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EFFECT OF CONFORMATION AND SPIN STATE ON SULPHYDRYL REACTIVITIES OF HEMOGLOBINS

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ABSTRACT. The pH-dependence of the second-order rate constant for the reaction of 2.2[1] -dithiobispyridine with the CysF9 (93)β sulphydryl group of hemoglobin in the R quaternary structure in the absence and presence of sodium *n*-dodecyl sulphate (SDS) is analysed in terms of proposed and tested model. The reactivity of the CysF9 (93)β sulphydryl group of rabbit hemoglobin (oxy, met) is 2.5 times faster than that of human hemoglobin A (oxy, met) in the absence and presence of SDS. It reveals that the CysF9 (93)β sulphydryl group of rabbit hemoglobin is in a more hydrophobic environment than that of human hemoglobin A. For the human haemoglobin A and rabbit hemoglobin derivatives (oxy, met) data are better fitted by the simpler equation. From the analysis pK_a values 5.7 and 8.7 are obtained for the ionization of groups coupled to the presumed reaction. The pK_a of 5.7 is assigned to His HC3 (146)β; and pK_a is assigned to the CysF9 (93)β sulphydryl group in the absence of SDS. In the presence of SDS, the thiolate anion is the modulating factor. The combination of SDS with hemoglobin promotes a conformational change, but the change is apparently independent of spin state.

INTRODUCTION

The reactivity of the CysF9 (93)β sulphydryl group of hemoglobin has been employed as a monitor of tertiary and quaternary structure change [1-5]. Studies of the reactivity of this sulphydryl group as a function of pH have shown that two types of pH-dependence profiles are obtainable, 2,2[1] -dithiobispyridine (2-DTP) with namely: (1) a monoprotic acid and (2) a diprotic acid [6-8]. Such simple pH-dependence profiles are convenient for determining the nature and the number of amino acid residues that influence the reactivity of the CysF9 (93)β sulphydryl group [5, 8, 9]. These residues have been shown to be His HC3(146)β and the CysF9 (93)β sulphydryl itself, with pK_a values of *ca.* 6.0 and 8.5, respectively [5, 8, 9].

In this paper the question of coupling of spin state and conformation on the sulphydryl reactivities of hemoglobins in the absence and presence of sodium *n*-dodecyl sulphate (SDS) is examined with a spectrometric method.

MATERIALS AND METHODS

Blood from normal donors was obtained from the Blood Bank at the University College Hospital, Ibadan and Rabbits were purchased from Rabbitry Unit, Department of Animal Science, University of Ibadan, Ibadan. 2-DTP was purchased from Sigma and was used without any further treatment. SDS was purchased from British Drug Houses and use as supplied.

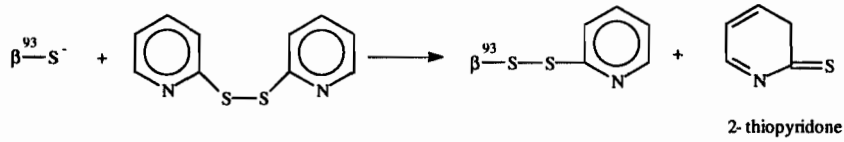
2-DTP solution was prepared by dissolving 22 mg of 2-DTP in 20% ethanol to make

0.01 M solution in a 10 mL volumetric flask [10]. Its concentration was also determined spectrophotometrically at 281 nm in phosphate buffer (pH 7.0, I = 0.2 M), using an absorptivity of 9.73 mM^{-1} cm^{-1} for 2-thiopyridone [11]. The pH of the solutions were determined on a Beckman-TM 50 pH/ISE meter.

2-DTP kinetics. The kinetics were studied on a Shimadzu computerized double beam UV-160 spectrophotometer at 25 °C. Solutions of hemoglobin (10 μM) were prepared in phosphate buffers (pH 5.6 to 8.0) and borate buffers (pH 8.0), each of total ionic strength 0.05 M.

