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Biophysical Chemistry 119 (2006) 196 – 204

Biophysical Chemistry

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Reversible reaction of 5,5′-dithiobis(2-nitrobenzoate) with the hemoglobins of the domestic cat: Acetylation of NH_3^+ terminal group of the β chain transforms the complex pH dependence of the forward apparent second order rate constant to a simple form

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Received 8 July 2005; received in revised form 14 September 2005; accepted 14 September 2005
Available online 19 October 2005

Abstract

We demonstrate kinetically that the reaction of 5,5′-dithiobis(2-nitrobenzoate) with the CysF9[93]β sulfhydryl group of domestic cat hemoglobins is a reversible process. In the major hemoglobin, in which the NH_3^+ terminal group of GlyNA1[1]β is free, k_f, the apparent forward second order rate constant, has a complex pH dependence profile. In the minor hemoglobin, the NH_3^+ terminal group of SerNA1[1]β is acetylated, and the pH dependence profile of k_f is simple. These results support the proposal that the positively charged groups at the organic phosphate binding site are electrostatically linked to CysF9[93]β. Quantitative analyses of the complex profiles enabled us to estimate pK_as of 7.47±0.3; 6.53±0.03 and 8.49±0.3 for GlyNA1[1]β, HisH21[143]β and other histidines within 2 nm of the sulfhydryl, and CysF9[93]β, respectively, of the major hemoglobin. Analyses of the simple profiles gave pK_as of 6.33±0.17 and 8.54±0.5 for HisH21[143]β and other histidines within a distance of 2 nm of the sulfhydryl, and CysF9[93]β of the minor hemoglobin, respectively.

Keywords: Cat hemoglobin; 5,5′-dithiobis(2-nitrobenzoate); Reversible kinetics; pH dependence

1. Introduction

Over the past four decades considerable interest has been generated by the CysF9[93]β sulfhydryl group of hemoglobin because its reactivity changes with changes in tertiary or quaternary structure [1–9]. Using 5,5′-dithiobis(2-nitrobenzoate) – DTNB – as sulfhydryl reagent, we found that at an ionic strength of 50 mmol dm^{-3} the apparent forward second order rate constant, k_f, for the reaction of DTNB with CysF9[93]β of human hemoglobins A and S varies with pH in a complex manner [10,11]. At an ionic strength of 200 mmol dm^{-3} the complex profile becomes simple, resembling the titration curve of a diprotic acid. Furthermore, at an ionic strength of 50 mmol dm^{-3}, but in the presence of the organic phosphate inositol hexakisphosphate, the complex profile also becomes simple. These findings led us to propose [10,11] that at ionic strength 50 mmol dm^{-3} CysF9[93]β of human hemoglobin is electrostatically linked to ValNA1[1]β, HisNA2[2]β and HisH21[143]β, the positively charged ionisable groups at the organic phosphate binding site [12,13]. We also proposed that this linkage gives rise to the observed complex pH dependence of k_f.

The hemoglobins of the domestic cat (felis catus) provide an opportunity to test these propositions. These hemoglobins are remarkable because, compared to human hemoglobin, they have mutations involving some of the amino acid residues at the organic phosphate-binding site [14]. In both the major and minor hemoglobins, HisNA2[2]β of human hemoglobin is substituted by phenylalanine. This substitution should cause no difference between the two cat hemoglobins. Additionally, in the major hemoglobin, ValNA1[1]β of human hemoglobin is substituted by glycine [14]. The substitution NA1[1]$\beta^{Val\rightarrow Gly}$ does not involve a change in charge because the positive charge on the NH_3^+ terminal group is retained. In the minor

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doi:10.1016/j.bpc.2005.09.009

hemoglobin the substitution is NA1[1]$\beta^{Val\rightarrow Ser}$, and it is noteworthy that *the terminal NH_3^+ group of SerNA1[1]β is acetylated and therefore has no charge* [14].

With this difference between the two cat hemoglobins in mind, we have undertaken a comprehensive investigation of the reaction between DTNB and the cat hemoglobins at ionic strength 50 mmol dm^{-3} to determine what effect the difference in charge at the organic phosphate-binding site would have on the nature of the pH dependence profile of k_f, the forward apparent second order rate constant. We obtained complex pH dependence profiles for the oxy, aquomet and carbonmonoxy derivatives of the major hemoglobin. By contrast, we obtained simple profiles for the corresponding derivatives of the minor hemoglobin. These results support the proposition that the positively charged organic phosphate binding groups are electrostatically linked to CysF9[93]β.

An important question regarding the reaction of DTNB with hemoglobin is whether it is reversible or not. A kinetic study of the reactions of DTNB with simple thiols indicates that these reactions are not reversible [15]. On the other hand, the equilibrium constant for the reaction of 4,4′-dithiobis(benzenesulfonic acid) with cysteine, a simple thiol compound, has been determined [16]. [4,4′-dithiobis(benzenesulfonic acid) is a disulfide with a structure closely resembling that of DTNB.] To the best of our knowledge, there is no report in the literature that shows unequivocally whether the reaction of DTNB with a thiol group in a biological system is reversible or not. We have therefore undertaken a kinetic study to clear up this point. Our data strongly indicate that the reactions of the major and minor cat hemoglobins with DTNB are reversible.

2. Experimental

Cat blood was obtained by decapitating domestic cats purchased from the local market. The blood was collected in bottles containing freshly prepared acid–citrate–dextrose anticoagulant. Hemoglobin was prepared by normal laboratory procedures, except that the isotonic saline used to wash the red blood cells contained 11.5 rather than 9.5 g NaCl dm^{-3}. It was stored as the carbonmonoxy derivative.

