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Effect of inositol hexakisphosphate on sulphydryl reactivities of low (cat) and high (human) oxygen affinity haemoglobins

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Abstract

The effect of organic phosphate binding on the reactivities of low-affinity cat haemoglobin in comparison to high-affinity human haemoglobin was determined. Kinetics of the reaction of the CysF9[93]β sulphydryl groups in both haemoglobins with 5,5′-dithiobis(2-nitrobenzoate) – DTNB – which has been linked to oxygen binding, was used to probe this effect. Plots of the pseudo-first order rate constant, k_{obs}, as a function of the DTNB concentration were linear and had positive intercepts. This is indicative of the fact that the organic phosphate used, inositol hexakisphosphate (inositol-P_6), does not abolish reversibility in both haemoglobins. Cat haemoglobins gave simple pH-dependence profiles for the apparent second order forward rate constants, k_F. The presence of inositol-P_6 increased k_F, hence reactivity, by two-fold throughout the experimental pH range for cat oxyhaemoglobin. In contrast, inositol-P_6 decreased k_F for human oxyhaemoglobin. Around the physiological pH, k_F decreased from a maximum value of 31.9 ± 0.6 $mol^{-1}dm^3s^{-1}$ to 26.7 ± 0.3 $mol^{-1}dm^3s^{-1}$ for human haemoglobin, while it increased from a minimum of 17.2 ± 0.4 $mol^{-1}dm^3s^{-1}$ to 20.6 ± 0.6 $mol^{-1}dm^3s^{-1}$ for cat minor oxyhaemoglobin.

Keywords: Cat and human haemoglobins; oxygen affinities; sulphydryl reactivities; organic phosphate; 5,5′-dithiobis (2-nitrobenzoate).

Introduction

Haemoglobin is a classical example of an allosteric protein. It possesses, among other properties, multiple interacting binding sites; the ability to bind non-covalently to a primary ligand (oxygen); homotropic effects (quaternary conformation changes induced upon binding the primary ligand); and heterotropic effects (regulation of the primary ligand binding by a secondary effector molecule binding at a different site). The changes in structure that accompany binding of oxygen to haemoglobin have been the subject of extensive analyses. 2,3-bisphosphoglycerate (2,3-BPG), an anionic compound that is present in human red blood cells at approximately the same concentration as haemoglobin, is an important heterotropic effector molecule that helps in regulating the affinity of haemoglobin for oxygen [1, 2]. The crystal structure of haemoglobin shows that a molecule of 2,3-BPG binds to a positively charged central cavity ('pocket') between the β chains. This binding stabilizes the T (tense) conformation and reduces its affinity for oxygen [3, 4]. The haemoglobin molecule is thus able to unload its oxygen. A close look at the binding-site in human haemoglobin reveals that three positively charged amino groups on each β chain interact directly with this heterotropic effector molecule. They are HisNA2[2]β, LysEF6[82]β and HisH21[143]β. A fourth residue, ValNA1[1]β, is also 'indirectly' implicated in the binding of BPG to haemoglobin [2]. Inositol hexakisphosphate (inositol-P_6) is a close analogue of inositol pentakisphosphate, the physiological effector in avian erythrocytes. It is one of the most effective organic phosphates that regulate the affinity of haemoglobin for oxygen [4]. The very high overall negative charge of inositol-P_6 gives it the ability to effectively neutralize positive charges on residues which are present at the organic phosphate binding site; it is therefore the most widely used organic phosphate in experimental studies. Moreover, both 2,3-BPG and inositol-P_6 bind to the same amino acid residues [4].

Compared to human haemoglobin, cat haemoglobins have mutations in some of the amino acid residues at the organic phosphate-binding site [5]. In both major and minor cat haemoglobins, phenylalanine replaces histidine of human haemoglobin at position NA2[2]β. In addition, ValNA1[1]β of human haemoglobin is substituted by a glycine in the major haemoglobin of cat. In the minor haemoglobin, ValNA1[1]β of human haemoglobin is replaced by SerNA1[1]β, with its terminal amino group acetylated (therefore has no charge). These remarkable differences result in an overall reduction of charges at the organic phosphate binding sites of the domestic cat compared to human haemoglobin and has also led to differences in the oxygen affinities of the two haemoglobins.