The solutions were equilibrated at 25 °C. A 1 mL aliquot of each solution was pipetted into a 1 x 1 cm cuvette which was subsequently placed in the compartment of the spectrophotometer. A few microliters of 2-DTP of known concentration was measured with a Finn pipette into a glass rod shaped in a shallow spoon rod. The rod was used to add the 2-DTP solution and to stir the 2-DTP-hemoglobin mixture. The change in absorbance was followed at 343 nm for the reaction of hemoglobin with 2-DTP in the absence and presence of SDS. The colour produced is due to the formation of 2-thiopyridone. The same procedure was repeated for the reaction of 2-DTP with hemoglobin in the absence of SDS. Each kinetic run was repeated twice under identical experimental conditions.

In case of denaturation, each buffer solution contains 10 mM SDS which is the critical micelle concentration of SDS for proteins [12]. Hemoglobin solution (10 μM) was added to each buffer solution containing SDS. The hemoglobin solutions were allowed to equilibrate for one hour before employing the 2-DTP kinetic procedure. Each kinetic run was repeated twice under identical experimental conditions. The reaction of 2-DTP with hemoglobin may be represented as follows:



2- thiopyridone (TP)

TP concentration was determined according to Lambert-Beer's law:

$$A = \varepsilon.c.\ell \qquad (1)$$

where A is the absorbance, ε is the molar absorptivity for TP, c is the concentration of TP and ℓ is path length. After integration of second order equation we obtain the second order rate constant k (eqution 2):

$$k = \frac{1}{t(a-b)} \ln \frac{b(a-x)}{a(b-x)} \qquad (2)$$

where t is the time, a is the 2-DTP concentration, b is the hemoglobin concentration, and x is the TP concentration. The apparent second order rate constants, k_{app}, were calculated with the second order equation. The molar absorptivity of 2-thiopyridone previously determined was used. It was independent of pH and temperature [10].

RESULTS AND DISCUSSION

The titration of hemoglobin with 2-DTP is limited to pH < 9 due to the hydrolysis of the disulphide bonds [7]. It is known that above pH 9 complications arise in account of the increased rate of hydrolysis of disulphide bonds. This would result in uncertainty about the intergrity of 2-DTP results above this pH.

It is generally accepted that the reactions of sulphydryl groups of simple compounds and in proteins is via nucleophilic attack by the thiolate anion. It has been suggested that the reactivity of the sulphydryl group depends on two factor: the conformation of the charged ionizable groups on the proteins and the pK of the sulphydryl group [10].

Analysis of simple profiles in the absence of SDS. At an ionic strength of 50 mM the apparent second-order rate constant, k_{app}, for the reaction of 2-DTP with the Cys F9 (93)β sulphydryl group of the oxy, and methemoglobin derivatives of human hemoglobin A and rabbit hemoglobin varies with pH in a simple manner as shown in Figure 1. Figure 1 profiles resemble diprotic acid profiles. All the hemoglobin derivatives used in this study are in the R (relaxed) quaternary structure. We previously analysed simple profiles like those [5, 8, 9] shown in Figures 1 and 2 with the following equations:

$$k_{app} = k_1 \frac{K_1}{K_1 + [H^+]} + k_2 \frac{K_2}{K_2 + [H^+]} \qquad (3)$$

In this equation, k_1 is the limiting apparent second order rate constant at high pH for the 2-DTP reaction when the reactivity of the CysF9 (93)β sulphydryl group is linked to the ionization of His HC3 (146)β, with ionisation constant K_1; k_2 is the limiting apparent second order rate constant at high pH when the sulphydryl reactivity is linked to the ionisation of CysF9 (93)β with ionisation constant K_2.