2.1. Separation of hemoglobins

Cat hemolysate contains two hemoglobins, major and minor. The separation of the hemoglobins was achieved as follows in a cold room at 5 °C. A 3 cm (diameter) by 30 cm column of Whatman CMC-52 carboxymethylcellulose, a microgranular, preswollen cation exchanger, was used. The resin was pre-equilibrated with 10 mmol dm^{-3} phosphate buffer, pH 6.5. The minor hemoglobin was completely eluted with the pH 6.5 buffer, whereas the major component remained bound to the resin. The major hemoglobin was eluted at room temperature with 140 mmol dm^{-3} phosphate buffer, pH 7.0. The hemoglobins were stored in the freezer and thawed when required. Prior to use for experiments, each hemoglobin was passed through a Dintzis ion exchange column [17] to remove endogenous organic phosphates and undesired ions.

2.2. Determination of the number of reactive sulfhydryl groups

The total number of reactive thiol groups was determined by Boyer titration [18] of the carbonmonoxy derivative in phosphate buffer, pH 7.6 (ionic strength 50 mmol dm^{-3}), using p-hydroxymercuri(II)benzoate. The number of sulfhydryl groups reacting with DTNB was determined as previously described [19].

2.3. Kinetics

The kinetics of the reaction of DTNB with the cat hemoglobins were monitored at 412 nm on a Pye-Unicam Heλioseα uv-visible spectrophotometer coupled to an on-line data acquisition system. The reaction was carried out under pseudo-first order conditions, with DTNB in at least 30-fold excess over the concentration of (DTNB-reactive) sulfhydryl groups. Each run was repeated at least three times under the same experimental conditions and was allowed to proceed to near completion. The data were analyzed with a 1990 update of DISCRETE, a computer program for the analysis of multiple exponential signals [20,21]. A slight modification of DISCRETE allowed the determination of the absorbance reading at the end of the reaction. The standard error in the determination of the pseudo-first order rate constant was about 5 %.

3. Results

3.1. Number of reactive sulfhydryl groups

According to their amino acid sequences [14], each of the cat hemoglobins has 12 sulfhydryl groups, identical in pairs, per tetramer molecule. These are located at positions A11[13],

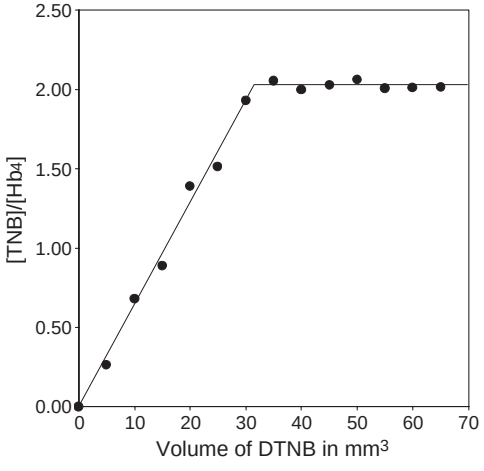


Fig. 1. Titration of the major cat carbonmonoxyhemoglobin with 5,5′-dithiobis(2-nitrobenzoate), DTNB: ratio of the concentration of 5-thio-2-nitrobenzoate (TNB) produced to the concentration of hemoglobin tetramer (Hb_4) as a function of the volume of DTNB mixed with 3 cm^3 of hemoglobin. Conditions: hemoglobin concentration, 10 μmol (heme) dm^{-3} (5 μmol dm^{-3} in reactive sulfhydryl groups); stock concentration of DTNB, 0.5 mmol dm^{-3}; phosphate buffer pH 7.6, ionic strength 50 mmol dm^{-3} (added salt, NaCl). A similar result was obtained with the minor hemoglobin.

B15[34], G11[104] and G18[111] on each of the two α chains and at positions F9[93] and G14[112] on each of the two β chains. Of these, those at positions G11[104]α and G14[112]β are known not to react with any sulfhydryl reagent because they are at the $\alpha_1\beta_1$ subunit interface and are therefore "masked" [22]. That leaves only eight sulfhydryl groups per tetramer that can react with sulfhydryl reagents. We found by Boyer titration [18] that all eight reacted with p-hydroxymercuri(II)benzoic acid (data not shown). Surprisingly, however, only two reacted with DTNB (Fig. 1). Repeated titrations of the major and minor hemoglobins gave a mean of 2.1±0.1 sulfhydryl groups per tetramer. In order to be certain that this result was not an artefact, we carried out a DTNB titration on pigeon hemoglobin under the same conditions as for cat hemoglobin. We obtained four sulfhydryl groups per pigeon hemoglobin tetramer, in agreement with our previous result. [19]. We therefore conclude that the value of 2.1 obtained for cat hemoglobin is correct.

