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Ligand-dependent reactivity of the CysB5[23]β sulfhydryl group of the major haemoglobin of chicken

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Chicken haemoglobin contains eight reactive sulfhydryl groups per (tetramer) molecule, as determined by Boyer titration with *p*-chloromercury(II)benzoic acid. However, only four of these sulfhydryls are reactive towards 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). They are at the F9[93] and B5[23] positions of each of the two β subunits in the molecule. The time course of the DTNB reaction is biphasic. With oxyhaemoglobin, k_{app}, the apparent second-order rate constant of the fast phase, increases monotonically with pH, the simple profile resembling the titration curve of a diprotic acid; the pH-dependence of k_{app} for the slow phase is bowl-shaped. With carbonmonoxyhaemoglobin and aquomethaemoglobin, k_{app} for the fast phase is bowl-shaped whilst k_{app} for the slow phase increases monotonically with pH. Quantitative analyses of the simple profiles show that the reactivity of the sulfhydryl group to which they may be attributed is subject to the influence of two ionizable groups on the molecule, with mean pK_a values of 6.4 ± 0.1 and *ca.* 8.4 ± 0.3. These pK_a values are assigned to HisHC3[146]β and CysF9[93]β, respectively. Quantitative analyses of the bowl-shaped profiles show that the reactivity of the sulfhydryl group to which they may be attributed is subject to the influence of two ionizable groups on the protein, with mean pK_as of 6.85 ± 0.05 and 8.3 ± 0.2. These values are assigned to HisG19[117]β and CysB5[23]β, respectively. It is highly significant that the CysB5[23]β sulfhydryl groups of carbonmonoxy- and aquomet-haemoglobin react *ca.* 100 times faster than that of oxyhaemoglobin. By contrast, the difference in the reactivities of the CysF9[93]β sulfhydryls of the three haemoglobin derivatives is no more than four-fold. This indicates that, in chicken haemoglobin, changes in the haem ligand give rise to structural changes in the neighbourhood of the CysB5[23]β sulfhydryl which are far more significant than those in the neighbourhood of the CysF9[93]β sulfhydryl.

Over the past 30 years the CysF9[93]β sulfhydryl group of haemoglobin has been used as a probe of tertiary and quaternary structure.[1–10] We have shown that, at low ionic strength, reaction of this sulfhydryl group with 5,5′-dithiobis(2-nitrobenzoate) (DTNB) is subject to the electrostatic influence of the basic groups at the organic phosphate binding site of human haemoglobins, and we have calculated the pK_as of these and other groups from pH-dependence profiles of the kinetics of the DTNB reaction.[11,12]

We have also found that interesting structural information can be obtained from the reactivities of sulfhydryl groups located at other positions in the haemoglobin molecule.[13–15] Recently, we reported a pH-dependence study of the reaction of pigeon haemoglobin with DTNB. Our results showed that, of the eight sulfhydryl groups per molecule reacting with *p*-chloromercury(II)benzoate, (*p*CMB), only the four at positions F9[93] and B5[23] on each of the two β chains react with DTNB.[15] We found that the sulfhydryl groups at position F9[93]β are one to two orders of magnitude faster reacting than those at the B5[23]β position. We interpreted this result on the basis of the X-ray crystal structure of haemoglobin. This shows that, whereas the F9[93]β position is relatively exposed to the solvent, the B5[23]β position is in a tight and highly hydrophobic region between helices B and G.

The α and β chain amino acid sequences of chicken haemoglobin are very similar to those of pigeon.[16–20] In the present report we extend our previous study on pigeon to the haemoglobin of chicken. (Chicken blood contains two haemoglobins. Unless otherwise indicated, all references to chicken haemoglobin are to the major component.)

Materials and Methods

Separation of chicken haemoglobins

Chicken haemolysate contains two haemoglobins. These were separated using the procedure outlined by Brygier *et al.*,[21] except that Whatman CM 52 microgranular preswollen cation exchanger was used in place of CM 11. The major fraction was eluted last. Each fraction was deionized by passage through a Dintzis ion-exchange column.