If the sulphydryl group is the only modulating factor, equation 3 may be expressed in a simpler form as:

$$k_{app} = k_2 \frac{K_2}{K_2 + [H^+]} \qquad (4)$$

Figure 1 shows that both rabbit hemoglobin derivatives (oxy and met) react faster with 2-DTP then human haemoglobin A (oxy and met). 2-DTP is a neutral sulphydryl reagent which is sensitive to the pK of the sulphydryl group of the protein. Acid-alkaline methemoglobin transition exists in a methemoglobin. At low pH, the transition shifts to the aquo form while at high pH, it shifts to the hydroxymethemoglobin form. It implies that 2-DTP reacts faster with hydroxymethemoglobin than with aquohemoglobin as shown in Figure 1.

The velocity of combination of 2-DTP with rabbit hemoglobin derivatives is considerably higher than for the human hemoglobin A derivatives. Since 2-DTP is very reactive in an hydrophobic environment, it implies that CysF9 (93)β sulphydryl group of rabbit haemoglobin is in a more hydrophobic environment than that of human hemoglobin A. Rabbit hemoglobin has a higher net charge (per tetramer molecule) of +4 than human haemoglobin A [13] which confirms that rabbit hemoglobin has more non-polar amino acid residues than that of human hemoglobin A. The difference in

reactivity between rabbit hemoglobin and human hemoglobin A is about 2.5 fold. This magnitude of difference is typical of the hydrophobic environment of the CysF9 (93)β sulphydryl group of the hemoglobin.

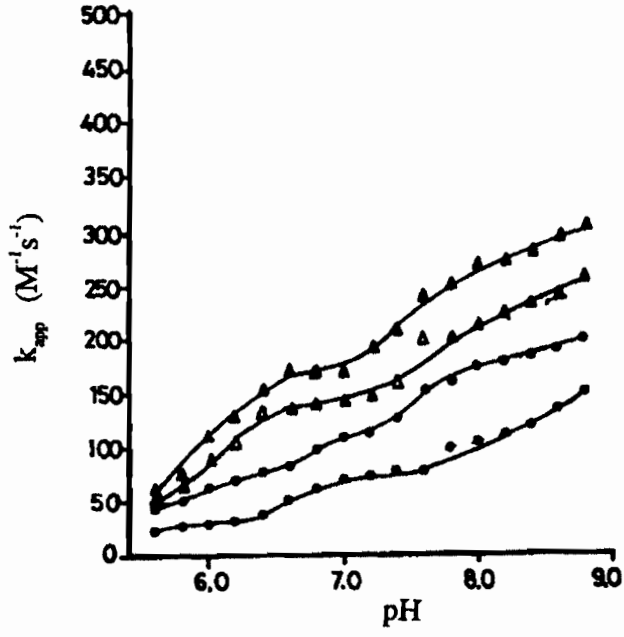


Figure 1. Dependence of k_{app} on pH for the reaction of 2-DTP with the CysF9 (93)β sulphydryl group of hemoglobin in the absence of SDS; (∘) oxyhemoglobin A; (•) aquomethemoglobin A; (△) rabbit oxyhemoglobin; and (▲) rabbit aquomethemoglobin. Conditions: phosphate buffers, $5.6 \leq$ pH ≤ 8.0; borate buffers, pH > 8.0; ionic strength, 0.05 M (added salt, NaCl); temperature, 25 ^{0}C; hemoglobin concentration, 10 μM heme. Each experimental point is the mean of three determinations and is subject to a standard error of *ca.* 5%. The lines through the standard points are theoretical best-fit lines drawn with the parameters reported in Table 1 using equation 3.

Figure 1 was analysed with equation 3 and 4. The lines through the data points in the Figure 1 are theoretical best fit lines calculated with equation 3 using the best fit parameters reported in Table 1. The pK_1 value is 5.8 ± 0.20 and pK_2 value is 8.5 ± 0.10. The pK_1 and pK_2 for such simple profiles were previously assigned to His HC3(146)β and CysF9(93)β of various hemoglobins [5, 8, 9], respectively and we, therefore, tentatively make the same assignments for human hemoglobin A and rabbit hemoglobin at 25 °C. Equation 4 gave poor fit. It shows that His HC3(146)β influences the sulphydryl reactivity of hemoglobin.