Some haemoglobins have high oxygen affinity (high-affinity) while others have low oxygen affinity (low-affinity). High-affinity haemoglobins bind organic phosphates strongly and this lowers their oxygen affinities and their CysF9[93]β sulphydryl reactivities. Low-affinity haemoglobins on the other hand, bind organic phosphates weakly, and their oxygen affinities are hardly affected [6]. Consequently, an assumption which is yet to be verified is that the reactivity of their CysF9[93]β is not affected by organic phosphates. The cat haemoglobins are classed among the low (oxygen) affinity haemoglobins, whereas human haemoglobin A is classed among the high (oxygen) affinity haemoglobins [7].

The CysF9[93]β sulphydryl group is conserved in most mammalian and avian haemoglobins; it is one of the important residues that have been extensively studied. It has been used as a probe in the detection of ligand-induced conformational changes which occur in the protein moiety as a result of oxygen binding. This is because the reactivity of the CysF9[93]β sulphydryl group changes with changes in tertiary or quaternary structure, as haemoglobin loads and unloads oxygen [8-10]. The question that arises is whether the classification based on oxygen affinity will be reflected as differences in sulphydryl reactivity. It has been previously observed that inositol-P_6 *increases* the rate of the reaction of DTNB with the CysF9[93]β sulphydryl group of derivatives of cat major haemoglobins [20]. Since this result is contrary to previous results on the effect of inositol-P_6 on other haemoglobins it seemed necessary to ascertain these findings using cat minor oxyhaemoglobin on which the effect of inositol-P_6 has not been checked.

Materials and methods

Sample preparation

Screened human blood was obtained from non-smoking adult donors with genotype AA. Cat blood was obtained from domestic cats purchased from a local market in Ibadan, Nigeria. In each case, the blood was collected into freshly prepared acid-citrate-dextrose anticoagulant. Haemoglobin was prepared using standard laboratory procedures [11]. For human haemoglobin, an isotonic saline of 9.5 g NaCl dm^{-3} was used to wash the red blood cells while 11.5 g dm^{-3} was used for the cat haemoglobins. Low molecular weight impurities contained in the haemoglobins were removed by dialysis against 10 mmol dm^3 phosphate buffer ($pH \approx 6.5$) at 5°C. This procedure was repeated two more times. Oxyhaemoglobin prepared was stored in a freezer at far lesser than 0°C and was used up within a week to prevent denaturation of the haemoglobin.

Since cat haemolysate comprises of two haemoglobin types: the major ($\approx 60\%$) and the minor haemoglobin ($\approx 40\%$), there is need for its separation prior to use. The separation was carried out at 5°C using a pre-washed carboxymethyl cellulose (CMC-52) resin packed into a column (a modified method of Taketa *et al* [12]). The minor haemoglobin was eluted first with 10 mmol dm^{-3} phosphate buffer pH 6.5 while the major haemoglobin was eluted with a 10 mmol dm^{-3} phosphate buffer pH 8.0 ($I = 0.2$ mol dm^{-3}). After separation, the major and minor fractions of the haemoglobin were further dialysed twice at 5°C (for three hours per dialysis) against 10 mmol dm^{-3} phosphate buffer (pH = 6.8) to remove unwanted chloride and phosphate ions.

Kinetics

One of the advantages of 5,5'-dithiobis (2-nitrobenzoate), DTNB, over other sulphydryl reagents is that the kinetics of its reaction with haemoglobin sulphydryl groups can be monitored on a simple UV-Visible spectrophotometer [13]. Furthermore, DTNB is sensitive to the electrostatic environments that characterize the reactivity of the CysF9[93]β sulphydryl group [14]. The reactions of DTNB with both the stripped human oxyhaemoglobin and inositol-bound haemogloin solutions, were monitored at 412 nm on a Varian Cary 400Scan UV-Visible spectrophotometer under pseudo-first order conditions. This was achieved by reacting each of the haemoglobin samples with at least sixty-fold excess of DTNB per sulphydryl group. 3 cm^3 aliquot of a 10 μmol (haem) dm^{-3} (5 μmol dm^{-3} reactive sulphydryl groups) haemoglobin solution at a particular pH was reacted with a calculated volume of 29.07 mmol dm^{-3} DTNB solution. Temperature was maintained at 25°C. To determine the effect of inositol-P_6, a 4:1 molar ratio of [inositol-P_6] : [Hb] was used. Each kinetic run was repeated at least three times under identical

experimental conditions. The progress of the reaction was recorded as kinetic traces (*absorbance vs time*) and its corresponding data on an external recording unit.