3.2. Kinetics

An important question regarding the reaction of DTNB with hemoglobin is whether the reaction is reversible or not. In order to clear up this point, we assume that the reaction of DTNB with cat hemoglobins is reversible. Bearing in mind that DTNB reacts only with the anion form of a sulfhydryl group [15,23,24], we formulate the reaction as follows:

$$\mathrm{HbSH} + \mathrm{DTNB} \overset{K_{\mathrm{SH}}}{\rightleftharpoons} \mathrm{H^+} + \mathrm{HbS^-} + \mathrm{DTNB} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{H^+} + \mathrm{HbS.TNB} + \mathrm{TNB^-} \overset{K_{\mathrm{TNB}}}{\rightleftharpoons} \mathrm{HbS.TNB} + \mathrm{TNBH}. \quad (1)$$

In Eq. (1) HbSH is hemoglobin with the CysF9[93]β sulfhydryl in its protonated (unreacting) form; $\mathrm{HbS^-}$is the corresponding (reacting) anion form; HbS.TNB is the mixed disulfide formed after the reaction of hemoglobin with DTNB; $\mathrm{TNB^-}$is 5-thio-2-nitrobenzoate, the anionic, chromophoric

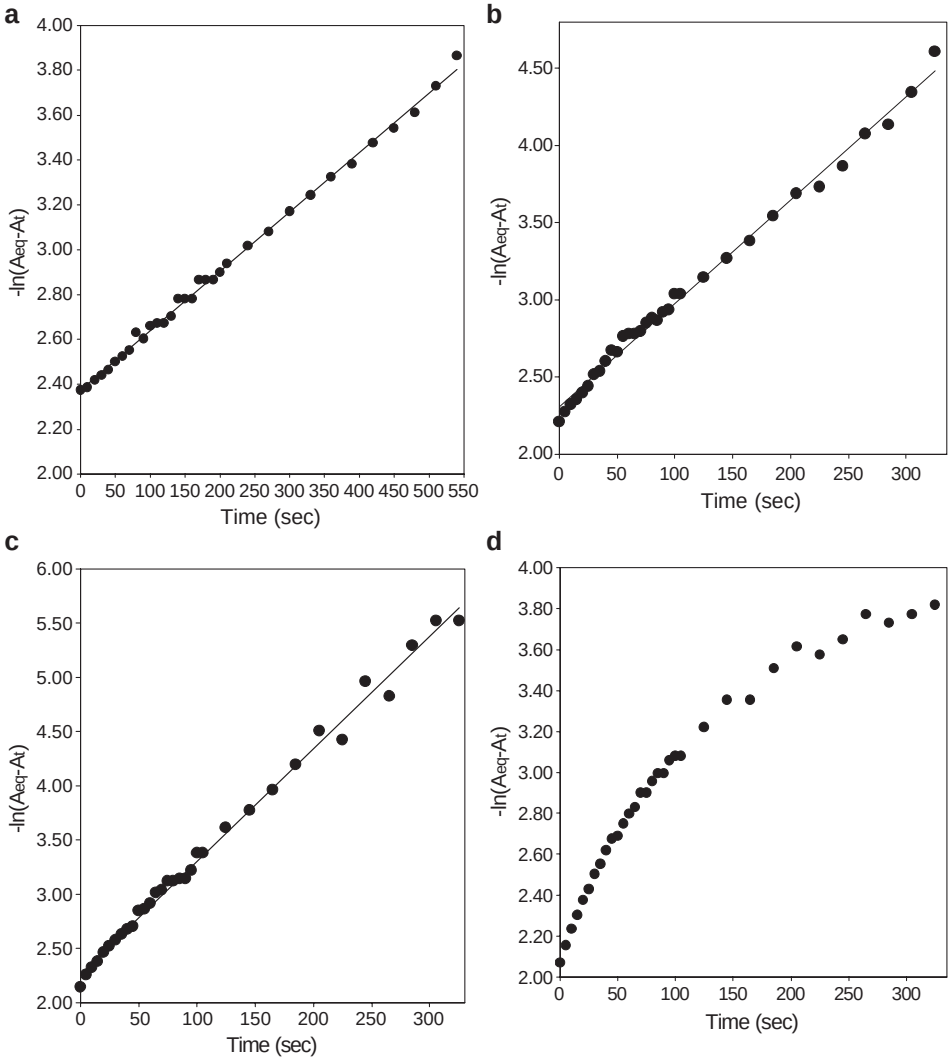


Fig. 2. Semi-logarithmic plots of the time courses for the reaction of DTNB with derivatives of the minor cat hemoglobin at 25 °C: (a) oxyhemoglobin at pH 6.10; (b) aquomethemoglobin at pH 7.12; (c) carbonmonoxyhemoglobin at pH 8.43; (d) aquomethemoglobin at pH 8.78. Conditions: hemoglobin concentration, 10 μmol (heme) $\mathrm{dm^{-3}}$ (5 μmol $\mathrm{dm^{-3}}$ in reactive sulfhydryl groups); DTNB concentration, 150 μmol $\mathrm{dm^{-3}}$; phosphate buffers, $5.6 \leq \mathrm{pH} \leq 8.0$; borate buffers, $\mathrm{pH} > 8.0$; $\lambda = 412$ nm. Similar results were obtained with the major hemoglobin.

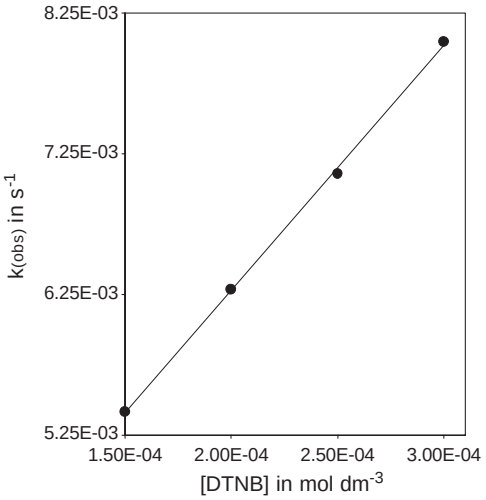


Fig. 3. Dependence of the pseudo-first order rate constant, k_{obs}, on the DTNB concentration for the reaction of DTNB with CysF9[93]β of the major cat oxyhemoglobin at 25 °C. Conditions: phosphate buffer pH 7.45 (ionic strength 50 mmol dm^{-3}; added salt, NaCl). Other conditions as in Fig. 2. Each experimental point is the mean of at least three determinations. The standard error on each point is *ca* ±10% of the mean.

product of the reaction; TNBH is the protonated form of TNB^-; k_f and k_r are the apparent second order rate constants for the forward and reverse reactions, respectively; and K_{SH}, K_{TNB} are equilibrium constants for the ionisations of CysF9[93]β and TNBH, respectively. In what follows, it is assumed that the protolytic steps of Eq. (1) are several orders of magnitude faster than the forward and reverse reaction steps [25–27].