Figure 2 shows the pH-dependence studies of the reaction of 2-DTP with human hemoglobin A and rabbit hemoglobin derivatives (oxy and met) in the presence of SDS. It should be noted that SDS is an anionic surfactant that denatures protein and interacts

with positively charged groups of the protein [12] . In this case, SDS must have ruptured and broken the hydrogen bonds and salt bridges holding the α- and β-chains together thereby exposing the buried hydrophobic moeities to the solvent. 2-DTP reacts faster with the rabbit hemoglobin than with human hemoglobin A. The reason is simply that the CysF9 (93)β sulphydryl group of rabbit hemoglobin is in a more hydrophobic environment than that of human hemoglobin A. In addition, rabbit hemoglobin has a higher net charge (per tetramer molecule) of +4 than human hemoglobin A. Within the pH range of study, SDS will interact with the following cationic groups: ValNa1(1)β; His NA2 (2)β, His H21 (143)β and His HC3 (146)β. This cationic group is therefore also ionizable in the range of our experimental pH.

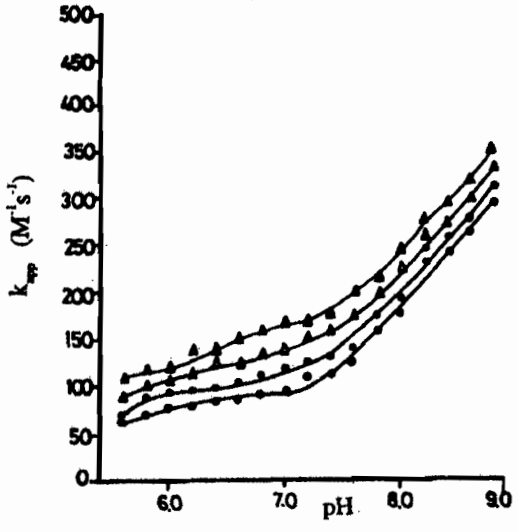


Figure 2. Dependence of k_{app} on pH for the reaction of 2-DTP with the Cys F9 (93)β sulphydryl group of hemoglobin in the presence of SDS; (∘) oxyhemoglobin A; (•) aquomethemoglobin A; (△) rabbit oxyhemoglobin; and (▲) rabbit aquomethemoglobin. Conditions: Phosphate buffers, $5.6 \leq pH \leq 8.0$; borate buffers, $pH > 8.0$; ionic strength, 0.05 M (added salt, NaCl); temperature, 25 °C; hemoglobin concentration, 10 μM heme; SDS micelle concentration, 10 mM. Each experimental point is the mean of three determinations and is subject to a standard error of *ca.* 5%. The lines through the experimental points are theoretical best-fit lines drawn with the parameters reported in Table 2 using equation 4.

Figure 2 was analysed with equations 3 and 4. The lines through the data points in the Figure 2 are theoretical best-fit lines calculated with equation 4 using the best-fit parameters reported in Table 2. The pK_2 value is 8.9 ± 0.10. It shows that the pK of the thiolate anion is the only modulating factor. Equation 3 gave poor fit.

Steric hinderance to access to the sulphydryl group. It is known that access to the CysF9 (93)β sulphydryl group of T-state (tense) hemoglobin is hindered by the formation of a salt bridge between His HC3 (146) β and AspFG1 (94) β [14]. There is a strong

experimental evidence that this salt bridge is also formed in R-state hemoglobin [15].

Table 1. Reaction of 2-DTP with the CysF9 (93) β sulphydryl group of hemoglobins in the absence of SDS.

Derivative	pK_1	pK_2	$k_1(M^{-1}s^{-1})$	$k_2(M^{-1}s^{-1})$
human oxyhemoglobin	5.6	8.5	45.01	140.30
human aquomethemoglobin A	5.7	8.5	70.25	175.50
rabbit oxyhemoglobin	5.9	8.7	90.40	230.40
rabbit aquomethemoglobin	6.0	8.6	110.70	275.20

Table 2. Reaction of 2-DTP with the CysF9 (93)β sulphydryl group of hemoglobins in the presence of SDS.

