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Full Length Research Paper

Effect of High Ionic Strength and Inositol Hexakisphosphate on the Reactivity of CysF9[93]β of Rabbit Haemoglobin

Adedayo Akinkunmi Fodeke[1], Jonathan Oyebamiji Babalola[2] and Christopher Olumuyiwa Aboluwoye[3]

1- *Department of Chemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria.*
2- *Department of Chemistry, Faculty of Science, University of Ibadan, Ibadan, Nigeria.*
3- *Department of Chemical Sciences, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria.*

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Corresponding Author
Adedayo A. Fodeke
Department of Chemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria.

Abstract
At ionic strength of 50 mmol dm^{-3} the pH dependence profile of the apparent second-order rate constant, k_{app}, for the reaction of 5,5'-dithobis(2-nitrobenzoate), DTNB, with the CysF9[93]β of stripped (i.e free of organic phosphate) rabbit haemoglobin are complex. Quantitative analyses of the complex profiles indicate that pK_a values of groups linked to the reactivity are; 6.20 ± 0.05, 7.00 ± 0.20 and 8.4 ± 0.05 assigned to HisH21[143]β, ValNA1[1]β and the sulfhydryl group of CysF9[93]β respectively. At an ionic strength of 50 mmol dm^{-3} in the presence of organic phosphate inositol hexakisphosphate (inositol-P_6) and at an ionic strength of 200 mmol dm^{-3} the complex profiles are converted to simple profiles; the rates of the DTNB reaction were also greatly reduced. Quantitative analyses of the simple profiles gave pK_1 values ranging from 5.80 to 6.50, and pK_2 ranging from 7.84 to 8.94 depending on derivatives and whether the reaction was at ionic strength 200 mmol dm^{-3} or in the presence of inositol-P_6. These pK_as were assigned to the ionization of HisHC3[146]β and CysF9[93]β, respectively. The rates of reactions of stripped rabbit hemoglobin were faster than for corresponding human hemoglobin by at least a factor of 3. These differences were reduced or even reversed in the presence of inositol-P_6 or ionic strength 200 mmol dm^{-3}.

Keywords: *Haemoglobin, sulphydryl group, ionizable groups, 5,5'-dithiobis(2-nitrobenzoate).*

Introduction

The CysF9[93]β sulfhydryl group of haemoglobin is invariant in all animals but in fish and amphibian hemoglobin's [1]. This sulfhydryl has served as a probe to structure change over the past five decades [2-9]. The nature and number of the groups that influence the reactivity of CysF9[93]β in the R quaternary structure have been determined from studies of the pH dependence of the reactivity of the sulfhydryl [10 – 15].

It has previously been demonstrated [14] that the reactivities of CysF9[93]β of human hemoglobin A and S towards 5,5'dithiobis(2-nitrobenzoate) (DTNB), a reagent carrying two negative charges, are different. This differences was accounted for largely on the basis of differences in the positive electrostatic field produced at the F9[93]β site by the ionizable amino acid residues on each protein.

Hemoglobin A and S differ by only a single amino acid mutation at the A3[6] site on each of their two β subunits where a negatively charged glutamic acid residue in haemoglobin A is replaced by a neutral valine residue in haemoglobin S [16]. Since the differences between haemoglobins A and S is small, we now extend the study to the DTNB reaction of rabbit hemoglobin, whose amino acid sequence [17, 18] differs from that of human haemoglobin [19] by several amino acid changes. We present here a report of a detailed pH dependence kinetic study of the reaction of four rabbit haemoglobin derivatives with DTNB.

Materials and methods

Oxyhaemoglobin was prepared from freshly drawn blood of rabbit collected into anticoagulant using standard laboratory procedure. The oxyhaemoglobin was dialysed against distilled water and was converted into the carbonmonoxy and aquomet derivatives. All the haemoglobin derivatives were stripped (freed of ions and endogenous organic phosphate) by passage through a beds of ion exchangers (Dintzis column) [20]. The concentrations of the oxy and aquomet derivatives were determined spectrophotometrically at 540 nm as the cyanomet derivative using a molar extinction coefficient (per haem) of 10.9 x 10^3 $M^{-1}cm^{-1}$. A molar absorptivity of 1.4 x 10^4 $M^{-1}cm^{-1}$ at 537.5 nm was used for the carbonmonoxyhaemmoglobin. Rabbit azidomet was prepared as previously described [10, 12], using published values of azide binding constants [21].

