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Full Length Research Paper

The reactivity of CysF9[93]β sulphydryl group of des-HisHC3[146]β human haemoglobin A

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The pH dependence of the second order rate constant of the reaction of 5,5′-dithiobis(2-nitrobenzoate) (DTNB) with CysF9[93]β sulphydryl group of human haemoglobin A at 50 mmol dm^{-3} is complex. The removal of the terminal HisHC3[146]β of haemoglobin A by enzymatic cleavage with carboxypeptidase A breaks the salt bridge between HisHC3[146]β and AspFG1[94]β and reduces the strain on CysF9[93]β sulphydryl group. The pH dependence profiles of the second order rate constant for the reaction of DTNB with CysF9[93]β sulphydryl group of the modified haemoglobin A derivatives at 50 mmol dm^{-3} became simple with significant reduction in the reaction rates contrary to expectations. The implication is that CysF9[93]β becomes occluded and hence less reactive. The mean pK_as of the ionizable groups linked to the reactivity of CysF9[93]β sulphydryl group of des-HisHC3[146]β human haemoglobin A were 5.52 ± 0.01 and 8.29 ± 0.1. These values are assigned to HisH21[143]β and CysF9[93]β amino acid residues, respectively.

Key words: Haemoglobin, CysF9[93]β, carboxypeptidase A, 5,5′-dithiobis(2-nitrobenzoate), salt bridge.

INTRODUCTION

Haemoglobin is the most studied protein, yet it remains evergreen (Bettati et al., 2009) because of the lack of agreement on the mechanisms underlying the fine regulations of its structural and functional properties. The reactivity of CysF9[93]β sulphydryl group has been of used as indicator of tertiary and quaternary structure changes in haemoglobin in the past decades (Guidotti, 1965; Antonini and Brunori, 1969; Gibson, 1973; Perutz, et al., 1974; Hensley et al., 1975; Heidner et al., 1976; Baldwin, 1980; Shanaan, 1983; Okonjo et al., 2008, 2009; 2010). The reactivity of CysF9[93]β sulphydryl group has been shown to be affected by the amino sequence of the haemoglobin, increased ionic strength and the presence of allosteric effectors such as inositol hexakisphosphate (Okonjo et al.,1995, 1996, 2008, 2010).

The pH dependence of the apparent second order rate constant of the reaction of 5,5′-dithiobis (2-nitrobenzoate) (DTNB) with CysF9[93]β of human haemoglobin A at ionic strength of 50 mmol dm^{-3} gives a complex profile (Okonjo, et al., 1995; 1996). However, at an ionic strength of 200 mmol dm^{-3}, the complex profile becomes simple, resembling the titration curve of a diprotic acid.

This is because the electrostatic environment of the sulphydryl group has been screened off. A similar observation was obtained when organic phosphate, inositol hexakisphosphate, was added. This organic phosphate is known to bind to haemoglobin at the amino acid groups, ValNA1[1]β, HisNA2[2]β, LysEF6[82]β and HisH21[143]β, at the dyad axis. The change in profile observed in the presence of organic phosphate is believed to have been caused by the reduced exposure of CysF9[93]β to sulphydryl reagent induced by the organic phosphate. However, Bonaventura et al. (1972) had earlier reported that addition of inositol hexakisphosphate reverses the characteristics of normal haemoglobin, which were lost upon the removal of the HisHC3[146]β residues. HisHC3[146]β is known to contribute about 50% to the Bohr effect observed in human haemoglobin A (Perutz, et al., 1980). The cleavage of HisHC3[146]β is therefore expected to remove the major part of the ion pair effect and to ease the strain on AspFG1[94]β and subsequently

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its neighbor CysF9[93]β sulphydryl group.