Let '*a*' depict the total concentration of (DTNB-reactive) sulfhydryl groups, '*b*' the total DTNB concentration, and '*x*' the concentration of TNB produced at time *t*. The rate of the reaction (see Eq. (1)) is given by:

$$-d(a-x)/dt = k_f(a-x)(b-x) - k_r x^2 \quad (2)$$

Under pseudo-first order conditions, $b \gg a$, and with the equilibrium constant $K_{eq}=k_f/k_r$, Eq. (2) becomes

$$dx/dt = -k_f\{bx - (1 - 1/K_{eq})x^2\} \quad (3)$$

If $(1 - 1/K_{eq})x^2 << bx$, Eq. (3) can be simplified to Eq. (4):

$$dx/dt = -k_f bx \quad (4)$$

Integration of Eq. (4) gives

$$-\ln x = k_f bt + \text{constant} \quad (5)$$

In terms of absorbance changes, Eq. (5) becomes

$$-\ln(A_{eq} - A_t) = k_f bt + \text{constant} \quad (6)$$

where A_{eq} is the absorbance when the reaction has come to equilibrium and A_t is the absorbance at any time t. A plot of $-\ln(A_{eq}-A_t)$ versus time should give a straight line of slope $k_f b$, usually denoted by k_{obs}, the pseudo-first order rate constant. Under conditions in which $(1-1/K_{eq})x^2$ of Eq. (3) is no longer negligible compared to bx, such as when the equilibrium constant K_{eq} is very small, a plot according to Eq. (6) should no longer give a straight line.

Fig. 2(a–c) show, for the minor hemoglobin, typical pseudo-first order rate plots for the reaction of DTNB with the oxy, aquomet and carbonmonoxy derivatives at various pH values. It is seen that straight-line plots are obtained, in accordance with Eqs. (5) and (6). For both the major and minor hemoglobins, similar plots were obtained at all pH values for each of the three derivatives in the range $5.6 \leq pH < 8.6$. In each case the plot is linear for up to *ca* 2.5 to 3 half-lives. Moreover, R^2, the square of the correlation coefficient, is at least 0.99 in each case. However, at pH > 8.6 the plots are no longer linear (Fig. 2d). This indicates that $(1 - 1/K_{eq})x^2$ is no longer negligible compared to bx (see Eq. (3)) at pH > 8.6, and Eqs. (5) and (6) can no longer account for the kinetic data. This finding supports our initial assumption that the reaction of DTNB with the cat hemoglobins is a reversible process, at least in the range pH > 8.6.

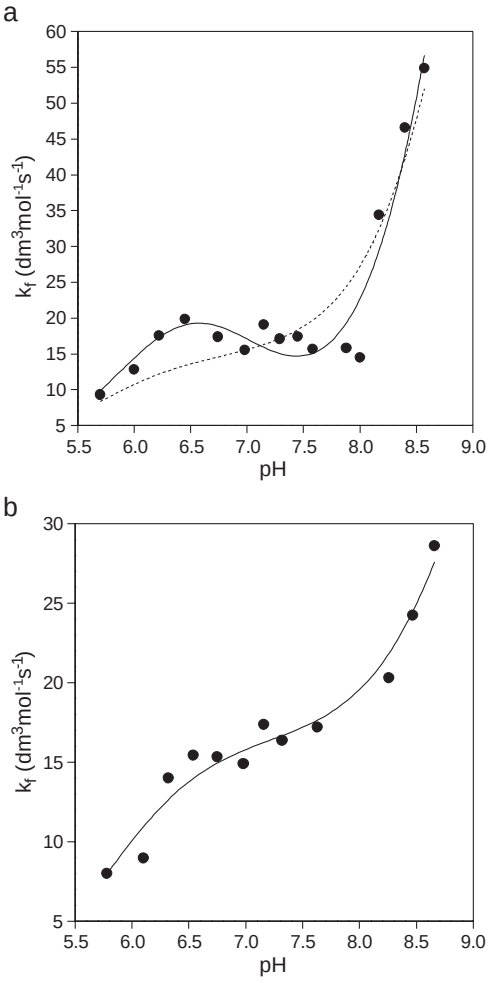


Fig. 4. Dependence of k_f, the apparent forward second order rate constant, on pH for the reaction of the CysF9[93]β sulfhydryl group of cat oxyhemoglobin with DTNB at 25 °C: (a) the major hemoglobin; the dotted line is the best-fit line drawn using Eq. (7) of the text with $n=1$; the full line is the best-fit line drawn with $n=2$ using the best-fit parameters reported in Table 1; (b) the minor hemoglobin.; the line through the data points was drawn with Eq. (7) with $n=1$, using the best-fit parameters reported in Table 2. Conditions: as in Fig. 2.