The reaction of cat haemoglobin with DTNB was carried out in a similar manner on a Cecil CE2501 BioQuest® UV-Visible spectrophotometer.

Results and discussion

The complete reaction of haemoglobin with 5,5′-dithiobis(2-nitrobenzoate) – DTNB – may be depicted as [11, 15]:

$$PSH + DTNB \underset{}{\overset{Q_{SH}}{\rightleftharpoons}} H^+ + PS^- + DTNB \underset{k_R}{\overset{k_p}{\rightleftharpoons}}$$
$$H^+ + PS.ST + TNB^- \overset{Q_{TNB}}{\rightleftharpoons} PS.ST + TNBH \quad \ldots . \text{(Eq. 1)}$$

In Eq 1, PSH is haemoglobin with the CysF9[93]β sulphydryl group in its protonated (unreacting form); PSÉ is its corresponding DTNB-reacting form; PS.ST is the mixed disulphide formed after the reaction of the sulphydryl group with DTNB; TNBÉ is 5-thio-2-nitrobenzoate, the anionic chromophoric product of the reaction; TNBH is obtained from the protonation of TNBÉ; the ionization constants of CysF9[93]β and TNBH are given as Q_{SH} and Q_{TNB} respectively; k_F is the apparent (forward) second order rate constant for the DTNB reaction step in which PS.ST is formed; and k_R is the corresponding reverse rate constant.

A typical kinetic trace for the reaction of DTNB with human oxyhaemoglobin at 412 nm is shown in Figure 1. Similar traces were obtained over the pH range 5.6 d≤ pH d≤ 9.0 for both stripped and inositol-bound human oxyhaemoglobins. Figure 2 is the corresponding trace for cat oxyhaemoglobins. The data were analysed with the SigmaPlot® Systat software. The analyses of the traces gave monophasic kinetics. This depicts that only two sulphydryl groups per tetramer are reactive towards DTNB in both human and cat haemoglobins respectively.

The corresponding pseudo-first order plot is shown in Figure 3. A straight line graph was obtained in each case throughout the experimental pH range for human haemoglobins. For cat minor oxyhaemoglobin, linear plots of k_{obs} were obtained over the range 5.6 ≤ pH ≥ 8.5. At pH ≥ 8.6, the kinetics appear to be no longer pseudo-first order for both stripped and inositol-bound cat haemoglobins. This is because the plots obtained over the range 8.6 ≤ pH ≤ 9.0 were non-linear. A typical example of such non-linear plot is shown in Figure 4. Similar results have been observed by Okonjo and Fodeke in 2006 [16]. The apparent forward second order rate constant, k_F, was determined from the least square slopes of plots of the pseudo-first order rate constant, k_{obs}, against the DTNB concentration [16].