Kinetics

The procedure for carrying out the kinetic experiment has been described before in detail [9]. Transmittance reading obtained at a wavelength of 412 nm was converted to absorbance. Since the reaction of DTNB with CysF9[93]β sulfhydryl group of haemoglobins a second-order process, the data were fed into a computer programmed to calculate the second-order rate constant, k_{app}, from the second-order rate equation. The concentration of 5-thio-2-nitrobenzoate (TNB) produced at a given time was calculated using published values of the molar absorption coefficient of TNB at 412 nm as a function of pH. [22]

Curve fitting

The curve fitting of the pH dependence profiles of the apparent second-order rate constant, k_{app}, was performed with computer programme written on Matlab software.. The direct approach to the curve fitting problem (the full variable approach) minimizes the objective function. Chisquared, with respect to variation of both the rate constants k_j and the ionization constants K_j. Another approach (the restricted variable approach) expresses the rate constants (which occur linearly in the equation to be fitted) in terms of the ionization parameters by solving a least-squares problem; it then minimizes the objective function with respect to the ionization parameters alone. Both approaches were supported by the software used. In the analysis, an automatic non-negative constraint is imposed by working directly with the pK_j values of the ionization parameters. The software supports the Powell conjugate direction algorithm [23, 24] and Brent's parabolic interpolation algorithm [28]. It also supports several hybrid combinations of these algorithms obtained by using the result from one algorithm as the initial guess for another algorithm.

Results

Data at ionic strength 50 mmol dm^{-3} in stripped haemoglobin (free of organic phosphate)

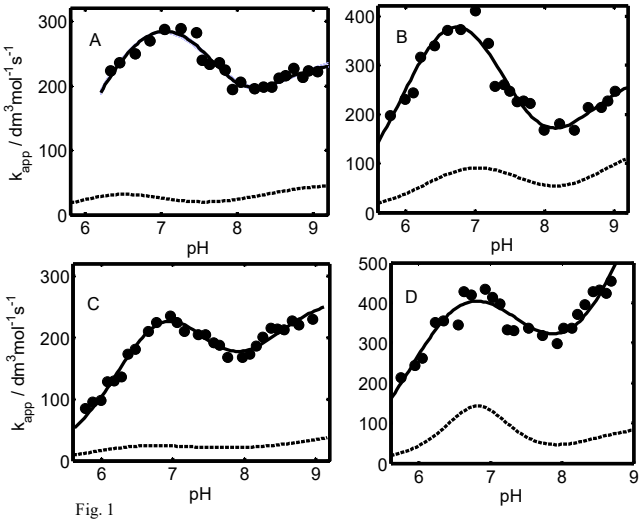


Fig. 1

Dependence of k_{app} on pH for the reaction of DTNB with the CysF9[93]β sulfhydryl group of rabbit haemoglobin stripped of organic phosphate (experimental points): (a) Oxyhaemoglobin, (b) carbonmonoxhaemoglobin, (c) azidomethaemoglobin, (d) aquomethaemoglobin. Dashed curves are the theoretical best-fit curve through data for human haemoglobin A calculated with the parameters reported in Table 2 of ref. 13. Condition: phosphate buffers, $5.6 \leq pH \leq 8$; borate buffers, $pH > 8.0$; ionic strength 50 mmol. dm^{-3} (added salt NaCl); temperature, 20°C; haemoglobin concentration, 10 µmol. (haem) dm^{-3} (5 µmol. dm^{-3} in reactive sulfhydryl group). Each experimental point is the mean of three determinations subject to a standard error of about 10 %. The curve through the experimental point are the theoretical best-fit curves calculated with parameters reported in Table1 [compare with scheme 1 and eqn. (1) with n = 2 for (a) – (c) and n = 3 for (d)].

Fig. 1a shows the dependence of k_{app}, the apparent second-order rate constant, on pH for the reaction of stripped (that is, free of organic phosphates) rabbit oxyhaemoglobin with DTNB at ionic strength of 50 mmol dm^{-3}. Each experimental point is the mean calculated from three kinetic runs and subjected to a standard error of about 10 %. It is seen that the pH dependence profile is complex. The experimental points show a peak of reactivity around pH 7.0, with a drop on either side of this pH and then a rise on going from pH 8.0 to 9.0. Similar results are shown for the carbonmonoxy- (Fig. 1b), azidomet- (Fig. 1c) and aquomet- (Fig. 1d) derivatives, with peaks at pH about 6.8 in each case. Such complex profiles have been obtained before for human haemoglobins [13, 14].

Data at ionic strength 50 mmol dm^{-3} in the presence of inositol hexakisphosphate

Fig. 2a reports k_{app}, as a function of pH for the reaction of DTNB with CysF9[93]β of four rabbit hemoglobin derivatives at an ionic strength of 50 mmol. dm^3 in the presence of inositol-P_6. For each derivative, the complex profile obtained for stripped haemoglobin (Fig. 1) is a dramatically altered by the organic phosphate to the form of the titration curve of a simple acid. Inositol-P_6 also causes a dramatic decrease in the reactivity of the sulfhydryl group (compare Figs. 1 and 2). In the low pH range this is particularly noticeable for the carbonmonoxy and aquomet derivatives, for which the decrease in reactivity is by two orders of magnitude (compare data points in Fig. 1b and d with those in Fig. 2b and d, respectively).