Titration with sulfhydryl reagents

We determined the number of sulfhydryl groups reacting with *p*CMB according to the method described by Boyer[22] and carried out titrations with DTNB as previously described.[15]

Kinetics

We studied the kinetics of the DTNB reaction at 412 nm under pseudo-first-order conditions, as previously described.[11,15] The data were recorded on a Philips PM 8261 Xt chart recorder. All reactions were allowed to proceed to completion. Each experiment was repeated at least twice. After converting transmittance readings to absorbance, the data were analysed with a 1990 update of DISCRETE, a computer program for the analysis of multiple exponential signals.[23] The analyses gave two kinetic phases, fast and slow. The pseudo-first-order rate constant for each phase, k_{obs}, was converted to k_{app}, the apparent second-order rate constant, by dividing by the DTNB concentration. The standard error in the determination of k_{obs} was *ca.* 10% for the fast phase and *ca.* 20% for the slow phase. Reactions were carried out at 20 °C in phosphate ($5.6 \leqslant pH \leqslant 8$) and borate ($pH \geqslant 8$) buffers of ionic strength 50 mmol dm^{-3}, at a haemoglobin concentration of 10 μmol (haem) dm^{-3} (10 μmol dm^{-3} in DTNB-reactive sulfhydryl groups). The DTNB concentration was at least 100 μmol dm^{-3} in each experiment. With oxyhaemoglobin it was not possible to observe any kinetics at 10 μmol (haem) dm^{-3} and a DTNB concentration of 100 μmol dm^{-3}. We, therefore, worked at concentrations of 20 μmol (haem) dm^{-3} and 500 μmol dm^{-3}, respectively.

Results and Discussion

Titration with sulfhydryl reagents

The sulfhydryl groups in chicken haemoglobin are located at

the following positions: G11[104]α, H13[130]α, H4[126]β, F9[93]β and B5[23]β.[17–20] Since there are two α and two β chains per molecule, this gives a total of 10 sulfhydryl groups per molecule. CysG11[104]α is in the $\alpha_1\beta_1$ subunit contact region and is therefore inaccessible to any sulfhydryl reagent.[24] This leaves eight sulfhydryl groups that may be accessible to sulfhydryl reagents.

Titration of chicken haemoglobin with *p*CMB[22] gave eight reactive sulfhydryl groups per (tetramer) molecule (data not shown). On the other hand, titration with DTNB gave only 3.8, that is, approximately four sulfhydryl groups per molecule (Fig. 1). These results are similar to those obtained previously for pigeon haemoglobin[15] and imply that four of the sulfhydryl groups accessible to *p*CMB are not accessible to DTNB. On the basis of the 3D structure of haemoglobin, and because DTNB reacts only with the thiolate anion form of a sulfhydryl group,[13,25] we showed, previously, for pigeon haemoglobin that the reacting sulfhydryls are CysF9[93]β and CysB5[23]β.[15] Since chicken and pigeon have closely similar primary structures,[16–20] we assume that the same sulfhydryls react with chicken haemoglobin.

Kinetics

The kinetics of the reaction of DTNB with all the chicken haemoglobin derivatives are biphasic. Fig. 2(a) shows the pH dependence of k_{app} for the fast phase of the reaction of DTNB with oxyhaemoglobin. It is seen that k_{app} increases monotonically with pH, the simple profile resembling the titration curve of a diprotic acid. In contrast, the pH dependence of k_{app} for the slow phase [Fig. 2(b)] is bowl-shaped. These results are similar to those obtained previously with pigeon haemoglobin.[15] A comparison of the data shows that the fast phase is between 2 (pH ⩽ 6.2) and 90 (pH 9) times faster than the slow phase.

The data for carbonmonoxyhaemoglobin are shown in Fig. 3. In complete contrast to the oxy data the profile of the fast phase [Fig. 3(a)] is bowl-shaped, while that of the slow phase [Fig. 3(b)] has the simple form of the titration curve of a diprotic acid. The fast phase is between 3 (pH 7.8) and 80 (pH 5.6) times faster than the slow phase.

The fast phase of chicken aquomethaemoglobin also has a bowl-shaped pH dependence profile [Fig. 4(a)], while the slow phase has a simple profile resembling the titration curve of a diprotic acid [Fig. 4(b)]. Moreover, the fast phase is between 3 (pH 7.6) and 85 (pH 5.6) times as fast as the slow phase.