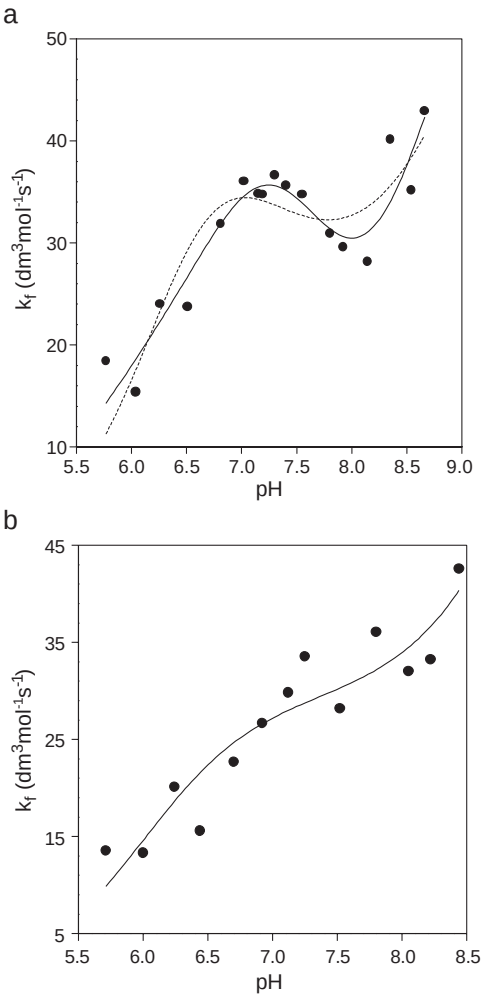


Fig. 5. Dependence of k_f, the apparent forward second order rate constant, on pH for the reaction of the CysF9[93]β sulfhydryl group of cat aquomethemoglobin with DTNB at 25 °C: (a) the major hemoglobin; the dotted line is the best-fit drawn using Eq. (7) of the text with $n=2$; the full line is the best-fit drawn with $n=3$; best-fit parameters reported in Table 1; (b) the minor hemoglobin.; the line through the data points was drawn with Eq. (7) with $n=2$, using the best-fit parameters reported in Table 2. Conditions: as in Fig. 2.

For the oxy derivative of the major hemoglobin, Fig. 3 reports the dependence of k_{obs}, the pseudo-first order rate constant, on the DTNB concentration at pH 7.45. Similar plots were obtained for all three derivatives of the major and minor hemoglobins at all pH values below 8.6. For each plot R^2 is at least 0.9. Values of k_f, the forward second order rate constant for the DTNB reaction (see Eq. (1)), were obtained from the least squares slopes of such plots. Calculation shows that each of these plots has a non-zero intercept (unlike the data reported for the reaction of DTNB with simple thiol compounds [15]). This finding indicates that the reaction of DTNB with the cat hemoglobins is a reversible process in the range $5.6 \leq pH < 8.6$.

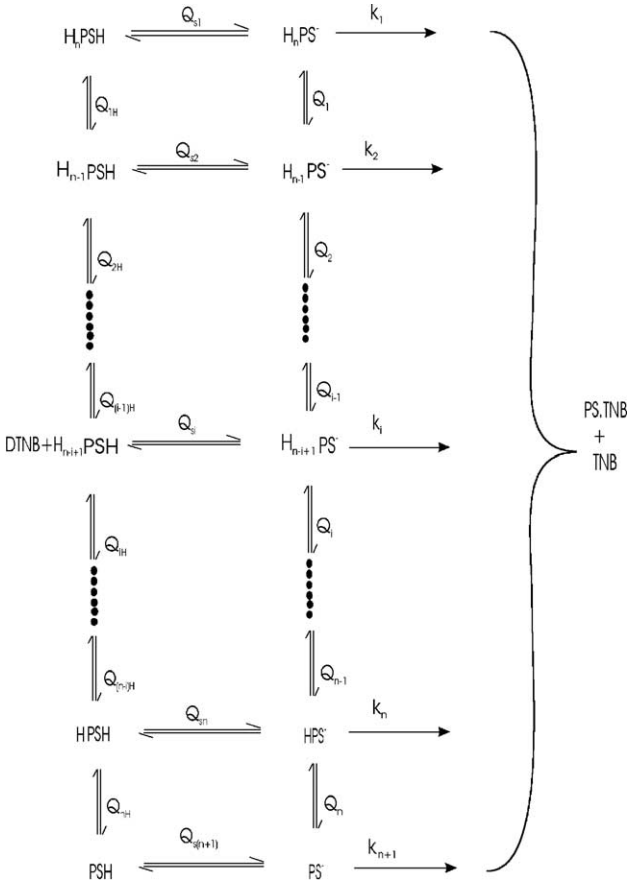
3.3. Analyses of k_f versus pH profiles

Fig. 4a reports the dependence of k_f on pH for the oxy derivative of the major hemoglobin. It is obvious that the profile is complex, like those of human hemoglobins [10,11]. Fig. 4b reports the corresponding plot for the minor hemoglobin; the profile is simple. Fig. 5a and b report the corresponding profiles for the aquomet derivative, and Fig. 6a and b the corresponding profiles for the carbonmonoxy derivative. It is seen in each case that the profile for the major hemoglobin is complex while that of the minor hemoglobin is simple.

