HAEMOGLOBINS WITH MULTIPLE REACTIVE SULFHYDRYL GROUPS: REACTIONS OF 5,5'-DITHIOBIS(2-NITROBENZOIC ACID) WITH CysF9[93]β AND CysH3[125]β OF GUINEA PIG HAEMOGLOBIN

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(Received July 31, 2001; revised May 1, 2002)

ABSTRACT. Guinea pig haemoglobin has six sulfhydryl groups, one at each of the positions F9[93]β, G11[104]α and H3[125]β, which appear in pairs. Titration with p-hydroxymercuri(II)-benzoic acid and with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) indicate that four of the six sulfhydryls are reactive. The time course of the DTNB reaction is triphasic at pH > 7. The fast phase is about 2 orders of magnitude faster than the intermediate phase and can be clearly separated from it. The intermediate phase, where it exists, is about 3-4 times faster than the slow phase. However, the amplitude of the intermediate phase, which has a maximum of about 15% of the total signal amplitude, becomes negligible as it approaches pH 7, so that the kinetics eventually becomes biphasic.

The pH dependence profile of \( k_{app} \), the apparent second-order rate constant, for the fast phase resembles the titration curve of a diprotic acid. Quantitative analysis indicates that the reactivity of the sulfhydryl group to which this phase may be attributed is linked to two ionizable groups with \( pK_a \)s of 6.4 ± 0.1 and 7.8 ± 0.2. These values are assigned to HisH3[125]β and CysF9[93]β, respectively. The pH dependence profile of \( k_{app} \) for the slow phase resembles the titration curve of a monoprotic acid. Quantitative analysis indicates that the sulfhydryl group to which this phase may be attributed is linked to a single ionizable group with a \( pK_a \) of 6.1 ± 0.2. Examination of the structure of guinea pig haemoglobin near the H3[125]β position shows that there is a lysine residue, LysA5[8]β, which is only 4 Å (0.4 nm) away from CysH3[125]β. The presence of this lysine lowers the \( pK_a \) of CysH3[125]β from the normal value for sulfhydryl groups (between 8 and 8.5) to 6.1.

KEY WORDS: Haemoglobin, Sulfhydryl groups, Guinea pig, 5,5'-Dithiobis(2-nitrobenzoic acid), CysF9[93]β, CysH3[125]β, p-Hydroxymercury(II)-benzoic acid, Lysine

INTRODUCTION

In the last three decades the CysF9[93]β sulfhydryl group of haemoglobin has been employed as an indicator of tertiary and quaternary structure [1-5]. In the recent past we demonstrated that there are interesting correlations between the reactivities of sulfhydryl groups (at positions other than F9[93]β) and the structures of their immediate environments [6-8].

Two regions of the haemoglobin molecule that would be interesting objects of study are the \( \alpha_1\beta_1 \) and \( \alpha_1\beta_2 \) subunit interfaces, through which the interaction between the subunits, the so-called haem-haem interaction, is transmitted on oxygen binding [9]. Unfortunately, sulfhydryl groups located at these interfaces are occluded and therefore do not react with sulfhydryl reagents [10].

Guinea pig haemoglobin has a sulfhydryl group located at position H3[125]β. In deoxyhaemoglobin this position is at the \( \alpha_1\beta_1 \) subunit interface and is therefore occluded; but in liganded haemoglobin position H3[125]β is not occluded [11]. Therefore CysH3[125]β would

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be expected to be reactive in liganded haemoglobin. Static titration experiments on guinea pig oxyhaemoglobin indicate that four sulphhydryls are reactive; that is, in addition to the normally reactive CysF9[93]β another type of sulphhydryl group is reactive in this haemoglobin.

In order to gain a better understanding of the nature of the reacting sulphhydryl groups, we have undertaken a comprehensive pH dependence kinetic study of the reaction of guinea pig haemoglobin with 5,5'-dithiobis(2-nitrobenzoate) (DTNB). We find that the reaction is so fast that it can only be followed with a stopped-flow instrument.

**EXPERIMENTAL**

Male guinea pigs were purchased from the local market at Ibadan. Haemoglobin was prepared from blood according to well-known laboratory procedures. The only exception is that, prior to the lysis step, the red blood cells were washed thrice with a cold isotonic saline solution containing 11.5 g NaCl per dm$^3$ instead of the usual 9.5 g per dm$^3$, since the cells lysed prematurely at the latter concentration. The oxyhaemoglobin so prepared was converted to the carbonmonoxy derivative and stored frozen. Prior to use for experiments each haemoglobin derivative was "stripped", that is, deionized by passage through a Dintzis ion-exchange column [12].