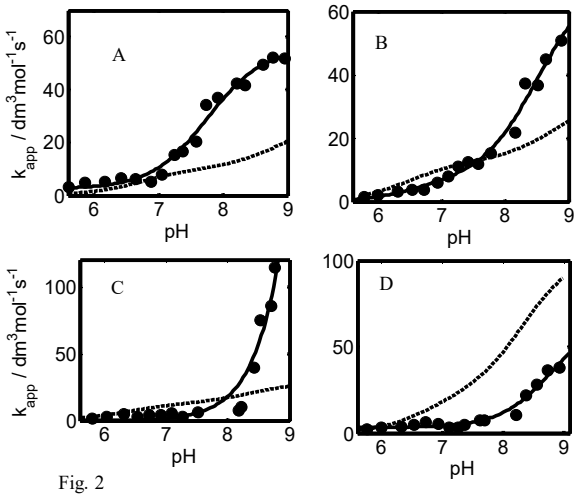


Fig. 2

Dependence of k_{app} on pH for the reaction of DTNB with the CysF9[93]β sulfhydryl group of rabbit haemoglobin in the presence of inositol-P_6. (a) Oxyhaemoglobin, (b) carbonmonoxhaemoglobin, (c) azidomethaemoglobin, (d) aquomethaemoglobin. The conditions were the same as in Fig. 1 except that 10 μmol. dm^{-3} of inositol-P_6 was added. The curve through the experimental data points are theoretical best-fit curve calculated with parameters reported in Table 2, using eqn. (3), [(a) and (d)] or eqn. (2), b. A theoretical fit was not possible for azidomet data, (c). Data for human haemoglobin A (dotted curves) are calculated with the parameters reported in Table 1 of ref. 13

Data at ionic strength 200 mmol. dm^{-3}

Fig. 3 reports data for the reaction of DTNB with rabbit haemoglobin at an ionic strength of 200 mmol. dm^{-3}.

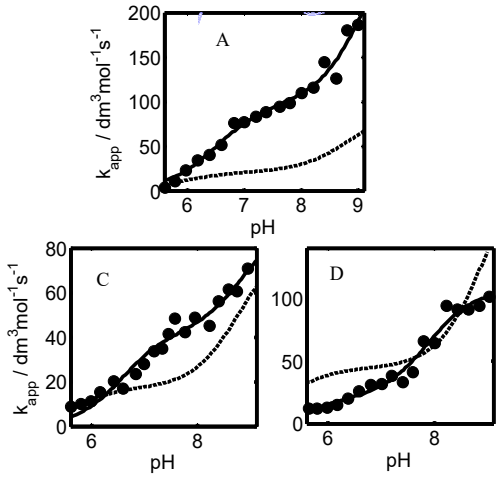


Fig. 3

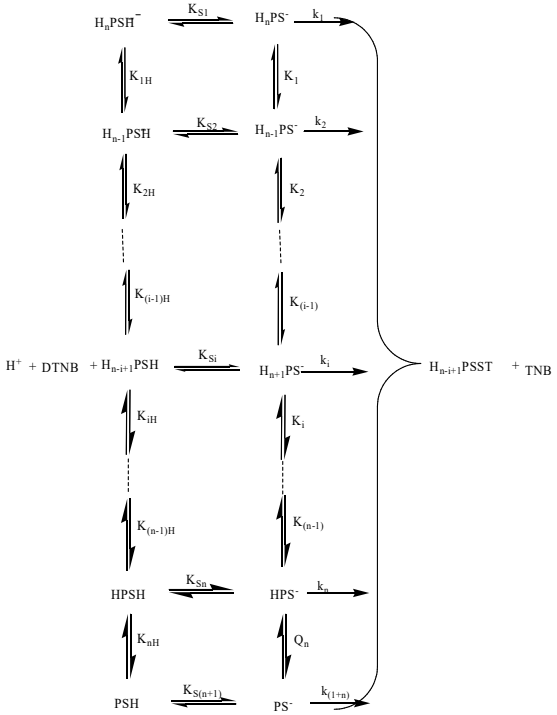
Dependence of k_{app} on pH for the reaction of DTNB with the CysF9[93]β sulfhydryl group of rabbit haemoglobin (stripped of organic phosphates) at ionic strength 200 mmol. dm^{-3}. Apart from increased salt concentration other conditions were as in Fig.1. (a) Oxyhaemoglobin, (b) carbonmonoxhaemoglobin, (c) azidomethaemoglobin, (d) aquomethaemoglobin. The curve through the experimental data points are theoretical best-fit curve calculated with parameters reported in Table 3 using eqn. (2). Dashed curves are theoretical best-fit curve through the data for human haemoglobin A calculated with the parameters in Table 6.

It is seen that at a higher salt concentration the complex profiles obtained at ionic strength 50 mmol. dm^{-3} (Fig. 1) are converted to profiles resembling titration curve of a simple acid. There is also dramatic drop in reactivity arising from the increase in salt concentration (compare data points in Fig. 1 and 3). Similar, but not so drastic, results have been obtained for human haemoglobins [13, 14].

Discussion

Quantitative analyses of the complex profiles

Complex profiles like those in Fig. 1 arise as a consequence of electrostatic interaction between CysF9[93]β site and the basic group at the organic phosphate binding site [13,14]. Such profiles have been accounted for previously with the following Scheme shown [13, 14].