The influence of the ion pair formation between HisHC3[146]β and AspFG1[94]β on the reactivity of CysF9[93]β sulphydryl group should be prominent since AspFG1[94]β is next to the CysF9[93]β in the amino acid sequence. Therefore, this study was aimed at investigating the effect of this ion pair on the reactivity of CysF9[93]β, since it is possible to remove the ion pair by enzymatic cleavage of the terminal HisHC3[146]β by carboxypeptidase-A (CPA) hydrolysis of haemoglobin A. It is expected that when the strain of the salt bridge is removed from CysF9[93]β, the amino acid will assume a relaxed condition, lower energy configuration and react faster with DTNB.

MATERIALS AND METHODS

Sample preparation

Blood sample of non-smoking healthy adult with homozygous haemoglobin A was obtained from the blood Bank. Haemoglobin was prepared from the blood as previously described by Okonjo et al. (1995). Enzymatic cleavage was carried out on the oxy haemoglobin A by sequential digestion according to the method of Bettati et al. (1997) using carboxypeptidase A (CPA). The samples purity was confirmed using the methods adopted by Bettati et al. (1997). Des-HisHC3[146]β haemoglobin samples were stored in the freezer and thawed when required. The various derivatives of haemoglobin were prepared as earlier described by Okonjo et al. (1995). Prior to use for experiments, each haemoglobin sample was passed through a Dintzis ion exchange column to remove endogenous compounds and undesired ions.

Kinetic experiment

The kinetics of the reaction of DTNB with des-HisHC3[146]β haemoglobin samples were monitored at 412 nm on a Shimadzu 1600PC double beam UV–Visible spectrophotometer interfaced to a computer and thermostated with a Lauda 30D table Cryostat. The reaction was carried out under a second order reaction conditions as described by Okonjo et al. (1995).

RESULTS AND DISCUSSION

Cysteine is the most reactive amino acid in haemoglobin. Human haemoglobin A has only one reactive sulphydryl group located at the position nine on the F helix of the β-chain, CysF9[93]β. The other sulphydryl group on position 104 of the α-chain is at the sub-unit interface, hence it is occluded and does not react. Cysteine only reacts in the thiolate form of DTNB. Its reactivity has been an indicator for both the tertiary and quaternary changes in haemoglobin.

The pH dependence profile of the apparent second order rate constant, k_{app}, for the reaction of des-HisHC3[146]β oxyhaemoglobin A with DTNB is shown in Figure 1. The profile is simple and resembles the titration curve of a diprotic acid. This means that there are two ionizable amino acids that are linked to the reactivity of CysF9[93]β. A comparison of the reaction of des-HisHC3[146]β HbA to that of normal haemoglobin A (inset of Figure 1) shows that the complex pH dependence profile obtained for normal haemoglobin A has been made simple for des-HisHC3[146]β. The rate of reaction was also drastically reduced. This implies that the salt-bridge between HisHC3[146]β and AspFG1[94]β, which has been removed by the enzymatic hydrolysis HbA to form des-HisHC3[146]β HbA has great implication on haemoglobin structure. This is not surprising because HisHC3[146]β has been reported to contribute about half of the Bohr effect in haemoglobin (Perutz et al., 1980).

In order to check the effect of ligand binding to the haem on the reactivity of des-HisHC3[146]β HbA, the pH dependence profile on k_{app} for the reaction of DTNB with des-HisHC3[146]β carbonmonoxyhaemoglobin A is reported in Figure 2. The profile is similar to that obtained for des-HisHC3[146]β oxyhaemoglobin A except that the rate was slightly higher. The increase in the rate compared with that of des-HisHC3[146]β oxyhaemoglobin is not surprising because the reaction of normal carbonmonoxyhaemoglobin with DTNB is also higher than that of oxyhaemoglobin. The inset in Figure 2 shows a comparison of the reaction of normal carbonmonoxyhaemoglobin A with DTNB to that of des-HisHC3[146]β carbonmonoxyhaemoglobin A. Apart from the change in the shape of the pH dependence profile, carbonmonoxyhaemoglobin A reacted between six and 20 times faster than des-HisHC3[146]β carbonmono-xyhaemoglobin A.