Dintzis column is a mixed bed of generated forms of Amberlite and Zerolit DM-F resins purchased from the British Drug House. The hydrogen form of the resin was generated by washing a known volume of Amberlite IR 120 resin packed in a column with about 10 times its volume of 3 M HCl slowly. The resin was then washed with distilled water until the effluent was acid free - neutral to litmus. The acetate form was generated by washing Amberlite IRA 400 packed in a column with about 10 times its volume of 3 M HCl slowly. The resin was then washed with distilled water until the effluent was acid free - neutral to litmus. About 10 times its volume of 3 M sodium acetate was then passed through the column. The resin was again washed with distilled water until the effluent gave no precipitate with silver nitrate solution. The ammonium form was generated by slowly passing 100 mL of 3 M NaCl through a packed column of Amberlite IR 120 resin. The resin was washed with distilled water until the effluent gave no precipitate with silver nitrate. Thereafter ten times its volume of 3 M NH$_4$Cl was slowly passed through the column and washed with distilled water until the effluent gave no precipitate with AgNO$_3$ solution. Each of the mixed form and mixed indicator form of Zerolit DM-F resins were simply washed with distilled water, without further treatments. A 50-mL burette with a glass wool plugged end, served as the column. About 2.5 cm length of the generated hydrogen form was first packed into the burette containing distilled water. The tap was opened while the column was being filled with 7.5 cm length each of mixed indicator, mixed form, acetate and ammonia forms of the resin in that order. The column was then washed thoroughly with distilled water [12].

DTNB was purchased from Sigma and $p$-hydroxymercuri(II)benzoic acid (pMB) was purchased as the sodium salt from Aldrich. DTNB was prepared as a 50 mM stock solution in absolute ethanol. The pMB stock solution was prepared with 0.1 M NaOH and diluted in buffer to a concentration of 840 µM, prior to use. Determinations of the number of reactive sulphhydryl groups with pMB were carried out according to the method of Boyer [13]. Determinations with DTNB were carried out as previously described [7] using a 2.5 mM stock solution in the appropriate buffer.

Kinetic experiments were performed at 20 °C in phosphate (pH ≤ 8) and borate (pH > 8) buffers. All buffers employed in the experiments were of ionic strength 50 mM. The kinetics of the reaction between haemoglobin and DTNB were studied under pseudo-first order conditions, with DTNB in 20-fold excess over haemoglobin. The reactions were monitored at 450 nm on a
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Dionex stopped-flow apparatus coupled to an on-line data acquisition system. Kinetic traces were evaluated with the OLIS KINFIT set of programs (On Line Instruments). Each kinetic run was repeated four times under the same experimental conditions. The standard error in the determination of $k_{\text{obs}}$, the pseudo-first order rate constant, was 5% or less for the fast and slow phases but was larger (ca 10%) for the intermediate phase, owing to its small amplitude. Apparent second order rate constants, $k_{\text{app}}$, were obtained from $k_{\text{obs}}$ by dividing the latter by the DTNB concentration. Unless otherwise indicated, the final concentrations of haemoglobin and DTNB after mixing were 10 µM (haem) and 200 µM, respectively.

Curve-fitting

pH-dependence profiles of $k_{\text{app}}$ were fitted with user-friendly computer software. The direct approach to the curve-fitting problem (the full variable approach) minimizes the objective function chi-squared with respect to both the rate constant $k_i$ and the ionization constants $K_j$ (see equation (1) of "Results"). For cases where the ionization constants do not appear linearly in the equation to be fitted (for example, the equations used to fit the complex profiles obtained with human haemoglobins [14, 15]), the approach used is to express the rate constants, which appear linearly in the equation to be fitted, in terms of the ionization parameters by solving a least squares problem. Thereafter the objective function is minimized with respect to the ionization parameters alone. Our software supports both approaches. An automatic non-negativity constraint is also imposed by working directly with the negative logarithm of the ionization parameters, that is, pH and $pK_j$.

Our software supports the Powell conjugate direction algorithm [16, 17], the Levenberg-Marquardt algorithm [18], the Nelder-Mead Downhill Simplex algorithm [19] and, for one-dimensional restricted-variable problems, the Golden Search algorithm [20] and Brent's Parabolic Interpolation algorithm [21]. It also supports several hybrid combinations of these algorithms obtained by using the result from one algorithm as the initial guess for another.

