudes of each subunit ( $\delta E_{iL}$ ), with and without inositol- $P_6$ , have been plotted against subunit fractional saturation with thiocyanate,  $Y$ . The shapes of these plots are expected to be convex upwards since the amplitude should increase from a low value at low  $Y$ , through a maximum of  $Y \approx 0.5$ , to a low value as  $Y \rightarrow 1.0$ . The values of  $Y$  were calculated for each subunit with the equation:

$$Y_i = K_{iL} C_L / (1 + K_{iL} C_L) \quad (5)$$

Eqn. (5) is valid for  $C_L \gg C_{Hb}$ , where  $C_{Hb}$  is the total methaemoglobin concentration. Under our experimental conditions, the lowest thiocyanate concentration, 1 mmol  $\text{dm}^{-3}$ , is in 17-fold excess of  $C_{Hb}$  (58  $\mu\text{mol dm}^{-3}$ , haem basis).

Fig. 3 shows that the expected convex curves were obtained for both the  $\alpha$  and  $\beta$  subunits in the absence of inositol- $P_6$ . However, in its presence there was a drastic reduction of  $\delta E_{\beta L}$  values to the levels of  $\delta E_{\alpha L}$ ; the latter being unaffected, particularly at high fractional saturations. This clearly indicates that the  $\beta$  subunit is perturbed in such a way that its absorption coefficient is diminished. Thus, the inositol- $P_6$ -induced spectral changes reported by Perutz *et al.*<sup>18</sup> for the thiocyanate-methaemoglobin complex at 427

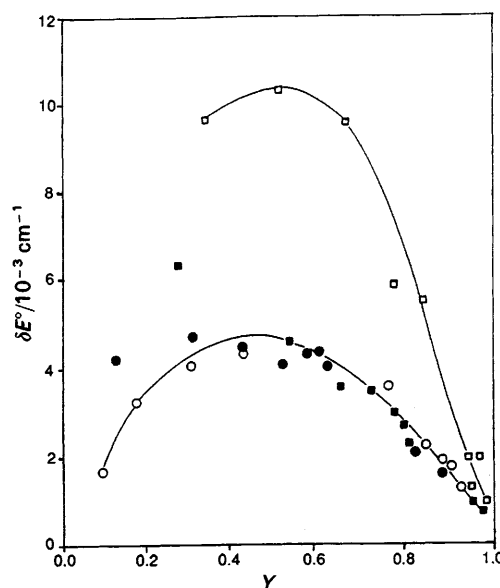


**Fig. 2** Dependence of reciprocal relaxation time,  $1/\tau$ , on inositol- $P_6$  concentration at 27 °C. Conditions: 20 mmol  $\text{dm}^{-3}$  tris-maleate buffer (pH 6.44), ionic strength, 0.25 mol  $\text{dm}^{-3}$  (added salt, NaCl); methaemoglobin concentration, 58  $\mu\text{mol dm}^{-3}$  (haem basis); thiocyanate concentration, 11 mmol  $\text{dm}^{-3}$ ; observation wavelength, 427 nm. Standard errors from three temperature jumps are indicated by the error bars.

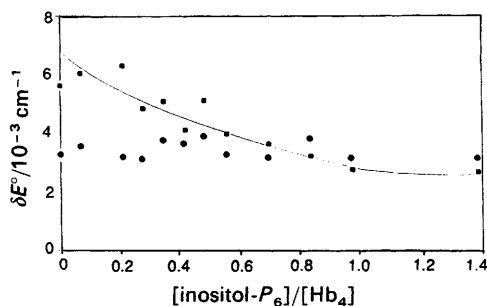
nm, our observation wavelength, are confined to the  $\beta$  subunits.

The effect of inositol- $P_6$  on the spectral properties of the  $\beta$  subunits on the methaemoglobin thiocyanate complex may be observed in another way. We have determined the effect of increasing inositol- $P_6$  concentrations on the amplitude, at a fixed thiocyanate concentration. The thiocyanate concentration selected for this purpose was 11 mmol  $\text{dm}^{-3}$ . At this concentration, the  $\alpha$  subunits are ca. 60%, and the  $\beta$  subunits ca. 80%, saturated with ligand [compare with eqn. (5) and the binding constants of Table 1]. From Fig. 3 we predict that, at low inositol- $P_6$  concentrations,  $\delta E_{\alpha L}$  ( $Y = 0.6$ ) will be lower than  $\delta E_{\beta L}$  ( $Y = 0.8$ ), but at high inositol- $P_6$  concentrations  $\delta E_{\alpha L}$  ( $Y = 0.6$ ) will be higher than  $\delta E_{\beta L}$  ( $Y = 0.8$ ). We also predict that  $\delta E_{\alpha L}$  will be unaffected by the inositol- $P_6$  concentration.

Fig. 4 shows that these predictions are fully borne out. As expected from Fig. 3,  $\delta E_{\alpha L}$  ( $Y = 0.6$ ) fluctuates in the region  $(3.1-3.9) \times 10^{-3}$  without any definite trend being observed over the range of inositol- $P_6$  concentrations used. In sharp contrast to this,  $\delta E_{\beta L}$  ( $Y = 0.8$ ) decreases from  $6 \times 10^{-3}$  at zero inositol- $P_6$  concentration to  $2.5 \times 10^{-3}$  at the highest inositol- $P_6$  concentration, an absorbance change of ca.  $3.5 \times 10^{-3}$  which is in excellent agreement with the inositol- $P_6$ -induced spectral change of the  $\beta$  subunits at 80% thiocyanate saturation (compare with Fig. 3). This result obtained



**Fig. 3** Subunit relaxation amplitude,  $\delta E_{iL}$ , as a function of fractional saturation with thiocyanate,  $Y$ . Values of  $Y$  were calculated from eqn. (5) of the text using the binding constants reported in Table 1. Open symbols: stripped methaemoglobin, 58  $\mu\text{mol dm}^{-3}$  haem, pH 6.44. Filled symbols: methaemoglobin (58  $\mu\text{mol dm}^{-3}$  haem) plus 145  $\mu\text{mol dm}^{-3}$  inositol- $P_6$ , pH 6.44. ( $\square$ ,  $\blacksquare$ ) fast (or  $\beta$  subunit) relaxation; ( $\circ$ ,  $\bullet$ ) slow (or  $\alpha$  subunit) relaxation. Other conditions as in Fig. 1.