We previously accounted for complex profiles like those in Figs. 4a, 5a and 6a with Scheme 1 [10,11]. In Scheme 1 n ionizable groups are electrostatically linked to the CysF9[93]β site. H_nPSH, $H_{n-1}PSH$, . . ., $H_{n-i+1}PSH$, . . ., HPSH and PSH are hemoglobin species having n, (n − 1), . . ., (n − i + 1),. . ., 1 and 0 protons bound to the electrostatically thiol-linked ionisable groups. Each of these species has its thiol group protonated. H_nPS^-, $H_{n-1}PS^-$, . . ., $H_{n-i+1}PS^-$,. . ., HPS^- and PS^- are the corresponding thiolate anion forms of the various species. (The various Q terms signify ionization constants.) Since only the thiolate anion forms are reactive towards DTNB [15,23,24], the relationship between k_f and the parameters of Scheme 1 is given by the expression [10,11]:

$$k_f = \frac{k_{n+1} + \sum_{i=1}^{n} k_i (H^+)^{n-i+1} \left(\prod_{j=i}^{n} Q_j \right)^{-1}}{\left\{ 1 + \sum_{i=1}^{n} (H^+)^{n-i+1} \left(\prod_{j=i}^{n} Q_j \right)^{-1} + \frac{(H^+)}{Q_{s(n+1)}} \left[1 + \sum_{i=1}^{n} (H^+)^{n-i+1} \left(\prod_{j=i}^{n} Q_{jH} \right)^{-1} \right] \right\}} \quad (7)$$

In analysing data on human hemoglobins A and S at ionic strength 50 mmol dm^{-3} we assumed that complex profiles like



Scheme 1.

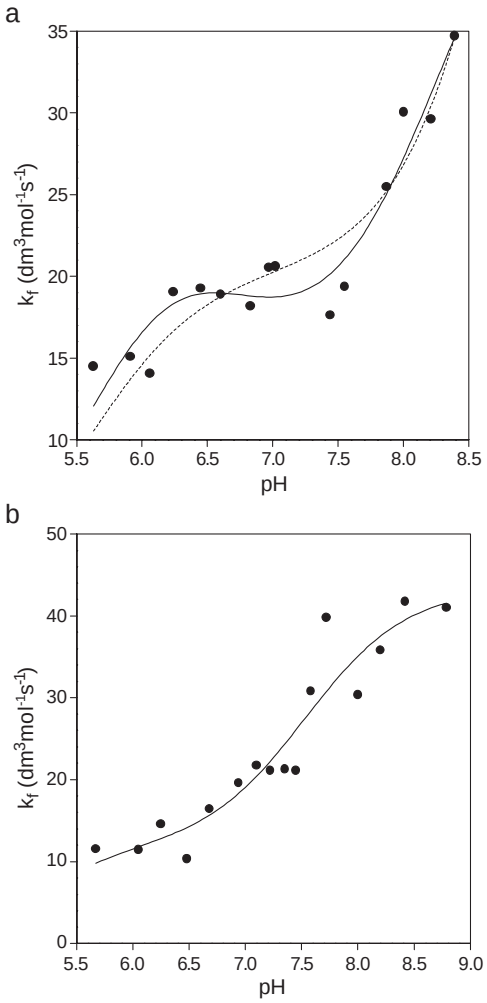


Fig. 6. Dependence of k_f, the apparent forward second order rate constant, on pH for the reaction of the CysF9[93]β sulfhydryl group of cat carbonmonoxyhemoglobin with DTNB at 25 °C. (a) the major hemoglobin; the dotted line is the best-fit using Eq. (7) of the text with $n=1$; the full line is the best-fit drawn with $n=2$ using the best-fit parameters reported in Table 1; (b) the minor hemoglobin.; the line through the data points was drawn with Eq. (7) with $n=1$, using the best-fit parameters reported in Table 2. Conditions: as in Fig. 2.

those in Figs. 4a and 5a arose from the electrostatic linkage of a number of ionisable groups to the CysF9[93]β site [10,11]. This assumption was justified by the fact that at high ionic strength (200 mmol dm^{-3}) the complex profiles obtained at ionic strength 50 mmol dm^{-3} were converted to simple profiles [10,11]. Moreover, at ionic strength 50 mmol dm^{-3} the organic phosphate inositol hexakisphosphate also causes the complex profiles to become simple [10,11]. Since inositol hexakisphosphate is known to bind to the basic groups at the organic phosphate binding site [12,13], thus neutralizing their charges, this latter finding led us to conclude that it is these basic groups that are electrostatically linked to the CysF9[93]β site [10,11].

In human hemoglobin the basic groups at the organic phosphate-binding site are ValNA1[1]β, HisNA2[2]β, HisH21[143]β and LysEF6[82]β [12,13]. Of these groups, only the first three are ionisable in the pH range 5.6 to 9 of our experiments. (LysEF6[82]β has a pK_a of about 10.5 and is therefore not ionisable in our experimental pH range.) Therefore, in analysing the complex pH-dependence profiles of k_f with Scheme 1 and Eq. (7) the value of n was tentatively assumed to be 3 [10,11]. However, HisNA2[2]β and HisH21[143]β were assumed to have closely similar pK_a values and the data were in fact analyzed with $n=2$ [10,11]. This assumption is not necessary with cat hemoglobin since the histidine at the NA2[2]β position in human hemoglobin is replaced by phenylalanine in cat hemoglobin [14].

Compared to human hemoglobin, each of the cat hemoglobins has two mutations at the organic phosphate-binding site: NA1[1]$\beta^{Val\rightarrow Gly}$ and NA2[2]$\beta^{His\rightarrow Phe}$ in the major hemoglobin and NA1[1]$\beta^{Val\rightarrow Ser}$ and NA2[2]$\beta^{His\rightarrow Phe}$ in the minor hemoglobin. The mutation NA1[1]$\beta^{Val\rightarrow Gly}$ in the major hemoglobin leaves the positive charge on the NH_3^+ terminus of the β subunit intact. By contrast, in the minor hemoglobin the NH_3^+ terminal group of SerNA1[1]β is acetylated and therefore has no charge. Consequently, the analyses of the major hemoglobin data (Figs. 4a, 5a and 6a) should require an n value greater than that required for the minor hemoglobin data.