RESULTS AND DISCUSSION

Static titrations

According to its amino acid sequence [22], guinea pig haemoglobin has 6 sulphhydryl groups per (tetramer) molecule. These are located at positions F9[93]β, G11[104]α and H3[125]β, which appear in pairs per haemoglobin tetramer. Position G11[104]α is at the $\alpha_1\beta_1$ subunit interface in both deoxy- and liganded haemoglobin [11]. It is known that sulphhydryl groups located at the subunit interfaces are occluded [10]. Consequently, CysG11[104]α does not react with any sulphhydryl reagent. CysH3[125]β is at the $\alpha_3\beta_2$ subunit interface in deoxyaehemoglobin but is outside this interface in liganded haemoglobin [11]. It should therefore be reactive in liganded haemoglobin. Since CysF9[93]β is reactive in all haemoglobins in which it is present, it is reasonable to expect that guinea pig haemoglobin will have a total of four reactive sulphhydryl groups per tetramer.
Figure 1. Titration of guinea pig oxyhaemoglobin with (a) pMB: ratio of the concentration of complex produced to the concentration of haemoglobin tetramer (Hb₄) as a function of the volume of pMB mixed with 3 mL of haemoglobin. Conditions: [haemoglobin] 20.5 µM (haem); stock [pMB] 840 µM; phosphate buffer pH 7.6 (ionic strength 50 mM; added salt, NaCl). The complex concentration was calculated from the change in absorbance corrected for dilution at 250 nm (wavelength), using a molar absorptivity of 7,600 M⁻¹ cm⁻¹ [13]. (b) DTNB: ratio of the concentration of 5-thio-2-nitrobenzoate (TNB), the product of the DTNB reaction, to the concentration of haemoglobin tetramer (Hb₄) as a function of the volume of DTNB mixed with 3 mL of haemoglobin. Conditions: [haemoglobin] 10 µM (haem); stock [DTNB] 2.5 mM; phosphate buffer pH 7.6 (ionic strength 50 mM; added salt, NaCl). The concentration of TNB was calculated from the change in absorbance at 412 nm, after correcting for dilution, using a molar absorptivity of 13,600 M⁻¹ cm⁻¹ [13].

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Titration of guinea pig haemoglobin according to the method of Boyer [13] using p-hydroxymercuri(II)benzoate gave about 4 reactive sulphhydryl groups per haemoglobin tetramer as shown in Figure 1a. Figure 1b reports the results of the titration of guinea pig haemoglobin with 5,5'-dithiobis(2-nitrobenzoate) (DTNB). It is seen that about 4 sulphhydryl groups per tetramer are reactive towards DTNB. Titrations with DTNB at several pH values in the range 6 < pH < 9 gave the same results: approximately 4 sulphhydryl groups per tetramer. These results agree with expectation and indicate that CysF9[93]β and CysH3[125]β are reactive.

Kinetics

We have already demonstrated [23] that, of all sulphhydryl reagents, DTNB is the most sensitive to the environment of sulphhydryl groups in haemoglobins because it carries two negative charges. For this reason we have chosen this reagent for the kinetic study of the sulphhydryl groups of guinea pig haemoglobin. However, DTNB has one disadvantage, it contains a disulphide bond, and the rate of hydrolysis of disulphide bonds increases markedly above pH 9 [24]. Therefore it is not advisable to follow DTNB reactions above pH 9 because of uncertainty about the integrity of this reagent under these conditions.

The time course of the DTNB reaction is triphasic at pH > 7. The fast phase is about 2 orders of magnitude faster than the intermediate phase and can be clearly separated from it. The intermediate phase is about 3-4 times faster than the slow phase. Between pH 9 and 7 the highest amplitude of the intermediate phase is about 15% of the total signal amplitude. Below pH 7, only a two-component fit is good for the kinetic traces using the OLIS KINFIT program. For this reason, and because the amplitude of the intermediate phase is very small even at pH > 7, emphasis in this report will be placed on only the fast and slow kinetic phases.

Figure 2. Variation of observed pseudo-first order rate constant, k_{obs}, with the DTNB concentration for the reaction of DTNB with the sulphhydryl groups of guinea pig oxyhaemoglobin. Conditions: [haemoglobin], 10 µM (haem); phosphate buffer pH 6.92, ionic strength 50 mM (added salt, NaCl); 20 °C. (a) Fast phase, CysF9[93]β; the least squares slope is 7640 M^{-1}s^{-1}. (b) Slow phase, CysH3[125]β; the least squares slope is 52.7 M^{-1}s^{-1}.