Derivative	pK_2	$k_2(M^{-1}s^{-1})$
human oxyhemoglobin A	8.8	250.40
human aquomethemoglobin A	8.8	275.00
rabbit oxyhemoglobin	8.9	295.30
rabbit aquomethemoglobin	9.0	305.45

Figure 3 and 4 show the pH-dependence studies of the reaction of 2-DTP with human hemoglobin A and rabbit hemoglobin in the absence and presence of SDS for oxyhemoglobin and methemoglobin, respectively. These results show that 2-DTP reacts faster with hemoglobin in the presence of SDS than with native hemoglobin. Native hemoglobin is sterically hindered by the presence of salt bridge between His HC 3 (146) β and Asp FG1 (94)β. 2-DTP is very sensitive to the pK of the thiol group, conformation and hydrophobic nature of the environment around the reactive sulphydryl group. Since 2-DTP is a neutral sulphydryl reagent, it would be expected that it will react faster in an hydrophobic environment. The reactive sulphydryl group CysF9 (93)β of hemoglobin in the presence of SDS is in a more hydrophobic environment than that in the absence of SDS. It suggest that our results are in perfect agreement that sulphydryl reactivities of hemoglobins in the absence and presence of SDS cannot be accounted for on the basis of a difference in steric hinderance to access to the sulphydryl group [16].

Difference in pK_a of the sulphydryl groups. Non-mercurial sulphydryl reagents react only with the thiol anion form of the sulphydryl group [10]. Consequently, the reactivity of a given hemoglobin with 2-DTP, a non-mercurial reagent, should depend on the pK_a of the sulphydryl group. Table 1 shows that for hemoglobin this pK_a is 8.6 ± 0.10 at an ionic strength of 50 mM in the absence of SDS. At an ionic strength of 50 mM this pK_a is 8.9 ± 0.10 in the presence of SDS. From our results (Figures 3 and 4), the pK_a values of the sulphydryl groups were responsible for the difference in the reactivities with 2-DTP. The higher the pK_a values the greater were the rates of sulphydryl reactivities of haemoglobin with 2-DTP.

Dissociation of hemoglobin tetramers to dimers. When hemoglobin tetramer dissociate to dimers the reactivity of the Cys F9(93)β sulphydryl increase [17]. It may therefore be argued that rabbit hemoglobin has a higher (2-DTP) sulphydryl reactivity than human hemoglobin A because it may be more dissociated to dimers.

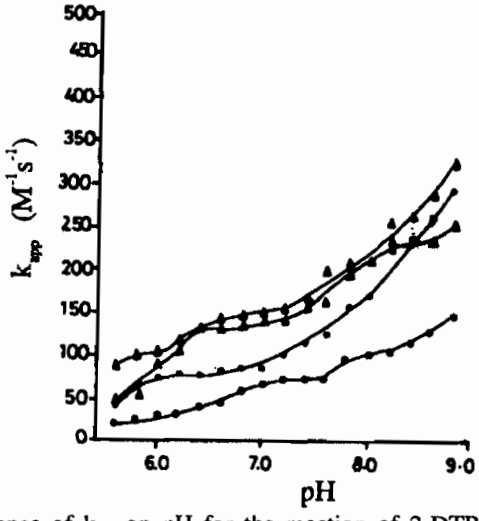


Figure 3. Dependence of k_{app} on pH for the reaction of 2-DTP with the CysF9(93)β sulphydryl group of oxyhemoglobin; (○) human oxyhemoglobin A without SDS; (●) human oxyhemoglobin A with SDS; (△) rabbit without SDS; and (▲) rabbit oxyhemoglobin with SDS.