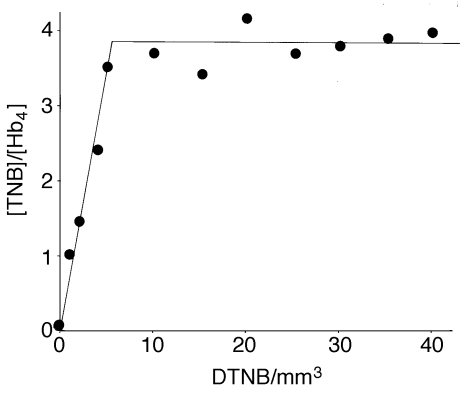

Fig. 1 Titration of chicken oxyhaemoglobin with DTNB: ratio of the concentration of 5-thio-2-nitrobenzoate (TNB) produced to the concentration of haemoglobin tetramer (Hb_4) as a function of the volume of DTNB mixed with 3 ml of haemoglobin. Conditions: [haemoglobin] 8.3 μmol (haem) dm^{-3}; [DTNB] 2.573 mmol dm^{-3}; phosphate buffer pH 7.6 (ionic strength 50 mmol dm^{-3}; added salt, NaCl). The TNB concentration was calculated from the change in absorbance, assuming a molar absorption coefficient of 13 600 dm^3 mol^{-1} cm^{-1}.

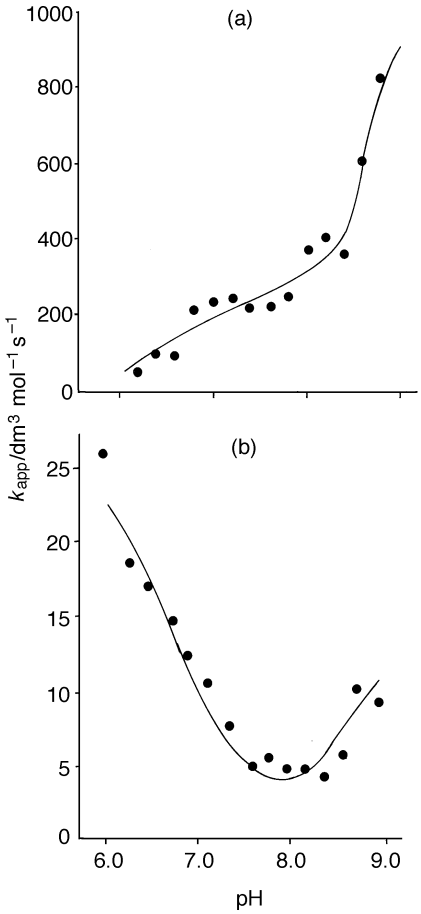

Fig. 2 Dependence of k_{app} on pH for the reaction of DTNB with (a) the CysF9[93]β and (b) the CysB5[23]β sulfhydryl group of chicken (major) oxyhaemoglobin. [Haemoglobin] 20 μmol (haem) dm^{-3} (20 μmol dm^{-3} in reactive sulfhydryl groups) and [DTNB] 500 μmol dm^{-3}. The lines through the experimental points are theoretical best-fit lines calculated with (a) eqn. (1) using the parameters reported in Table 1 and (b) eqn. (2) using the parameters reported in Table 2.

We recently reported the pH-dependence of the kinetics of the reaction of DTNB with the oxy, carbonmonoxy and aquomet derivatives of pigeon haemoglobin.[15] In each case, a simple pH-dependence profile was obtained for the fast phase, which was assigned to CysF9[93]β. A bowl-shaped pH-dependence profile was obtained for the slow phase in each case. This phase was assigned to CysB5[23]β for two reasons: (a) this residue is in a tight and highly hydrophobic region between helices B and G and would therefore not be expected to be very reactive, owing to steric constraints to the approach of DTNB. (b) We could only account for the bowl-shaped pH-dependence profile obtained by assuming that the sulfhydryl has a neighbouring cationic group (i) whose presence enables the sulfhydryl to form the thiolate anion and (ii) whose ionization is coupled to the reactivity of the sulfhydryl. From the 3D structure, we found that HisG19[117]β was the cationic group interacting with CysB5[23]β, since it is only 6 Å from the B5[23]β position.