In Figure 2 we report for the fast and slow phases the dependence of k_{obs}, the pseudo-first-order rate constant, on the DTNB concentration at pH 6.92. It is seen that k_{obs} varies linearly with the DTNB concentration and that the intercept on the ordinate axis is negligible for each
kinetic phase. The least squares slopes for the data in Figure 2a and 2b are in reasonably good agreement with those obtained at pH 6.92 by dividing $k_{obs}$ with the DTNB concentration (compare with Figure 3a and 4a, respectively). Consequently, for each kinetic phase we have determined the apparent second order rate constant, $k_{app}$, at each pH by dividing $k_{obs}$ with the DTNB concentration.

(a) Fast kinetic phase

Figure 3a reports the dependence of $k_{app}$ on pH for the fast kinetic phase of the reaction of DTNB with stripped oxyhaemoglobin, that is, oxyhaemoglobin freed from organic phosphates and other undesired ions by passage through a Dintzis ion-exchange column [12]. The corresponding data for the carbonmonoxy and aquomet derivatives are reported in Figure 3b and 3c, respectively. It is seen that each profile resembles the titration curve of a diprotic acid. These profiles resemble those previously reported for CysF9[93]$\beta$ [6-8, 23]. Therefore we tentatively assign them to this sulfhydryl.

It is known that in haemoglobin only the thiol anion form of a sulfhydryl group reacts with DTNB [25, 26]. For this reason we previously accounted for profiles similar to those reported in Figure 3 in terms of the fractional population of the thiol anion form of the sulfhydryl [6-8, 14, 15, 23]. In addition, we observed that a salt bridge is formed in R state haemoglobin between HisHC3[146]$\beta$ and AspFG1[94]$\beta$ [27]. This salt bridge sterically hinders access to CysF9[93]$\beta$ [9]. These considerations gave rise to the two-term equation (1) [23]

$$k_{app} = k_1\{K_1/(K_1 + [H^+])\} + k_2\{K_2/(K_2 + [H^+])\}$$

(1)

where $k_1$ is the limiting apparent second-order rate constant at high pH for the DTNB reaction when the reactivity of CysF9[93]$\beta$ is linked to the ionization of HisHC3[146]$\beta$, with ionization constant $K_1$; $k_2$ is the limiting apparent second-order rate constant at high pH when the reactivity of the same sulfhydryl is linked to its own ionization, with ionization constant $K_2$. The first fractional term is the fraction of the neutral form of the histidine; the second fractional term is the fraction of the thiol anion form of the sulfhydryl.

The chemical equation which defines $k_1$, $k_2$, $K_1$ and $K_2$ can be written as shown below,

\[
\begin{align*}
\text{Hb}^{\text{SH}} & \quad \text{ImH}^+ \\
\text{Hb}^{\text{ImH}^+} & \quad \text{S}^- \\
\text{Hb}^{\text{ImH}^+} & \quad \text{S}^- \\
\text{Hb}^{\text{ImH}^+} & \quad \text{+ DTNB} \\
\text{HbS.TNB} & \quad \text{+ TNB}
\end{align*}
\]

where Im is the imidazole group of HisHC3[146]$\beta$ and TNB is 5-thio-2-nitrobenzoate.

Using our curve-fitting software (see Experimental), we have employed equation (1) to analyze the profiles in Figure 3. The lines through the data points are theoretical best-fit lines calculated with the parameters reported in Table 1. It is seen that the fits are quite good: $pK_1$ varies between 6.2 and 6.5 and $pK_2$ varies between 7.5 and 8.0. Their mean values are 6.4 ± 0.1 and 7.8 ± 0.2, respectively.
Figure 3. Dependence of $k_{app}$, the apparent second order rate constant, on pH for the reaction of DTNB with CysF9[93]β of derivatives of stripped guinea pig haemoglobin at 20°C (fast kinetic phase). Each experimental point is the mean of 5 determinations and is subject to a standard error of 5% or less. Concentrations after mixing: haemoglobin, 10 µM (haem); DTNB, 200 µM. Buffers were all of ionic strength 50 mM. The lines through the data points are theoretical best-fit lines calculated with eqn. (1) using the parameters reported in Table 1. (a) Oxyhaemoglobin; (b) carbonmonoxyhaemoglobin; (c) aquomethaemoglobin.

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Table 1. Reaction of DTNB with CysF9[93]β of derivatives of stripped guinea pig haemoglobin (fast kinetic phase).