Scheme 1

In scheme I, n ionizable groups are electrostatically linked to the CysF9[93]β site of haemoglobin. H_nPSH, $H_{n-1}PSH$, ……, $H_{n-i+1}PSH$,….., HPSH and PSH are haemoglobin species having n, (n-1), …….., (n-i+1),……, 1 and 0 protons bound respectively, to the electrostatically thiol-linked ionizable groups; each of these species has its thiol group protonated. The corresponding thiolate anion forms are H_nPS^-, $H_{n-1}PS^-$, ……, $H_{n-i+1}PS^-$,….., HPS^-, and PS^- respectively. ki (i = 1, 2,…, n+1) are the apparent second-order rate constants for the reaction of DTNB with the thiolate anion forms of the various species. (Only thiolate anion forms are reactive towards DTNB [29, 30].) The K_j values (j = i, i+1,….,n) are the ionization constants of proton bound to various species of hemoglobin in which the ionizable groups are electrostatically linked to CysF9[93]β. These include carboxylic acids, histidines and terminal valines. The relationship between k_{app} and the parameters of scheme 1 is given by the expression [13, 14]:

$$k_{app} = \frac{k_{n+1} + \sum_{i=1}^{n} k_i (H^+)^{n-i+1} \left(\prod_{j=i}^{n} K_j \right)^{-1}}{\left\{ 1 + \sum_{i=1}^{n} (H^+)^{n-i+1} \left(\prod_{j=i}^{n} K_j \right)^{-1} + \frac{(H^+)}{K_{s(n+1)}} \left[1 + \sum_{i=1}^{n} (H^+)^{n-i+1} \left(\prod_{j=i}^{n} K_{jH} \right)^{-1} \right] \right\}} \quad ..(1)$$

We have fitted the data in Fig. 1 with eqn. (1) using the curve fitting software (see Materials and Methods). In fitting the oxy, carbornmonoxy and azidomet data with eqn. (1) we assumed that the organic phosphate binding groups; Val1NA[1]β, His2NA[2]β, and HisH21[143]β are the ionizable groups that constitute the electrostatic environment of CysF9[93]β. This assumption has been justified before [13, 14]. LysEF6[82]β is one of the basic group at the organic phosphate binding site. Nevertheless, it is excluded from consideration because its pK_a value of 10.5 is too high for it to be ionizable in the pH range 5.6 – 9.0 of the experiments. Therefore in Scheme (1) and eqn. (1) n has the value of 3. If the simplifying assumption is made that His2NA[2]β and His21[143]β have about the same pK_a, the value of n is reduced to 2. The curves in Fig. 1 a – c are theoretical best-fit curves calculated from eqn. (1). As can be seen, very good fits are obtained to all the data. The best-fit parameters are reported in Table 1. It is notable that the various pKj values in Table 1 are similar to those obtained previously for human haemoglobins [13, 14]

In fitting the aquomet data (Fig. 1d), cognizance was taken of the fact that the water molecule bound at the 6th coordination position of each iron (III) atom of aquomethaemoglobin confers a positive charge on each iron atom. It has been shown that this water molecule constitutes part of the electrostatic environment of CysF9[93]β [13,14]. Therefore the aquomet data were analyzed with eqn. (1) using a value of n = 3. At an ionic strength of 50 mmol. dm^{-3} the pK_a of water molecule is 8.02 for rabbit aquomethaemoglobin [31]. In fitting the aquomet data, this pK_a was assumed and treated as a non-adjustable parameter. The curve through the data points in Fig. 1d is the best-fit curve calculated with the fitting parameters reported in Table 1. It is seen that a very good fit is obtained to the data.

Quantitative analyses of the simple profiles

Profiles such as those in Figs. 2 and 3 have been previously analyzed [10 – 12] with the two - term equation,

$$k_{app} = k_1 \frac{K_1}{K_1 + [H^+]} + k_2 \frac{K_2}{K_2 + [H^+]} \text{ ----------(2)}$$

Eqn. (2) is based on the finding that there is a Bohr effect in R-state haemoglobin involving HisHC3[146]β [32]. This proves that the histidine forms salt bridge with AspFG1[94]β, resulting in steric hindrance by TyrHC2[145]β to the approach of reagent to CysF9[93]β [33]. At high pH, the histidine ionizes and the hindrance to the approach of DTNB to CysF9[93]β is eliminated. Consequently, the reactivity of the sulfhydryl should increase as the histidine increasingly ionizes with increasing pH. In eqn. (2) k_1 is the limiting apparent second-order rate constant at high pH for the DTNB reaction when the reactivity of CysF9[93]β is linked to the ionization of HisHC3[146]β, with ionization constant K_1, the first fractional term is the fraction of the neutral form of the histidine. k_2 is the limiting apparent second-order rate constant at high pH when the reactivity of the sulfhydryl group is linked to its own ionization, with ionization constant K_2. The second fractional term is the fraction of the thiol anion from of the sulfhydryl. Only this form reacts with DTNB [29, 30].