It is difficult to understand the differences in the shapes of the pH-dependence profiles of oxyhaemoglobin (Fig. 2), and carbonmonoxy- (Fig. 3) and aquomet-haemoglobin (Fig. 4), if the fast phases are to be associated with CysF9[93]β and the slow phases with CysB5[23]β. This difference cannot be attributed to the difference in haemoglobin concentration used for the two sets of experiments [20 μmol (haem) dm^{-3} for the oxy and 10 μmol (haem) dm^{-3} for the carbonmonoxy and aquomet derivatives], because the shape of the pH-dependence profile does not depend on the haemoglobin concentration.[12]

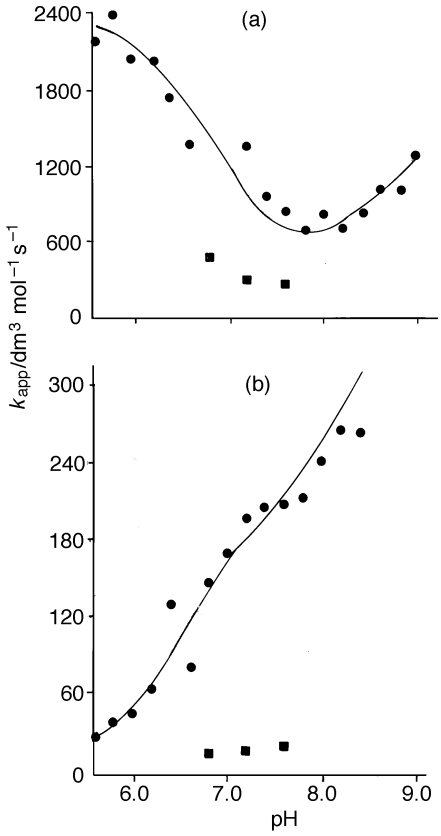

Fig. 3 Dependence of k_{app} on pH for the reaction of DTNB with (a) the CysB5[23]β and (b) the CysF9[93]β sulfhydryl group of chicken (major) carbonmonoxyhaemoglobin. The haemoglobin concentration was 10 μmol (haem) dm^{-3}. Other conditions as in Fig. 1. The lines through (●) are best-fit lines calculated with (a) eqn. (2) using the best-fit parameters reported in Table 2 and (b) eqn. (1) using the best-fit parameters reported in Table 1. (■) Haemoglobin plus 10 μmol dm^{-3} inositol-P$_6$.

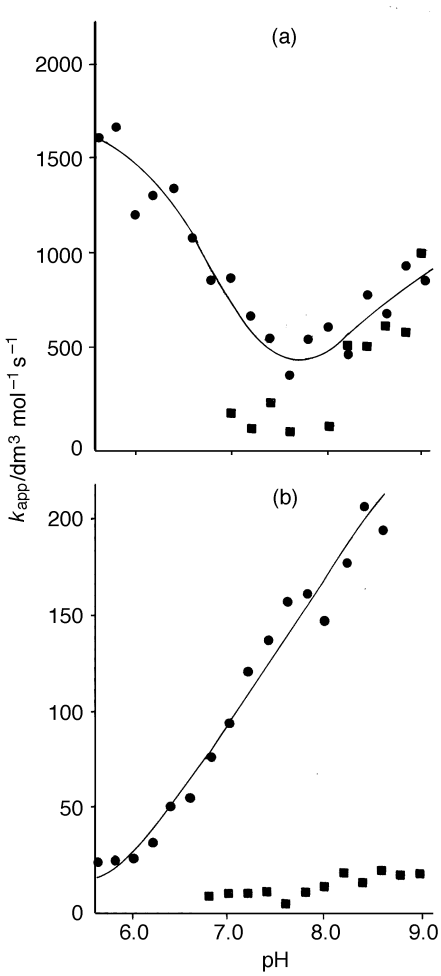

Fig. 4 Dependence of k_{app} on pH for the reaction of DTNB with (a) the CysB5[23]β and (b) the CysF9[93]β sulfhydryl group of chicken (major) aquomethaemoglobin. Conditions as in Fig. 2. The lines through (●) are theoretical best-fit lines calculated with (a) eqn. (2) using the best-fit parameters reported in Table 2 and (b) eqn. (1) using the best-fit parameters reported in Table 1. (■) Haemoglobin + 10 μmol dm^{-3} inositol-P$_6$.

We have attempted to gain an understanding of the apparent contradiction observed with the chicken major haemoglobin component by studying the reaction of DTNB with the carbonmonoxy derivative of the minor component.