<table>
<thead>
<tr>
<th>Derivative</th>
<th>pK₁</th>
<th>pK₂</th>
<th>k₁ (M⁻¹s⁻¹)</th>
<th>k₂ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxy</td>
<td>6.50</td>
<td>8.01</td>
<td>9500</td>
<td>15500</td>
</tr>
<tr>
<td>Carbonmonoxy</td>
<td>6.20</td>
<td>7.81</td>
<td>6980</td>
<td>16900</td>
</tr>
<tr>
<td>Aquomet</td>
<td>6.50</td>
<td>7.47</td>
<td>7210</td>
<td>9800</td>
</tr>
</tbody>
</table>

Best-fit parameters employed to fit the data in Figure 3 according to equation (1).

We assign these pKₐ's tentatively to HisHC3[146]β and CysF9[93]β, respectively. In fitting the various data, we found that we obtained the most consistent results for all haemoglobin derivatives with the Nelder-Mead Downhill Simplex algorithm [19].

(b) Slow kinetic phase

In Figure 4 we present the pH-dependence profiles of kₐpp for the slow kinetic phases of the reaction of DTNB with stripped oxyhaemoglobin and the carbonmonoxy and aquomet derivatives. Each curve resembles the titration curve of a monoprotic acid. This is the first time in our experience that we have obtained such simple profiles for haemoglobin, with the limiting value of kₐpp at high pH being observed experimentally below pH 9. We have fitted these profiles with the one-term equation (2)

\[ k_{\text{app}} = \frac{k K}{K + [H^+]} \]

(2)

where k is the limiting apparent second-order rate constant at high pH for the reaction of DTNB with the sulphydryl group whose ionization constant is K. The lines through the data points in Figure 4 are theoretical best-fit lines calculated with equation (2). It is seen that reasonably good fits are obtained to the data. The fitting parameters are reported in Table 2.

Table 2. Reaction of DTNB with CysH3[125]β of derivatives of guinea pig haemoglobin (slow kinetic phase).

<table>
<thead>
<tr>
<th>Derivative</th>
<th>pK</th>
<th>k₁ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxy</td>
<td>5.89</td>
<td>61.8</td>
</tr>
<tr>
<td>Carbonmonoxy</td>
<td>6.00</td>
<td>57.4</td>
</tr>
<tr>
<td>Aquomet</td>
<td>6.45</td>
<td>92.2</td>
</tr>
</tbody>
</table>

Best-fit parameters used to fit the data in Figure 4 according to equation (2).

The pKₐ of the reacting sulphydryl group varies between 5.9 and 6.4, with a mean value of 6.1±0.2.

(c) Assignment of kinetic phases

We have already excluded CysG11[104]α as a possible candidate for reaction with DTNB. We tentatively assign the fast kinetic phase to CysF9[93]β. This assignment may be justified as follows. First the pH-dependence profile of the fast kinetic phase (Figure 3) resembles those previously assigned to CysF9[93]β in other haemoglobins; [6-8, 14, 15] furthermore, the pK₁ value of 6.4 (Table 1) is that expected for a histidine [6-8, 14, 15, 23]. In view of the finding that there is a Bohr effect in R-state haemoglobin involving HisHC3[146]β [27], it is not
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It is unreasonable to assign the pK\textsubscript{1} of 6.4 to His\textsubscript{3C3}\beta. The pK\textsubscript{2} of 7.8 is somewhat lower than the usual pK\textsubscript{a} (8.0 - 8.5) of Cys\textsubscript{9F9}\beta in haemoglobins. Nevertheless, the difference (0.2 - 0.7 pK\textsubscript{a} unit) is not sufficient to invalidate the assignment of this pK\textsubscript{a} to Cys\textsubscript{9F9}\beta. We conclude that the fast kinetic phase (Figure 3) arises from the reaction of Cys\textsubscript{9F9}\beta with DTNB.

Figure 4. Dependence of \(k_{\text{app}}\) on pH for the reaction of DTNB with Cys\textsubscript{H3(125)}\beta of derivatives of stripped guinea pig haemoglobin (slow kinetic phase). Each point is the mean of 5 determinations and is subject to a standard error of 5% or less. The lines through the experimental points are theoretical best-fit lines calculated with equation (2) using the parameters reported in Table 2. Conditions as in Figure 3. (a) Oxyhaemoglobin; (b) carbonmonoxyhaemoglobin; (c) aquomethaemoglobin.