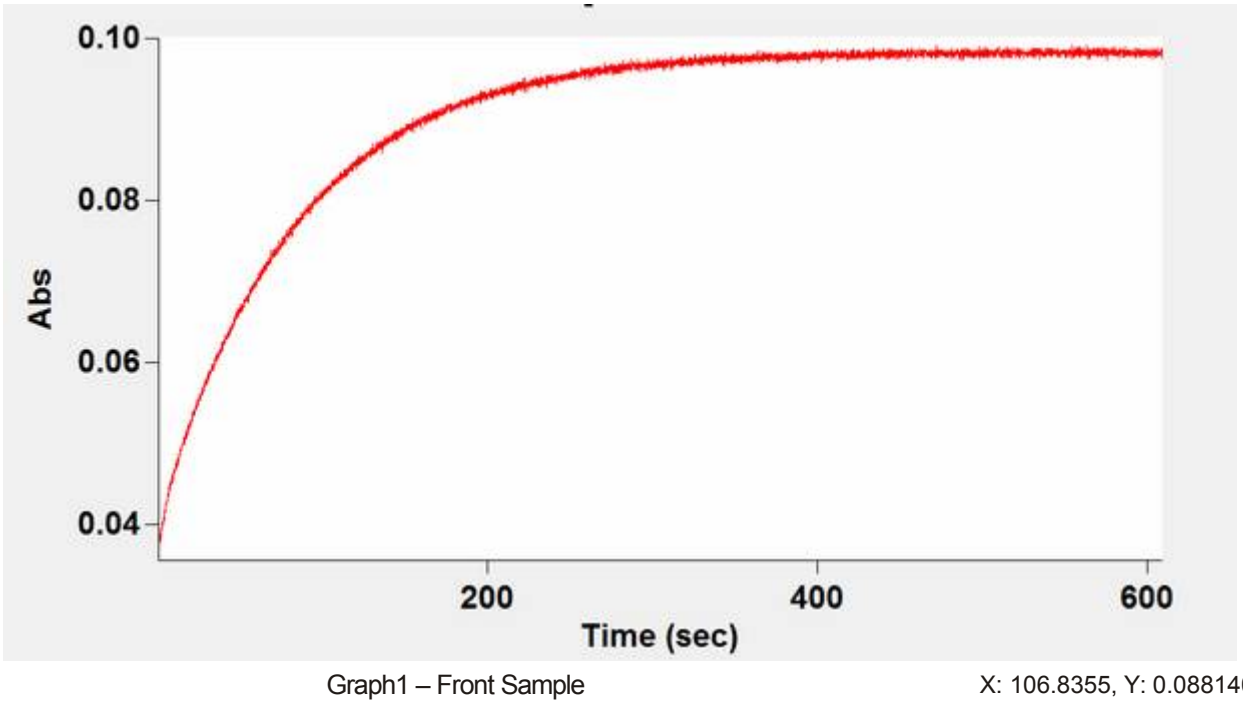


Figure 1. A kinetic trace for the reaction of 5,5'-dithiobis (2-nitrobenzoate) with the sulphydryl groups of **stripped human oxyhaemoglobin A** at 25°C and 412 nm: absorbance versus time.

Conditions: Phosphate buffer **pH 6.59** (ionic strength 50 mmol dm^{-3}, added salt, NaCl); haemoglobin concentration, 10 µmol (haem) dm^{-3} (5 µmol dm^{-3} in reactive sulphydryl groups); **[DTNB] = 300 µmol dm^{-3}**.

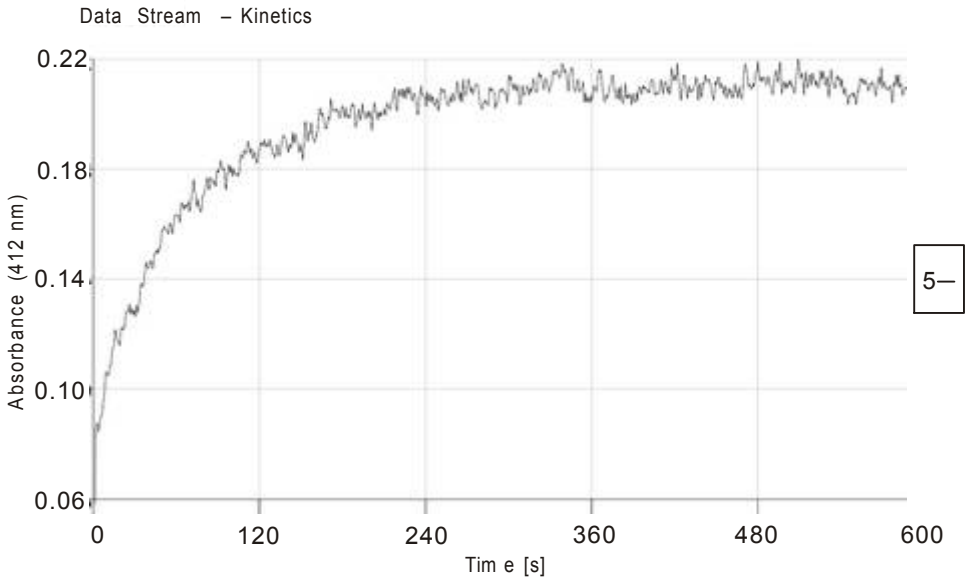
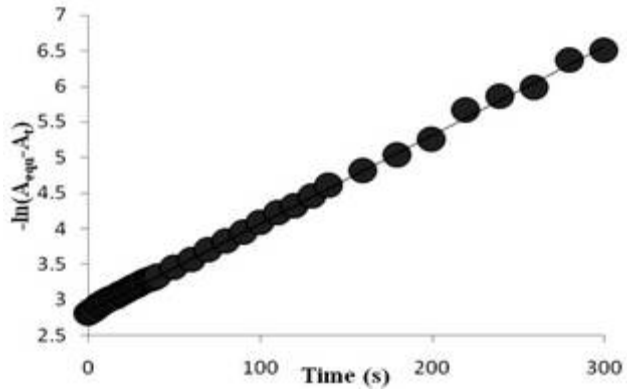