Chicken haemolysate contains two haemoglobins. The major component makes up 70% of the haemolysate; the minor component 25% and the remaining 5% is a non-haem fraction.[21] The two haemoglobins have identical β chains, but their α chain amino acid sequences are different. Bearing in mind that both components have identical β chains, and that the reacting sulfhydryl groups are located at the same positions on the β chains, we fully expected to obtain pH-dependence profiles for the minor haemoglobin qualitatively resembling those for the major component. To our surprise, this was not the case.

Fig. 5(a) shows the pH dependence of k_{app} for the fast phase of the reaction of DTNB with the carbonmonoxy derivative of the minor haemoglobin. The profile has a simple form resembling the titration curve of a diprotic acid. The pH dependence profile of the slow phase is shown in Fig. 5(b); it is bowl-shaped. Thus the carbonmonoxy derivative of the minor chicken haemoglobin has pH-dependence profiles for the fast and slow kinetic phases which are qualitatively different from those of the carbonmonoxy derivative of the major component (Fig. 3). They are, however, qualitatively similar to those of the oxy derivative of the major component (Fig. 2) and to those of all the derivatives of pigeon haemoglobin.

Analyses of pH-dependence profiles

Simple profiles. All the haemoglobin derivatives used in this study are in the R (relaxed) quaternary structure. We previously analysed simple profiles like those shown in Fig. 2(a), 3(b), 4(b) and 5(a) with the following equation:[26]

$$k_{app} = k_1 \frac{K_1}{K_1 + [H^+]} + k_2 \frac{K_2}{K_2 + [H^+]} \quad (1)$$

Eqn. (1) is based on the finding that there is a Bohr effect in R-state haemoglobin involving HisHC3[146]β.[27] This proves that the histidine forms a salt bridge with AspFG1[94]β, resulting in CysF9[93]β being sterically hindered by TyrHC2[145]β.[28] When the histidine ionizes, the salt bridge is broken and steric hindrance to the approach of DTNB to CysF9[93]β is removed. Consequently, the reactivity of this sulfhydryl should increase as the histidine ionises with increasing pH.

In eqn. (1), k_1 is the limiting apparent second-order rate constant at high pH for the DTNB reaction when the reactivity of the sulfhydryl group is linked to the ionization of HisHC3[146]β, with ionization constant K_1; k_2 is the limiting apparent second-order rate constant at high pH when the sulfhydryl reactivity is linked to the ionization of the sulfhydryl group itself, with ionization constant K_2. The first fractional term is the fraction of the neutral form of the histidine while the second fractional term is the fraction of the thiol anion form of the reacting sulfhydryl group.

We have analysed the data in Fig. 2(a), 3(b), 4(b) and 5(a) using eqn. (1). The lines through the data points in these figures are theoretical best fit lines calculated with eqn. (1)

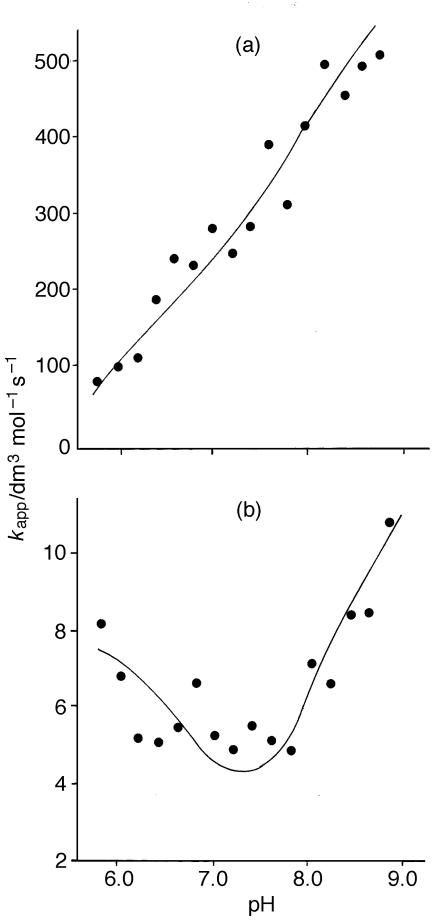

Fig. 5 Dependence of k_{app} on pH for the reaction of DTNB with (a) the CysF9[93]β and (b) the CysB5[23]β sulfhydryl group of chicken (minor) carbonmonoxyhaemoglobin. Conditions as in Fig. 2. The lines through the experimental points are theoretical best-fit lines calculated with (a) eqn. (1) using the best-fit parameters reported in Table 1 and (b) eqn. (2) using the best-fit parameters reported in Table 2.