Table 1: Reaction of stripped rabbit haemoglobin dervatives with DTNB at ionic strength 50 mmol. dm^{-3}

		derivative		
Parameter	**Oxy**	**Carbonmonoxy**	**Azidomet**	**Aquomet**
pK_{1H}	6.12	6.06	6.35	6.07
pK_1	6.09	6.19	6.23	6.27
pK_{2H}	7.71	7.35	7.34	7.50
pK_2	7.54	6.86	6.74	6.85
pK_{S3}	8.42	8.48	8.27	-
pK_{S4}	-	-	-	8.43
k_1	118143.0	330435.0	145671.0	338742.0
k_2	1032.0	2465.0	3323.0	5054.6
k_3	275.0	296.0	257.4	1157.0
k_4	-	-	-	575.7

[a]Best –fit parameters used to fit the rabbit data in Fig. 1 [cf. scheme 1 and eqn. (1)]. The units of k_i are $dm^3\ mol^{-1}s^{-1}$.

Attempts to analyze the profiles in Figs. 2 with eqn. (2) show that, with the exception of the carbornmonxy derivative (Fig. 2b), good fits could be obtained to the data only with pK_1 values of 5 or less. Since such low values are unsatisfactory for a histidine, the oxy and aquomet data were reanalyzed with simple one term equation:

$$k_{app} = k_2 \frac{k_2}{K_2 + [H^+]} + b \text{ ---------- (3)}$$

It was impossible to analyse the azidomet data with pK_2 value less than ca. 10. In eqn. (3) k_2 is the limiting apparent second-order rate constant at high pH for the DTNB reaction, which is subject only to the influence of the ionization of a single group with ionization constant K_2; b is a constant term equal to the base lines in Fig. 2a and d. The curve through the data points in these figures are the best-fit curves drawn through the experimental points. The best-fit parameters are reported in Table 2. Since eqn. (3) gave better fit to data reported in Fig. 2a and d, than eqn. 2, these curves represent the titration curve of a monoprotic acid. The best-fit parameters of the fit of eqn. (2) to the carbonmonoxy data (Fig. 2b) are also reported in Table 2.

Table 2: Reaction of rabbit haemoglobin derivatives with DTNB at ionic strength 50 mmol. dm^{-3} in the presence of inositol-P_6.

		Derivative		
Parameter	**[A]oxy**	**[B]carbonmonoxy**	**Azidomet**	**[A]aquomet**
pK_1	-	6.50	-	-
pK_2	7.90	8.53	-	8.60
k_1	-	6.60	-	-
k_2	65.00	56.80	-	14.00
baseline	5.00	-	-	2.50

[a]Best-fit parameters employed to calculate the curves in Fig. 3 (cf. eqn. (3)). [b]Fit with eqn. (2). The units of k_i are $dm^3\ mol^{-1}\ s^{-1}$.

It is seen that the fit to the data in Fig. 2a, b and d are good. The pK_1 value of 6.50 for carbomonoxyhaemoglobin, is assigned to HisHC3[146]β and pK_2 value of 7.90, 8.53 and 8.60 for oxy and aquomet derivatives respectively, are assigned to CysF9[93]β.
Eqn.2 was employed to fit the data in Fig 3. The curve through the data points are the best-fit curve calculated with the parameters reported in Table 3. It is seen that very good fits are obtained to all the data.

Table 3: [a]Reaction of stripped rabbit haemoglobin derivatives with DTNB at ionic strength 200 mmol. dm^{-3}.

		Derivative		
Parameter	**Oxy**	**Carbonmonoxy**	**Azidomet**	**Aquomet**
pK_1	6.44	6.00	6.55	5.81
pK_2	8.82	8.94	8.77	7.89
k_1	55.51	17.51	40.86	20.54
k_2	105.16	72.64	43.19	88.25

[a]Best-fit parameters employed to calculate the curves in Fig. 2 (cf. eqn. (2)). The units of k_i are $dm^3\ mol^{-1}\ s^{-1}$.

The mean value of pK_1 is 6.20 ± 0.20; that of pK_2 is 8.60 ± 0.30. These values are those to be expected for histidine and cysteine residue in haemoglobin, respectively. Following the previous assignments, [10 – 15] these pK_a values were assigned to HisHC3[146]β and CysF9[93]β, respectively.

Effect of inositol-P_6 on the shape of the pH dependence profile at ionic strength 50 mmol. dm^{-3}.
The profiles of oxy and aquomethaemoglobin in the presence of inositol-P_6 (Fig. 2a and d) are monophasic and best fitted with the one-term eqn. (3). The pK_as of the single ionizable group whose ionization is linked to the reactivity of CysF9[93]β in the two derivatives are 7.90 and 8.6 respectively (Table 2). These pK_as are readily assigned to CysF9[93]β. (Although the profile for azidomethaemoglobin could not be fitted with either eqn. (2) or (3), the trend of the data (Fig. 2c) looks monophasic.) There seems to be no indication that the ionization of HisHC3[146]β affects the reactivity of the sulfhydryl in the oxy, azidomet, and aquomet derivatives. On the other hand, the carbonmonoxyhaemoglobin profile is biphasic and was best fitted with two term equation; eqn. (2); and there is a clear indication that the ionization of HisHC3[146]β affects the reactivity of CysF9[93]β. This latter result conforms to previous results obtained on the effect of inositol-P_6 on the pH dependence profiles of human haemoglobin [13, 14].