We have analysed the data in Figs. 4a and 5a with Eq. (7). An attempt to fit the oxyhemoglobin data with an n value of 1 (dotted line in Fig. 4a) gave a bad fit. A better fit was obtained with an n value of 2 (full line in Fig. 4a). The fit of the aquomethemoglobin data with an n value of 2 is shown as a dotted line in Fig. 5a. It is seen that it is not good. Taking cognisance of the finding [10,11] that the water molecule at the 6th coordination position of the heme-bound Fe^{3+} ion of

Table 1
Reaction of the major cat hemoglobin with DTNB at an ionic strength of 50 mmol dm^{-3}

Parameter	Oxyhemoglobin	Aquomethemoglobin	Carbonmonoxyhemoglobin
pQ_{1H}	6.00	5.60	5.90
pQ_1	6.50	6.50	6.60
pQ_{2H}	7.00	7.00	6.63
pQ_2	7.00	7.50	7.91
$pQ_{s3/s4}$	*ca* 9.00	8.50	7.97
k_1 (mol^{-1})	9452.8	286.8	54.5
k_2 (mol^{-1} dm^3 s^{-1})	438.1	264.0	32.7
k_3 (mol^{-1} dm^3 s^{-1})	201.1	41.7	48.6
k_4 (mol^{-1} dm^3 s^{-1})	–	70.9	–

Best- fit parameters used to fit the data in Figs. 4a, 5a and 6a [compare with Scheme 1 and Eq. (7) of the text with $n=2$ (oxy- and carbonmonoxyhemoglobin) and with $n=3$ (aquomethemoglobin)].

aquomethemoglobin is electrostatically linked to CysF9[93]β, we fit the aquomethemoglobin data with an n value of 3 (full line in Fig. 5a), assuming a pK_a of *ca* 8.1 at 25 °C for the water molecule at the 6th coordination position of the iron atom [28]. It is seen that the fit with $n=3$ is much better than that with $n=2$.

As can be seen in Fig. 6a, the trend of the data points for carbonmonoxyhemoglobin appears to be less complex than those of oxy-and aquomethemoglobin (Figs. 4a and 5a): there appears to be no obvious peak. For this reason we attempted to fit the carbonmonoxy data with an *n* value of 1. The fit (dotted line) is obviously bad. We therefore fit the data with $n=2$ and obtained a better fit. The best-fit line is shown drawn as a solid line through the data points. The best-fit parameters used to draw the best-fit lines in Figs. 4a, 5a and 6a are reported in Table 1.

The simple profiles in Figs. 4b and 6b (oxy-and carbonmonoxyhemoglobin, respectively) were fit with Eq. (7) using an n value of 1 in each case. That of Fig. 5b (aquomethemoglobin) was fit with $n=2$ (because the ionisation of the water molecule at the 6th coordination position of the heme Fe^{3+} iron is linked to CysF9[93]β [10,11]). The best-fit lines through these figures are those drawn through the data points and the best-fit parameters are reported in Table 2. It is seen that the fits are quite good.

Calculation from the major hemoglobin data in Table 1 shows that the mean values are: $pQ_1=6.53\pm0.3$; $pQ_2=7.47\pm0.3$ and $pQ_{s3/s4}=8.49\pm0.3$. Following our assignments of pQ values for human hemoglobin [10,11], we assign these values to HisH21[143]β and other histidines within a distance of 2 nm of the reacting sulfhydryl, GlyNA1[1]β and CysF9[93]β of the major cat hemoglobin. The mean values of the parameters reported in Table 2 for the minor hemoglobin are $pQ_1=6.33\pm0.17$ and $pQ_{S2/S3}=8.5\pm0.5$. These values are assigned to HisH21[143]β and other histidines within a distance of 2 nm of the reacting sulfhydryl, and to CysF9[93]β, respectively, of the minor hemoglobin.

4. Discussion

4.1. Number of sulfhydryl groups reacting with DTNB

Titration of the cat hemoglobins with DTNB indicated that only 2 sulfhydryl groups per tetramer react with DTNB. An examination of the 3D structure of hemoglobin shows why only two of the eight sulfhydryl groups that react with p-hydroxymercuri(II)benzoate are reactive towards DTNB: CysA11[13]α appears to be in a hydrophobic environment, its nearest neighbor being the hydrophobic TrpA12[14]α; CysB15[34]α is at the $\alpha_1\beta_1$ subunit interface and, moreover, is in contact with ProH2[124]β, GlnH3[125]β and AlaH6[128]β, all of which are hydrophobic residues; CysG18[111]α interacts with the carboxyl group of GluB8[27]α, the carbonyl of ValG14[107]α and the hydroxyl of TyrB5[24]α, as it does in dog haemoglobin [23]. These contacts and interactions would make it difficult for these sulfhydryl groups to form the thiolate anion. Consequently, they cannot react with DTNB. This leaves CysF9[93]β as the only sulfhydryl that can react with DTNB. The finding, by static titration, that only one pair of identical sulfhydryl groups reacts with DTNB is supported by the monophasic kinetics observed for the DTNB reaction (Fig. 2(a – c)).