using the best-fit parameters reported in Table 1. The mean pK_1 value is 6.4 ± 0.1, and the mean pK_2 value is *ca.* 8.4 ± 0.3. pK_1 and pK_2 for such simple profiles were previously assigned to HisHC3[146]β and CysF9[93]β of various haemoglobins,[11,12,14,15,26] respectively and we, therefore, tentatively make the same assignments for chicken haemoglobins. The pK_2 value of 8.4 is approximate for two reasons: the pK_2 of 9.0 reported in Table 1 for oxyhaemoglobin [Fig. 2(a)] is approximate because it lies outside the data range. The pK_2 of 8.5 reported in Table 1 for carbonmonoxyhaemoglobin is also an approximation because of a plateau around pH 7.5 [Fig. 3(b)], which appears to be the end of the first transition. Consequently, the pK_2 value of 8.5 is probably an underestimate of the true value.

Bowl-shaped profiles were previously analysed with the following equation:[15]

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

Theoretically, such bowl-shaped profiles can only be accounted for by assuming that there is an ionizable cationic group close to the reacting sulfhydryl group. At low pH the cationic group is positively charged and the reaction of the negatively charged DTNB is fast. As the cationic group ionizes to its neutral form with increasing pH, the DTNB reaction slows down.

In eqn. (2) k_1 is the limiting apparent second-order rate constant at low pH for the DTNB reaction when the sulfhydryl reactivity is linked to the ionization of a neighbouring cationic group, with ionization constant K_1. The first fractional term in eqn. (2) is the fractional population of the cationic form of this group. The second fractional term is identical to that of eqn. (1), and the parameters are similarly defined. Eqn. (2) was used to fit the curves in Fig. 2(b), 3(a), 4(a) and 5(b). The lines in these figures are theoretical best-fit lines calculated with the parameters in Table 2. The mean value of pK_1 is 6.8 ± 0.1, and the mean value of pK_2 is 8.4 ± 0.2. We previously assigned pK_1 values for bowl-shaped profiles to HisG19[117]β and pK_2 values to CysB5[23]β[15] and tentatively make the same assignments here for chicken haemoglobins.

Assignment of kinetic phases to sulfhydryl groups

Our theoretical analysis assumes that the two kinetic phases observed for the reaction of DTNB with chicken haemoglobin are for two independent reactions, as justified previously.[15]

All the bowl-shaped profiles are qualitatively similar to those previously reported for pigeon haemoglobin (Fig. 5 of ref. 15). The latter were assigned to the reaction of the CysB5[23]β sulfhydryl group which is coupled to the ionization of HisG19[117]β; we tentatively make the same assignment here. Support is obtained from a comparison of the pK_1 values in Tables 1 and 2. The mean value of pK_1 for the bowl-shaped profiles is 6.8 ± 0.1 compared with a value of 6.4 ± 0.1 for the simple profiles. This represents a difference of 0.4 of a pK_a unit, identical to the difference of 0.4 of a pK_a unit observed for the corresponding parameters of pigeon haemoglobin.[15]

It is known that the reactivity of the CysF9[93]β sulfhydryl group is sterically hindered by TyrHC2[145]β when a salt bridge is formed between HisHC3[146]β and AspFG1[94]β.[28] In the presence of inositol hexakisphosphate (inositol-P_6), the pK_a of this histidine in human haemoglobin is increased by one pK_a unit, thus strengthening the salt bridge and reducing the reactivity of the CysF9[93]β sulfhydryl.[11,12] Human and chicken haemoglobin differ in a number of residues involved in inositol-P_6 binding:[29] HisH21[143]β and AspH17[139]β of human haemoglobin are replaced in chicken haemoglobin by arginine and histidine residues, respectively.[17] We therefore expect the negatively charged inositol-P_6 to bind very tightly to chicken haemoglobin and to produce a drastic reduction in the reactivity of CysF9[93]β . We are not aware of any corresponding mechanism for changing the reactivity of CysB5[23]β.

Table 1 Reaction of DTNB with the CysF9[93]β sulfhydryl group of chicken haemoglobins[a]

derivative	pK_1	pK_2	k_1 /dm^3 mol^{-1} s^{-1}	k_2 /dm^3 mol^{-1} s^{-1}
oxy[b]	6.5	*ca.* 9	230	1200
carbonmonoxy[b]	6.5	>8.5	200	250
aquomet[b]	6.5	8.0	110	130
carbonmonoxy[c]	6.1	8.0	200	400

[a] Best-fit parameters employed to fit the data in Fig. 2(a), 3(b), 4(b) and 5(a) according to eqn. (1). [b] Major haemoglobin; [c] minor haemoglobin.