There are two possible alternative explanations for the monophasic nature of the oxy, azidomet and aquomet data [Fig. 2a, c and d]: On binding of inositol-P_6, either (i) the pK_a of HisHC3[146]β becomes very low, below 5, so that its ionization has already taken place below the pH range of the experiment, $5.60 \leq pH \leq 9.00$; or (ii) the pK_a of histidine is raised considerably, so that its ionization cannot be distinguished from that of CysF9[93]β. The first alternative is highly unlikely because the pK_as of histidines are usually higher than 5. Moreover, it would imply that in low pH range the salt bridge formed between HisHC3[146]β and AspFG1[94]β does not exist in the oxy-, azidomet- and aquomet derivatives. Consequently, the sulfhydryl reactivity of these derivatives should be much higher than that of the carbonmonoxy derivative in which the salt bridge does exist, as demonstrated by the pK_1 of 6.5 (Table 2). Contrary to this expectation, there is no difference in the reactivities of the four derivatives in the low pH range (Fig. 2). The second alternative is a distinct possibility if inositol-P_6 binds very tightly to rabbit haemoglobin and so strengthens the salt bridge between HisHC3[146]β and AspFG1[94]β; thereby raising the pK_a of the histidine considerably and making it difficult for it to ionize. Indeed, while there is evidence that this histidine ionizes in carbonmonoxy derivative (Fig. 2b, Table 2), there seems to be no indication of ionization in the other derivatives (Fig. 2a, c, and d)

The somewhat unusual shape of the azidomet profile (Fig. 2c) requires comment. (This unusual shape made it impossible for us to carry out a quantitative analysis of the data). Between pH 5.8 and 8.2, k_{app} remains fairly constant at between 2.5 and 5.0 $dm^3\ mol^{-1}.\ s^{-1}$, and there seems to be no change in the reactivity of the sulfhydryl group. [Similar comments can be made for the oxy derivative (Fig. 2a) in the range $5.6 \leq pH \leq 7.0$ and for the aquomet derivative (Fig. 2d) in the range $5.6 \leq pH \leq 7.4$. for rabbit haemoglobin 2.5 $dm^3\ mol^{-1}.\ s^{-1}$ seems to be the lower limit of k_{app} when HisHC3[146]β/FG1[94]β salt bridge is fully formed. Above pH 8.2 however, the reactivity of the azidomet derivative increases considerably. The experiments were repeated at pH >8.2 and essentially the same result as before were obtained. They are not due to incomplete azidomet complex formation since the k_{app} values would have remained low, like those of aquomet derivative at pH >8.2 (Fig. 2d) if this was the case

Comparison of human and rabbit haemoglobins at ionic strength 50 mmol. dm^{-3}.
For the purpose of comparison with rabbit haemoglobin, the theoretical best-fit curve to the data for human haemoglobin A is included in Fig. 1 [13]. It is seen that the trend of the rabbit data is similar to that of the human data. Clearly, all the four rabbit haemoglobin derivatives react faster with DTNB than the corresponding human derivateives. The minimum reactivity difference occurs with the carbonmonoxy derivatives (Fig. 1b), a 2.5-fold difference at pH 9; the maximum reactivity difference, 11-fold, is found with oxy derivatives between pH 7.0 and 7.4. In an attempt to account for the higher reactivity of rabbit haemoglobin, the amino acid sequences of the β subunits of human and rabbit haemoglobins were compared, noting where differences in chare occur close to the F9[93]β site. It was discovered that human and rabbit β chains differ at F3[87]β position, where the neutral threonine residue in human haemoglobin is replaced in rabbit haemoglobin by a positively charged lysine residue. In the 3D structure of haemoglobin this lysine is only 5 Å (0.5 nm) away from CysF9[93]β. The positive electrostatic field created by this lysine at the F9[93]β site of rabbit haemoglobin must be absent in human haemoglobin. This account partially for higher reaction rate of rabbit haemoglobin with negatively charged DTNB compared to human haemoglobin.

Comparison of human and rabbit haemoglobins at ionic strength 50 mmol. dm^{-3} in the presence of inosition-P_6.
In Table 4 reports, as a function of pH, the results of calculations of $k_{app}(-) / k_{app}(+)$, the ratio of the apparent second-order rate constant for the DTNB reaction for stripped and inositol-P_6 bound rabbit haemoglobin. The corresponding data on human haemoglobin are shown in Table 5.