Figure 2. A kinetic trace for the reaction of 5,5'-dithiobis(2-nitrobenzoate) with the sulphydryl groups of **cat minor oxyhaemoglobin in the presence of inositol-P_6** at 25°C: absorbance versus time.

Conditions: Phosphate buffer **pH 7.12** (ionic strength 50 mmol dm^{-3}, added salt, NaCl); haemoglobin concentration, 10 µmol (haem) dm^{-3} (5 µmol dm^{-3} in reactive sulphydryl groups); **[DTNB] = 400 µmol dm^{-3}**; [inositol-P_6] =10 µmol dm^{-3}; observation wavelength, λ = 412 nm.

(a)



(b)

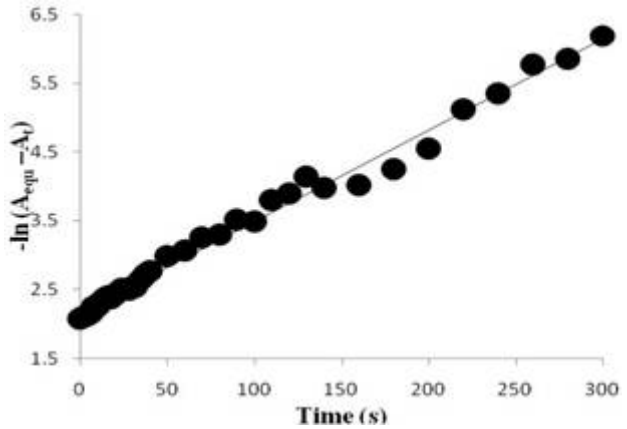


Figure 3. (a) Semi-logarithmic plot of the time course for the reaction 5,5'-dithiobis (2-nitrobenzoate) with **stripped human oxyhaemoglobin A** sulphydryl groups in phosphate buffer pH 6.59; k_{obs} = 12.0 (± 0.06) × 10^{-3} s^{-1}. The plot was linear for 5.2 half lives. **(b)** Semi-logarithmic plot of the time course for the reaction 5,5'-dithiobis(2-nitrobenzoate) with **cat minor oxyhaemoglobin** sulphydryl groups **in the presence of inositol-P_6**; phosphate buffer pH 7.12; [DTNB] = 400 µmol dm^{-3}; [inositol-P_6] = 10 µmol dm^{-3}; at 25°C; k_{obs} = 1.33 (± 0.04) × 10^{-2} s^{-1}. The plot was linear for 5.8 half lives.