4.2. Is the DTNB reaction with the cat hemoglobins reversible?

Our data support the conclusion that the reaction of the cat hemoglobins with DTNB is a reversible process: (i) The pseudo-first order rate plots at pH > 8.6 (Fig. 2d) are non-linear, indicating that Eqs. (5) and (6) are no longer sufficient to account for the kinetics and that Eq. (3), which includes the equilibrium constant, must be employed at high pH. (ii) Plots of k_{obs}, the pseudo-first order rate constant, against the DTNB concentration have non-zero intercepts (Fig. 3), a clear piece of evidence that the reaction is reversible.

The evidence presented in this paper shows that the reaction of DTNB with the hemoglobins of the domestic cat is reversible. That Eqs. (5) and (6) are no longer valid above pH 8.6 shows that these equations are no longer good approximations for Eq. (3), which contains the equilibrium constant. We are currently performing experiments to determine the equilibrium constant for the DTNB reaction (Eq. (1)), and there are indications that the equilibrium constant is much lower at high compared to low pH [K.O. Okonjo, A.A. Fodeke and T.A. Kehinde, unpublished data]. This explains why Eqs. (5) and (6) are valid at pH < 8.6 but invalid at higher pH.

Table 2
Reaction of the minor cat hemoglobin with DTNB at an ionic strength of 50 mmol dm^{-3}

Parameter	Oxyhemoglobin	Aquomethemoglobin	Carbonmonoxyhemoglobin
pQ_{1H}	5.68	5.99	5.60
pQ_1	6.50	6.00	6.50
pQ_{2H}	–	7.90	–
pQ_2	–	8.30	–
$pQ_{s2/s3}$	*ca*9.00	*ca* 9.00	7.61
k_1 (mol^{-1} dm^3 s^{-1})	4813.6	11533	198.1
k_2 (mol^{-1} dm^3 s^{-1})	55.1	201.45	44.3
k_3 (mol^{-1} dm^3 s^{-1})	–	82.37	–

Best- fit parameters used to fit the data in Figs. 4b and 6b [compare with Scheme 1 and Eq. (7) of the text with $n=1$] and 5b [compare with Scheme 1 and Eq. (7) of the text with $n=2$].

4.3. Organic phosphate binding groups and the nature of the pH-dependence profile

We found previously for human hemoglobins [10,11] that k_f first increases with increase in pH, attains a maximum value around pH 7, decreases to a minimum value around pH 8, and then increases again as the pH further increases. In the presence of high salt concentrations (ionic strength 200 mmol dm^{-3}) this complex profile becomes simple; at low ionic strength the organic phosphate, inositol hexakisphosphate, also converts the complex profile to a simple one [10,11]. We concluded from these results that this complex pH dependence arose from electrostatic interactions between CysF9[93]β and the positively charged, ionizable groups at the organic phosphate binding site: ValNA1[1]β, HisNA2[2]β and HisH21[143]β.

In the mutation NA2[2]$\beta^{His\rightarrow Phe}$, one of the ionisable histidines of human hemoglobin has been replaced by the non-ionisable phenylalanine in cat hemoglobin. As can be seen by comparing the profiles in Figs. 4a and 5a with those reported for human hemoglobins, the mutation NA2[2]$\beta^{His\rightarrow Phe}$ appears to have had little effect on the complex nature of the pH dependence profile. In Fig. 6a (the carbonmonoxy derivative) there seems to be no obvious peak in the k_f versus pH profile. Nevertheless, this profile (Fig. 6a) was best fit with the same value of n, 2, as the more complex-looking profile of oxyhemoglobin (Fig. 4a).

The effect on the nature of the pH dependence profile caused by the removal of the charge on the terminal NH_3^+ group of the β chain (by acetylation) is quite remarkable (compare Figs. 4a,b, 5a,b and 6a,b): each complex profile is converted to a simple profile. This provides strong support for the proposal that at ionic strength 50 mmol dm^{-3} or lower the basic groups at the organic phosphate binding site are electrostatically linked to the F9[93]β site. But over and above this, it is clear that the NH_3^+ terminal group of the NA1[1]β residue plays a prominent role in determining the complex nature of the pH dependence profile. Obviously, the reduced values of n required to fit the minor hemoglobin data (Figs. 4b, 5b and 6b) compared to those of the major hemoglobin (Figs. 4a, 5a and 6a) (compare Tables 2 and 1) are the consequence of the acetylation of SerNA1[1]β in the minor hemoglobin. This group is unmodified in the major hemoglobin [14].

We previously observed that complex k_f versus pH profiles were made simple by binding of the organic phosphate inositol hexakisphosphate or by increased ionic strength [10,11]. In the present paper we have demonstrated that chemical modification of the NH_3^+ terminal NA1[1]β residue, an organic phosphate binding group, also simplifies the profiles.

5. Conclusion

The evidence presented in this paper indicates that the reaction of DTNB with the hemoglobins of the domestic cat is reversible. Whether the reaction of DTNB with other hemoglobins is reversible is a matter for further investigation. Measurements of the equilibrium constant for the reaction of DTNB with cat hemoglobins will make it possible to determine the rate constant, k_r, for the reverse reaction (see Eq. (1)), a parameter that would otherwise be difficult to determine.

Our results strongly indicate that although all the organic phosphate binding groups are linked electrostatically to the F9[93]β site, the complex pH dependence profile of k_f for the DTNB reaction arises mainly from the charge on the amino terminal residue.

Acknowledgement

We are very grateful to the Alexander von Humboldt Foundation, Bonn, Germany for instrument support for our research spanning three decades. We acknowledge the partial financial support for our research given through Grant No. SRG/FSC/2000/53A of the Senate of the University of Ibadan. We thank Mr. Joel Niyi Ajayi for excellent technical assistance.

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