**Fig. 4** Subunit relaxation amplitude,  $\delta E_{IL}$ , as a function of the ratio total inositol- $P_6$ : total methaemoglobin tetramer. (■)  $\beta$  subunits; (●)  $\alpha$  subunits. Methaemoglobin concentration,  $58 \mu\text{mol dm}^{-3}$  haem; thiocyanate concentration,  $11 \text{ mmol dm}^{-3}$ . The thiocyanate concentration was sufficient to give ca. 60% saturation of the  $\alpha$  subunits and ca. 80% saturation of the  $\beta$  subunits [compare with Table 1 and eqn. (5) of the text]. Other conditions as in Fig. 2. Each  $\delta E_{IL}$  value plotted is the average from three experiments. (—) Trend of  $\delta E_{PL}$ .

with thiocyanate ion as ligand, suggests that at high concentrations of inositol- $P_6$ , the absorption spectra of the  $\alpha$  and  $\beta$  subunits of methaemoglobin are very similar.

### Discussion

The iron(III) ions in methaemoglobin are in a state of dynamic spin equilibrium, as has been demonstrated in temperature-jump studies.<sup>9,14</sup> A kinetic scheme, such as Scheme 1, in which the iron atoms assume the low-spin form to bind a ligand is readily understandable if a strong field ligand is involved. However, the situation becomes more curious for a ligand, such as the thiocyanate ion, that forms a high-spin complex with methaemoglobin. Our results show that Scheme 1 applies to thiocyanate binding even in the presence of inositol- $P_6$  which is known<sup>9,14,18</sup> to increase the high-spin population of methaemoglobin by 6–10%. This suggests that acquiring a thermodynamically favourable stereochemistry of the haem binding site, rather than the ligand field strength, controls the dynamics of ligand binding. Support for this idea comes from the finding, from X-ray crystallography, that in fluoromethaemoglobin the iron atom is either in the haem plane or  $0.8 \text{ \AA}$  on the distal side.<sup>12</sup> This is in spite of the fact that fluoromethaemoglobin is a high-spin complex. Note that the iron atom in the haem plane or towards the distal histidine is low spin, while it is high spin when out of plane towards the proximal histidine.<sup>19</sup> The  $^1\text{H}$  NMR study of Neya and Morishima,<sup>20</sup> in which common structural changes were observed for both high- and low-spin methaemoglobin–ligand complexes, in the presence of inositol- $P_6$ , further shows that irrespective of ligand field strength, the same kinetic scheme should hold.

In order to ensure that inositol- $P_6$  does not bind to the haem iron atoms, we carried out temperature-jump studies on methaemoglobin solution in the presence and absence of the organic phosphate monitored at spin-sensitive wavelengths.<sup>1</sup> A single relaxation spectrum was observed in each case which shows clearly that inositol- $P_6$  does not alter the haem environment. Thus the two relaxation phases observed in this work for thiocyanate binding to the inositol- $P_6$ –methaemoglobin complex are entirely due to the heterogeneity in the kinetics of the  $\alpha$  and  $\beta$  subunits.

This study shows that the relaxation amplitudes of thiocyanate binding to the  $\alpha$  subunit are not affected by the presence of inositol- $P_6$ . However, comparison of the  $\beta$  subunit amplitudes with and without phosphate, at the same frac-

tional saturation (with thiocyanate), shows that the  $\beta$  subunit amplitude is diminished by inositol- $P_6$ . Therefore, inositol- $P_6$  decreases the absorption coefficient of the  $\beta$  subunits. These findings agree with the NMR spectra<sup>20</sup> which show that the intensities of the methyl peaks of only the  $\beta$  subunits are reduced at low ligand (azide ion) concentrations in the presence of 4 mol inositol- $P_6$ /methaemoglobin tetramer. This suggests that inositol- $P_6$  perturbs the  $\beta$  subunit during ligand binding and may not have any effect on the  $\alpha$  subunit. Thus the inositol- $P_6$ -enhanced kinetics of ligand binding to  $\beta$  subunits usually reported<sup>4,5,10</sup> may be attributed to this perturbation.

### Conclusion

The pattern of kinetic and spectral relaxation amplitude differences between the subunits in the presence of inositol- $P_6$  shows a lack of cooperativity. This is consistent with the reports that the phosphate makes the methaemoglobin behave like an aggregation of four subunits;<sup>21</sup> with the same haem environment,<sup>9</sup> but with different polypeptide chains,<sup>7</sup> which react independently of each other, in a stable tetramer.<sup>9</sup> These are necessary conditions for effective decoupling of the  $\alpha$  and  $\beta$  kinetic phases in methaemoglobin reactions. Inositol- $P_6$ -saturated methaemoglobin is, therefore, suitable for studying the reaction of the protein with anionic ligands by chemical relaxation methods. Accordingly, what happens after a temperature jump is that the iron(III) ion spin states equilibrium relaxes to produce commensurate low-spin methaemoglobin species to which ligands preferentially bind.

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