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CysH3[125]β is at the αβ1 subunit interface in deoxyhaemoglobin but is outside this interface in liganded haemoglobin [11]. In view of the fact that this sulphhydryl is not likely to be as exposed to the solvent medium as CysF9[93]β is, it is reasonable to expect its reaction with DTNB to be slower. This justifies the assignment of the slow kinetic phase to CysH3[125]β. Moreover, the monophasic nature of the profiles in Figure 4 indicate that the reaction of the sulphhydryl group is not linked to the ionization of any ionizable group in the protein, apart from that of CysH3[125]β itself. We have already established that the reaction of CysF9[93]β is linked to the ionization of other groups in the haemoglobin molecule [6-8, 14, 15, 23]. We conclude that the slow kinetic phase is due to the reaction of CysH3[125]β with DTNB.

(d) Triphasic kinetics at pH > 7

We noted that at pH < 7 the kinetic signals for the reaction of guinea pig haemoglobin with DTNB are biphasic. They are, however, triphasic at pH > 7, although the intermediate phase only has a maximum amplitude that is 15% of the total signal amplitude. We cannot at present explain the origin of the intermediate phase. It is nevertheless noteworthy that the static titration experiments (using DTNB) over the range 6 ≤ pH ≤ 9 gave 4 sulphhydryl groups per tetramer. This strongly indicates that only the sulphhydryl groups at positions F9[93]β and H3[125]β react with DTNB.

(e) Unusually high rate of reaction of DTNB with CysF9[93]β of guinea pig haemoglobin

A remarkable aspect of our results is the very high rate of the reaction between DTNB and CysF9[93]β of guinea pig haemoglobin. At our experimental temperature (20 °C), the value of $k_{app}$ at high pH varies between about 17,000 M$^{-1}$s$^{-1}$ (aquomethaemoglobin) and 25,000 M$^{-1}$s$^{-1}$ (oxyhaemoglobin) (see Figure 3). These rates are comparable to the value of about 33,000 M$^{-1}$s$^{-1}$ obtained (at 25 °C) for the reaction of DTNB with glutathione by Palau and Daban [28]. In glutathione the sulphhydryl group is not restricted in any way and is thus quite reactive.

The reaction rates of CysF9[93]β of various derivatives of guinea pig haemoglobin are the highest we have measured for any haemoglobin; they are between two and three orders of magnitude higher than the rates reported for human haemoglobins A and S [14, 15]. These high values of $k_{app}$ for CysF9[93]β of guinea pig haemoglobin suggest that the sulphhydryl group does not suffer from the steric hindrance experienced by CysF9[93]β in other haemoglobins. This steric hindrance arises from the formation of a salt bridge between HisHC3[146]β and AspFG1[94]β [9]. When this salt bridge is present the pK$_a$ of the histidine lies between 6 and 6.5 [6-8, 14, 15]. Table 1 shows that the mean pK$_a$ of HisHC3[146]β of guinea pig haemoglobin is 6.4. We conclude that it is very likely that in guinea pig haemoglobin, just as in other haemoglobins, the HisHC3/AspFG1 salt bridge is intact. Consequently, the steric hindrance associated with the presence of this salt bridge must be effective in guinea pig haemoglobin. This leaves unexplained the very large difference in rates of reaction of CysF9[93]β in guinea pig and human haemoglobins.

(f) Unusually low pK$_a$ of CysH3[125]β

If our assignment of the fast kinetic phase to CysF9[93]β is correct, the slow kinetic phase must arise from the reaction of DTNB with CysH3[125]β. The mean pK$_a$ of 6.1 ± 0.2 obtained for this sulphhydryl (see Table 2) is unusually low and is about 2 pK$_a$ units lower than the normal pK$_a$s of sulphhydryl groups in haemoglobins. An examination of the 3D structure of guinea pig haemoglobin shows that a positively charged lysine residue, LysA5[8]β, is only 4 X (0.4 nm)
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away from the H3[125]β position. The presence of this positively charged group (pKₐ ca. 10.5) close to a sulphhydryl at this position should provide a positive electrostatic field that would drastically lower the pKₐ of CysH3[125]β.

ACKNOWLEDGEMENTS

The kinetic work reported in this paper was supported by a Royal Society of Chemistry Journals "Grant for International Authors" to KOO during a 2-month stay in Erlangen. The work also benefited from the continued support of the Alexander von Humboldt Foundation, Bonn, Federal Republic of Germany, Senate research grant (SRG/FSC/1996/30A) and the Postgraduate School Multidisciplinary research grant (Grant No 3-600-507) of University of Ibadan, Ibadan, Nigeria.

REFERENCES