Table 2 Reaction of DTNB with the CysB5[23]β sulfhydryl group of chicken haemoglobins[a]

derivative	pK_1	pK_2	k_1 /dm^3 mol^{-1} s^{-1}	k_2 /dm^3 mol^{-1} s^{-1}
oxy[b]	6.75	8.75	30	15
carbonmonoxy[b]	6.95	8.3	2400	1500
aquomet[b]	6.8	8.2	1700	1000
carbonmonoxy[c]	6.9	8.0	8	12

[a] Best-fit parameters employed to fit the data in Fig. 2(b), 3(a), 4(a) and 5(b) according to eqn. (2). [b] Major haemoglobin; [c] minor haemoglobin.

As a check on our assignment of the simple profiles to CysF9[93]β and the bowl-shaped profiles to CysB5[23]β, we determined which of these sulfhydryls has its reactivity drastically reduced by inositol-P_6. For this purpose we studied the reaction of chicken aquomethaemoglobin with DTNB in the presence of inositol-P_6. Fig. 4(b) shows that, between pH 7 and 9, k_{app} for the simple profile is reduced *ca.* 15-fold in the presence of inositol-P_6. In contrast [Fig. 4(a)], k_{app} for the bowl-shaped profile is reduced by a maximum of 7.5-fold (pH 7); above pH 8 the organic phosphate has virtually no effect on the value of k_{app}. Similar but more limited results were obtained for the carbonmonoxy derivative: for the simple profile, k_{app} is reduced 10-fold (pH 6.8) and 14-fold (pH 7.6) [Fig. 3(b)]; the corresponding reductions for the bowl-shaped profile [Fig. 3(a)] are 3- and 2.5-fold, respectively. These results allow us to assign the simple profiles to CysF9[93]β and the bowl-shaped profiles to CysB5[23]β.

These assignments are strengthened by comparison of the k_{app} values for the simple profiles. The difference in reactivity between the derivatives (oxy, carbonmonoxy and aquomet) is no more than 4-fold. This magnitude of difference is typical of the CysF9[93]β sulfhydryl group.[1,11,12,14,15,26]

Basis of high reactivity of CysB5[23]β in the CO and aquomet derivative of the major haemoglobin

Examination of the 3D structure of chicken haemoglobin shows that the CysB5[23]β sulfhydryl has the following hydrophobic residues as its nearest neighbours: TrpA15[18]β, ValG15[113]β, LeuE12[68]β (each at a distance of 5 Å) and PheGH1[118]β (at a distance of 9 Å). It also interacts with the carbonyl oxygen of HisG19[117]β. Therefore, the CysB5[23]β sulfhydryl should not be able to ionize to the thiol anion form but for the presence (at a distance of 6 Å) of the positively charged HisG19[117]β. This makes it possible for CysB5[23]β to form the thiolate anion and so to react with DTNB. In spite of the closeness of HisG19[117]β, the tight environment of the CysB5[23]β sulfhydryl implies that it should have a low reactivity, as has been found with the pigeon haemoglobin derivatives,[15] the oxy derivative of the major chicken haemoglobin [Fig. 2(b)] and the carbonmonoxy derivative of the minor haemoglobin [Fig. 5(b)].

It is indeed difficult to see how a residue in this region could exhibit the very high reactivities reported for the major chicken carbonmonoxy and aquomet derivatives, unless it is assumed that, in this haemoglobin, the change of ligand from O_2 to CO (or H_2O) gives rise to a significant change in tertiary structure in the neighbourhood of the CysB5[23]β residue. Examination of Table 2 shows that the pK_1 values (for the ionization of HisG19[117]β) of the major and minor carbonmonoxyhaemoglobins are virtually the same; but pK_2 (for the ionization of CysB5[23]β) is higher by 0.3 pK_a units for the major component. If one assumes that both haemoglobins have identical structures in the vicinity of this sulfhydryl, it would be reasonable to expect the major component to have a lower reactivity than the minor component, because the former has a higher pK_2 value. Contrary to this expectation, the major component has at least a 100-fold higher reactivity than the minor component [*cf.* Fig. 3(a) and 5(b)].