It is seen that inositol-P_6 drastically reduces the reactivity of CysF9[93]β of rabbit haemoglobin, and its effect in the low pH range is about an order of magnitude greater than is the case with human haemoglobin (compare Tables 4 and 5). Indeed the inositol-P_6 effect on rabbit haemoglobin is so great that, in spite of the higher reactivity of stripped rabbit compared to human haemoglobin (Fig. 1), at low pH, in the presence of inositol-P_6, the oxy (Fig. 2a) [and also the carbonmonxy (Fig. 2b)] derivatives of both haemoglobin have

about the same reactivity in the presence of the organic phosphate; between pH 6.0 and 8.2 the azidomet derivative of human haemoglobin is even more reactive than the corresponding rabbit derivative (Fig. 2c); and the human aquomet derivative is more reactive than the rabbit aquomet between pH 6.0 and 9.0. It should be noted that at ionic strength 50 mmol. dm^{-3} the positive electrostatic field provided by LysF3[87]β of rabbit haemoglobin at CysF9[93]β site is still present, since the distance between the two residues (5 Å) is less than the radius of the ionic atmosphere which, at this ionic strength, is ca. 20 Å [34].

Table 4: [a]Ratio of apparent second-order rate constant. $k_{app}(-) / k_{app}(+)$, as a function of pH at ionic strength of 50 mmol. dm^{-3}, for the reaction of DTNB with stripped and ionositol-P_6 bound rabbit hemoglobin derivatives.

		Derivative		
pH	**Oxy**	**Carbonmonoxy**	**Azidomet**	**Aquomet**
6.0	-	160.00	28.60	100.00
6.5	33.00	102.90	60.00	126.60
7.0	23.20	55.40	73.70	102.00
7.5	11.30	23.00	72.20	47.60
8.0	5.30	9.00	31.50	22.40
8.5	3.70	5.90	5.00	16.50
9.0	3.70	5.70	-	-

Table 5: [a]Ratio of apparent second-order rate constant. $k_{app}(-) / k_{app}(+)$, as a function of pH at ionic strength of 50 mmol. dm^{-3}, for the reaction of DTNB with stripped and ionositol-P_6 bound human hemoglobin A derivatives.

		Derivative		
pH	**Oxy**	**Carbonmonoxy**	**Azidomet**	**Aquomet**
6.0	12.00	10.80	4.60	10.00
6.5	6.80	11.70	1.80	12.30
7.0	3.60	9.20	2.10	8.50
7.5	2.20	6.40	1.50	2.60
8.0	2.10	3.80	1.20	1.10
8.5	2.20	3.50	1.20	1.00
9.0	2.00	4.10	1.20	-

[a]Data from the comparison of the relevant data in Ref. 13.

The above results indicate that inositol-P_6 binds more tightly to rabbit than human haemoglobin. Consequently the salt bridge formed between HisHC3[146]β and AspFG1[94]β should be stronger in rabbit than in human haemoglbin. A straightforward way to demonstrate that this is the case would be to show that the pK_a values calculated for this histidine in inositol-P_6 bound rabbit haemoglobin are higher than those of human haemoglobin [13]. These pK_as are, however, not available for all rabbit haemoglobin derivatives, except the carbonmonoxy derivative (see Table 2). For the rabbit carbomonoxy derivative the pKa value is 6.5, the same as that of human (Table 1 of Ref. 13). Nevertheless it is seen (Fig. 2b) that the human carbonmoxy derivative is more reactive than that of rabbit between pH 6 and 7.4.

Table 6: Reaction of stripped human haemoglobin A derivative with DTNB at ionic strength 200 mmol. dm^{-3}.

		Derivative		
Parameter	**[A]oxy**	**[A]carbonmonoxy**	**[B]azidomet**	**[B]aquomet**
Pk_1	5.70	5.60	5.61	5.50
Pk_2	9.00	9.00	8.81	9.50
K_1	19.90	16.00	17.00	46.00
K_2	95.30	105.00	63.60	470.00

[a]Best-fit parameters from Table 2 of ref 14; [b]data from present work

An indirect but more satisfactory way to prove that inositol-P_6 binds more tightly to rabbit than to human haemoglobin is to find a condition in which the pK_a of HisHC3[146]β can be determined for all the four derivatives of two haemoglobins. This has been done for stripped haemoglobin at ionic strength 200 mmol. dm^{-3}. The pK_as are given as pK_1 in Tables 3 and 6 for both haemoglobins. The mean pK_1 values are 6.20 ± 0.16 for rabbit and 5.60 ± 0.05 for human. The higher pK_a of HisHC3[146]β for rabbit haemoglobin proves that the salt bridge is stronger in rabbit than in human haemoglobin at ionic strength 200 mmol. dm^{-3}. It is not unlikely that this is also the case at ionic strength 50 mmol. dm^{-3}.