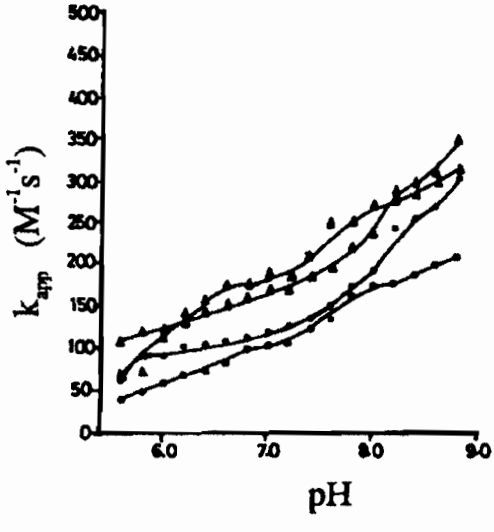


Figure 4. Dependence of k_{app} on pH for the reaction of 2-DTP with the Cys F9(93)β sulphydryl group of aquomethemoglobin; (○)human aquomethemoglobin A without SDS; (●) human aquomethemoglobin A with SDS; (△) rabbit aquomethemoglobin without SDS; and (▲) rabbit aquomethemoglobin with SDS.

At ionic strength of 50 mM, rabbit hemoglobin is more dissociated to dimers than human hemoglobin [18], and this results in the differences observed in the reaction rates (Figure 1). It is known that inositol-P_6 shifts the equilibrium between dimers and tetramers in favour of tetramers [18]. It has been reported that inositol-P_6 binds with the following amino acid residues: Val NA I (1)β, His NA 2 (2)β, Lys EF 6 (8,2)β, and His H21(143)β [18]. Inositol-P_6 is a negatively charged organic sulphate which is similar to SDS, a negatively charged surfactant. SDS is also expected to shift the tetramer-dimer equilibrium towards the tetramer state. If this assumption is valid, the results observed in Figures 3 and 4 show that in rabbit hemoglobin the rate of sulphydryl reactivities in the absence and presence of SDS within the pH range of 6.8 to 7.8 are approximately the same. We conclude that the change observed in not due to an increase in the population of tetramers. Human hemoglobin A or rabbit hemoglobin derivative (oxy and met) in the presence of SDS is 1.5 times faster with 2-DTP than with native hemoglobin throughout the experimental pH range (Figures 3 and 4).

Effect of high and low spin states. Extensive studies on methemoglobin have demonstrated the existence of iron in a mixture of high-spin and low-spin states in thermal equilibrium [19, 20]. X-ray crystallography has shown [21] that in methemoglobin, the high spin iron is out of the heme plane on the side of the proximal histidine while the low spin iron is within the heme plane.

A lowering of high spin content according to perutz *et al.* [22] implies a switch to the R conformational structure which has high binding affinity. From high to low spin transition, presupposes that the sulphydryl reactivities is very high (Figures 1 and 2). It is then likely that chemical modifications which contribute positive charges near the vicinity of the active sulphydryl lead to tertiary structure transitions which perturb the iron spin states equilibrium in favour of the low spin sate. This results into increase in sulphydryl reactivity of 2-DTP with hemoglobins (Figures 1 and 2). SDS causes little further increase in high spin character. SDS may influence the transition between aquo- and hydroxymethemoglobin at slightly alkaline pH, but this effect is at least partially non specific since the effect persists at ratios of SDS where binding is stoichiometric.

Oxyhemoglobin, which exists in the R-quaternary state, is low spin. In Figures 1 and 2, methemoglobin either in the absence or presence of SDS reacts 2.5 times faster with 2-DTP than that of oxyhemoglobin. If aquomethemoglobin-hydroxymethemoglobin transition shifts towards the low spin state at the high pH and oxyhemoglobin which exists in low spin states, it implies that high-low spin states transition in methemoglobin has no effect on the sulphydryl reactivities of hemoglobins with 2-DTP in the absence and presence of SDS.

We conclude that the combination of SDS with hemoglobin promotes a conformational change, but the change is apparently independent of spin state.

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