The major component has a higher net positive charge than the minor one, as demonstrated by electrophoresis.[30] Examination of the amino acid sequences of the two haemoglobins[17–20] shows that the α chain of the major component has a higher net charge of +3 than that of the minor component. Since the two haemoglobins have identical β chains, this is equivalent to a higher net charge of +6 per (tetramer) molecule. It may therefore be argued that this difference in net charge may be responsible for the 100-fold higher reactivity of the major component. Rabbit haemoglobin has a higher net charge (per tetramer molecule) of +4 than human haemoglobin; yet its CysF9[93]β sulfhydryl group has only a *ca.* 7-fold higher reactivity than that of human haemoglobin.[31] Therefore, it is highly unlikely that the observed 100-fold difference in the reactivity of CysB5[23]β of the major and minor haemoglobins could be attributed to the net charge difference of +6. In any case, even if one chooses to attribute the reactivity difference between the major and minor carbonmonoxyhaemoglobins to electrostatic effects, it would be difficult to explain on this basis the over 100-fold difference in the reactivities of the CysB5[23]β sulfhydryls of the CO and oxy derivatives of the major component, since there is no difference in net charge between these two derivatives.

It is known that, in some instances, apparently occluded sites, as judged from the crystal structure, are open for reaction because of the dynamic nature of the structure in solution. We propose that the drastically increased reactivities of the CO and aquomet derivatives of the major haemoglobin arise because the CysB5[23]β region is open for reaction in the CO and aquomet derivatives but not in the oxy derivative. That the presumed change in tertiary structure can be partially reversed is demonstrated by the effect of inositol-P_6 on the fast phases of the carbonomonoxy and aquomet derivatives: the organic phosphate reduces the CysB5[23]β reactivity. This implies that the tertiary structure of the major chicken haemoglobin in the vicinity of CysB5[23]β is sufficiently flexible to be modified by the addition of inositol-P_6.

Comparison of CysF9[93]β reactivities of major and minor chicken carbonmonoxyhaemoglobins

The major and minor chicken haemoglobins have identical β chains but different α chains.[17–20] On account of the identity of their β chains, it would have been expected that their CO derivatives would have similar sulfhydryl reactivities, since the reacting sulfhydryls are at identical positions on the β chains. We found, however, that the CysF9[93]β sulfhydryl of the minor haemoglobin is *ca.* twice as reactive as that of the major component. We now attempt to account for this difference in reactivity.

We have established that, at an ionic strength of 50 mmol dm^{-3} or less, the reaction of CysF9[93]β with DTNB is highly sensitive to electrostatic interactions.[11,12,26] Since the major haemoglobin is more positively charged than the minor component[17–20,30] it would be reasonable, on purely electrostatic grounds, to expect the reactivity of its CysF9[93]β sulfhydryl group to be higher than that of the minor haemoglobin. Contrary to the expectation, the minor haemoglobin has a two-fold higher reactivity.

Table 1 shows the values of pK_1 for HisHC3[146]β of both haemoglobins. It is seen that pK_1 for the major haemoglobin is 0.4 of a pK_a unit higher than that of the minor component (four times higher than the standard error). Since this histidine forms a salt bridge with AspFG1[94]β, the higher pK_1 value of the major haemoglobin represents an extra stabilization of the salt bridge formed in the major haemoglobin, compared to the minor haemoglobin. Therefore, steric hindrance by TyrHC2[145]β to the approach of DTNB to the F9[93]β site is more pronounced in the major haemoglobin. Additionally, Table 1 shows that pK_2 for the ionization of CysF9[93]β is 0.5 pK_a units higher for the major component. There is thus an extra barrier to the formation of the thiol anion in the major haemoglobin compared with the minor component. Since DTNB reacts only with the thiol anion form of haemoglobin sulfhydryl groups,[13,25] this is an additional reason for the lower reactivity of the major haemoglobin.

The finding that the CysB5[23]β (and also the CysF9[93]β) sulfhydryl groups of the chicken haemoglobins have different

reactivities is not totally surprising. It has been found that the major chicken haemoglobin has a lower O_2 affinity than the minor component, and its O_2 affinity is reduced by inositol-P_6 to a greater extent than that of the minor component.[30] The real surprise is the extent to which the reactivities of their CysB5[23]β residues differ.

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