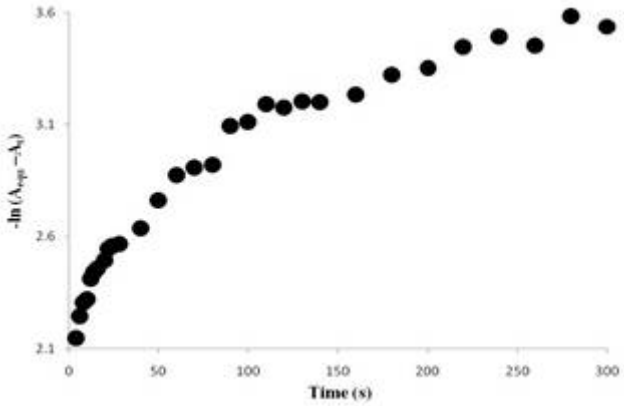
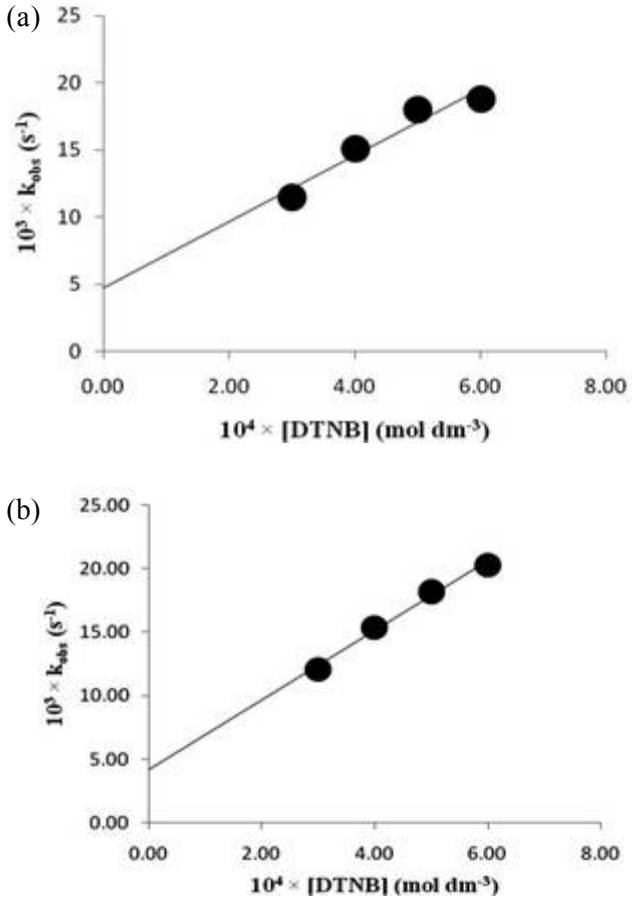


Figure 4: Semi-logarithmic plot of the time course for the reaction 5,5'-dithiobis(2-nitrobenzoate) with the **cat minor oxyhaemoglobin** sulphydryl groups **in the presence of inositol-P_6**; borate buffer **pH 8.60**; [DTNB] =300 µmol dm^{-3}; [inositol-P_6] = 10 µmol dm^{-3}.

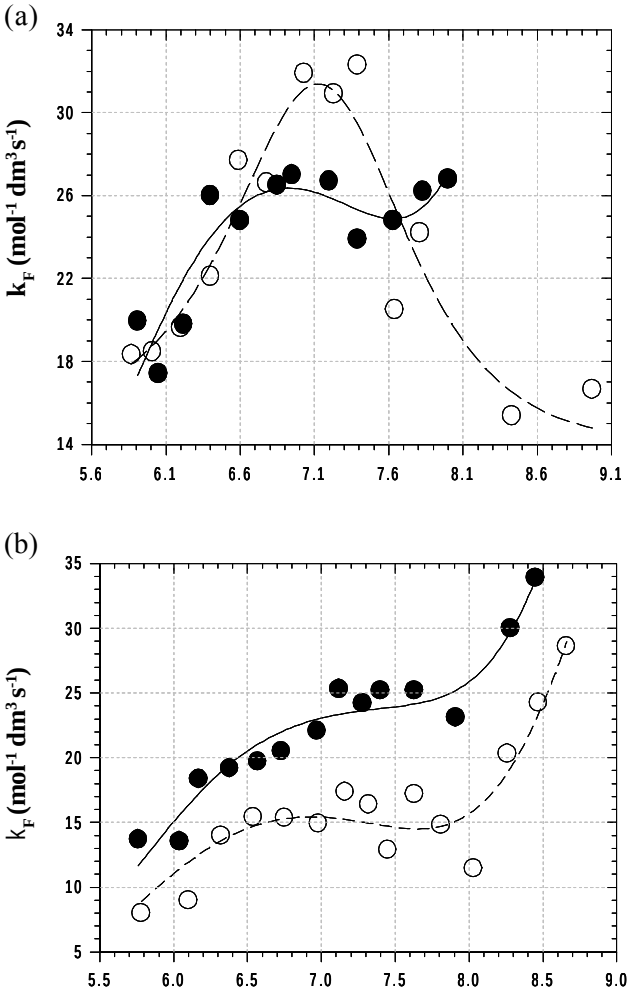
For both stripped and inositol-bound human oxyhaemoglobin A, plots of k_{obs} against the DTNB concentration were linear and had significant intercepts (Figure 5). This indicates that the reaction of DTNB with CysF9[93]β sulphydryl groups is a reversible process. While similar results were obtained at all pH values investigated for human haemoglobins, cat minor oxyhaemoglobin only gave similar results within the pH range $5.6 \leq pH \leq 8.5$.