Comparison of human and rabbit haemoglobin at ionic strength 200 mmol. dm^{-3}

For comparison with rabbit haemoglobin, the best-fit curves to the experimental data for human haemoglobin A [14] are shown as broken curves in Fig. 3. It is seen that the trends in the data are similar for both haemoglobins. A close examination of the data in Figs. 1 and 3 shows that, apart from the differences noted above for the stripped haemoglobins at ionic strength 200 mmol. dm^{-3}, there is yet

another remarkable difference between rabbit and human haemoglobins. In Table 7 we show, as a function of pH, comparison of k_{app} values obtained at ionic strengths of 50 and 200 mmol. dm^{-3} for rabbit haemoglobin, as ratio $k_{app}(50) / k_{app}(200)$. The corresponding ratios are shown for human haemoglbin [14] in Table 8. It is seen that the higher salt concentration has a much greater effect in reducing the rate of reaction of DTNB with rabbit than with human haemoglobin. At low pH the reduction in rate caused by the increased salt concentration is about an order of magnitude greater for rabbit than for human haemoglobin. Although this disparity is reduced at higher pH values, it persists up to pH 9.0 the highest experimental pH.

Table 7: Ratio of k_{app} at ionic strengths of 50 and 200 mmol. dm^{-3}, $k_{app}(50) / k_{app}(200)$, as a function of pH for rabbit haemoglobin.

		Derivative		
pH	**Oxy**	**Carbonmonoxy**	**Azidomet**	**Aquomet**
6.0	-	27.00	11.00	18.50
6.5	8.00	26.70	9.30	18.80
7.0	6.40	21.20	7.00	13.50
7.5	4.70	13.00	4.90	7.30
8.0	3.10	7.00	3.60	4.30
8.5	2.40	5.10	3.60	4.40
9.0	2.00	4.30	3.40	4.80

Table 8: Ratio of kapp at ionic strengths of 50 and 200 mmol. dm^{-3} $k_{app}(50) / k_{app}(200)$, as a function of pH for human haemoglobin A.

		Derivative		
pH	**Oxy**	**Carbonmonoxy**	**Azidomet**	**Aquomet**
6.0	1.60	3.20	1.30	1.70
6.5	1.90	6.50	1.30	2.90
7.0	1.50	5.00	1.30	2.50
7.5	0.70	4.10	1.00	1.10
8.0	1.10	2.10	0.70	0.65
8.5	0.80	1.90	0.60	0.70
9.0	0.70	1.30	0.50	0.60

[a]Data from ref. 14

Remarkably the effect of increased salt concentration in decreasing the sulfhydryl reactivity of rabbit haemoglobin is greater than the effect of inositol-P_6 in decreasing the sulfhydryl reactivity of human haemoglobin (compare Tables 5 and 7). The basis of the greater salt effect on rabbit compared to human haemoglobin is to be found in the amino acid differences at position F3[87]β: in human haemoglobin this position contains a neutral threonine; in rabbit haemoglobin, this is replaced by a positively charged lysine residue. At ionic strength 200 mmol. dm^{-3} the radius of ionic atmosphere is less than 5 Å (0.5 nm) [34], the distance of separation of lysine from CysF9[93]β. Therefore, at this ionic strength LysF3[87]β, which appears to be the major contributor to the reactivity difference between rabbit and human haemoglobins, is screened off electrostatically from CysF9[93]β. Consequently, its positive electrostatic field at the F9[93]β site of the reacting sulfhydryl group is eliminated, giving rise to much bigger reduction in reactivity of rabbit compared to human haemoglobin.

Examination of Fig. 3 shows that, except for carbonmonoxy derivatives (Fig. 3b), high salt concentration does not completely eliminate the reactivity differences between rabbit and human haemoglobin derivatives; for oxy derivatives (Fig. 3a), rabbit haemoglobin is still up to 2-fold more reactive at pH 8.0; for the azidomet derivatives (Fig. 3c) it is up to 2-fold more reactive at pH 7.6. On the other hand, for the aquomet derivatives (Fig. 3d), human haemoglobin is faster reacting below pH 7.4; but above this pH the reactivities appear to be about the same.

Conclusion

The rate of reaction of DTNB with CysF9[93]β should depend on at least five factors [4]: The equilibrium between the internal and the external conformations of the sulfhydryl group when HisHC3[146]β/AspFG1[94]β salt bridge is formed; the pK_a of the sulfhydryl group itself; the state of aggregation of the haemoglobin molecule, that is, the tetramer-dimer equilibirium; and the electrostatic interactions. The result in Figs. 2 and 3 when compared to Fig. 1 indicate that the major determinants of reactivity differences between rabbit and human haemoglobins are electrostatic interactions (compare Tables 7 and 8) and the existence of salt bridge formation (compare Tables 4 and 5). The extent of contribution of the equilibrium between the internal and external conformations of the sulfhydryl group to the reactivity difference is difficult to gauge, but it is not likely to be considerable. For most haemoglobin the pK_a for the ionization of CysF9[93]β is fairly constant at around 8.0 to 8.5; so the ionization of the sulfhydryl group should in most cases, make little contribution to the reactivity differences. The only likely additional contribution to the reactivity differences is the tetramer-dimer equilibrium. But even the effect of the equilibrium can be made negligible for most haemoglobins, as long as the

concentration of the haemoglobin is 10 μmol. (haem) dm^{-3}. In this concentration range, haemoglobin is to a very great extent, in the form of the tetramer, as it has been demonstrated [13], by calculations from tetramer-dimer equilibrium constant data [35].

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