The pH dependence profiles for stripped and inositol-bound human oxyhaemoglobin A and cat minor oxyhaemoglobin were compared in Figure 6. In human oxyhaemoglobin A, it is seen in Figure 6(a) that k_F is essentially the same between pH 5.8 and 6.8, implying that inositol-P_6 has no effect within this pH range. Between pH 6.9 and 7.5 however, the addition of inositol-P_6 significantly reduces k_F and hence, the reactivity. Since most experiments on sulphydryl reactivity were usually performed at the physiological pH (around pH 7.0), it was concluded that inositol-P_6 decreases the sulphydryl reactivity [6, 17-19]. Our results in Figure 5(a) indicate that this conclusion may not be valid at non-physiological pH values.



Figures 5.Typical plots for the dependence of the pseudo-first order rate constant, k_{obs}, on [DTNB] for the reaction of DTNB with CysF9[93]β of **(a) stripped human oxyhaemoglobin A (b) human oxyhaemoglobin A in the presence of inositol-P_6** at 25°C.

Conditions: Phosphate buffer **pH 6.59**; (ionic strength 50 mmol dm^{-3}; added salt, NaCl); haemoglobin concentration, 10 μmol (haem) dm^{-3} (5 μmol dm^{-3} in reactive sulphydryl groups). Each experimental point is the mean of at least three determinations.



Figures 6. Comparison of the pH-dependence profiles of k_F for the reaction of CysF9[93]β of **(a) human oxyhaemoglobin A (b)cat minor oxyhaemoglobin** with 5,52-dithiobis (2-nitrobenzoate): stripped haemoglobin (open circles); haemoglobin with inositol-P_6 (filled circles). Data for stripped cat haemoglobin obtained from Okonjo and Fodeke (2006).

For cat minor oxyhaemoglobin however, the kinetics gave a simple profile for the dependence of the apparent forward second order rate constant on pH with k_F increasing throughout the experimental pH range [Figure 6(b)]. In comparison to the stripped haemoglobin, k_F for the reaction in the presence of inositol-P_6 increases by a factor $\approx$ 2 between pH 5.76 and 8.45. This demonstrates that the reaction of CysF9[93]β of cat minor oxyhaemoglobin with DTNB in the presence of inositol-P_6 is faster than that of the stripped derivative. It also confirms the findings of Fodeke [20]. These results are indeed very interesting

because, to the best of our knowledge, previous works have reported the very opposite [6, 16, 19, 21].

Conclusion

Studies on the kinetics of the reaction of the CysF9[93]β sulphydryl group of some animal haemoglobins with DTNB have revealed that this reaction is reversible for stripped haemoglobin and inositol-bound haemoglobin: Plots of k_{obs}, the pseudo-first order rate constant, against the DTNB concentration were linear, with non-zero intercepts. In this current work, we have demonstrated that the reaction of DTNB with stripped human oxyhaemoglobin A is reversible. The plots have significant positive intercepts within the pH range 5.6-9.0. Addition of inositol-P_6 does not abolish the reversibility of the reaction.

Our results confirm that the reaction of cat minor oxyhaemoglobin with DTNB is faster in the presence of inositol-P_6 over the pH range for which pseudo-first order condition holds. This result is the reverse of what was obtained for human oxyhaemoglobin where the addition of inositol-P_6 reduced the reactivity especially within the physiological pH range. The increased reactivity of the cat haemoglobins in the presence of inositol-P_6 is most likely linked to mutations at their organic phosphate binding sites. In cat minor (and also the major) haemoglobin, HisNA2[2]β of human haemoglobin is substituted with PheNA2[2]β. In addition to this, the cat minor haemoglobin has a serine at the NA1[1]β position, and the terminal amino group of SerNA1[1]β is acetylated.

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