The pH dependence profile on k_{app} for the reaction DTNB with des-HisHC3[146]β aquomethaemoglobin A is reported in Figure 3. A similar profile to those of des-HisHC3[146]β oxy- and carbonmonoxyhaemoglobin A was obtained. This implies that the Fe^{3+} on the haem do not have any effect on the profile. The rate was only slightly higher than that of carbonmonoxy derivative. That is, the rate of the reaction of DTNB with the derivatives of des-HisHC3[146]β HbA was in the order; aquomet- > carbonmonoxy- > oxy-. The inset in Figure 3 shows the comparison with normal aquomethaemoglobin. The same explanations made for the oxy-derivative is also applicable. The effect of the removal HisHC3[146]β brought about a simplification of the reaction profile thereby removing the effect of the electrostatic environment linked to the reactivity of the CysF9[93]β sulphydryl group. The effect also led to reduction in the accessibility of the sulphydryl reagent to CysF9[93]β thereby leading to the drastic reduction in the reaction rate.

Analysis of pH dependence profiles

It has been suggested that the reactivity of sulphydryl group depends on the conformation of the sulphydryl group and the electrostatic effects of the charged

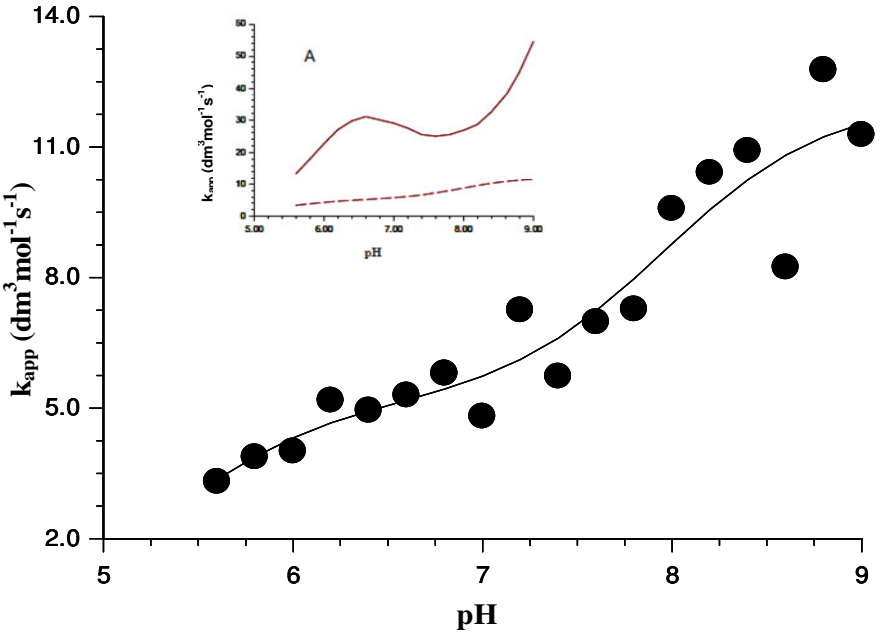

Figure 1. Dependence of k_{app} on pH for the reaction of DTNB with CysF9[93]β sulphydryl group of des-HisHC3[146]β oxyhaemoglobin A at 50 mmol dm^{-3} ionic strength. Inset is the comparison of the theoretical best fit line for normal human oxyhaemoglobin A to that of des-HisHC3[146]β oxyhaemoglobin A. Conditions: 10 µmol dm^{-3} haemoglobin concentration, (haem) (5 µmol dm^{-3} in reactive sulphydryl groups); 100 µmol dm^{-3} DTNB concentration; 5.6 ≥ pH ≥ 8.0 phosphate buffers; pH≥8 borate buffers; ionic strength made up with NaCl; temperature of 25°C.

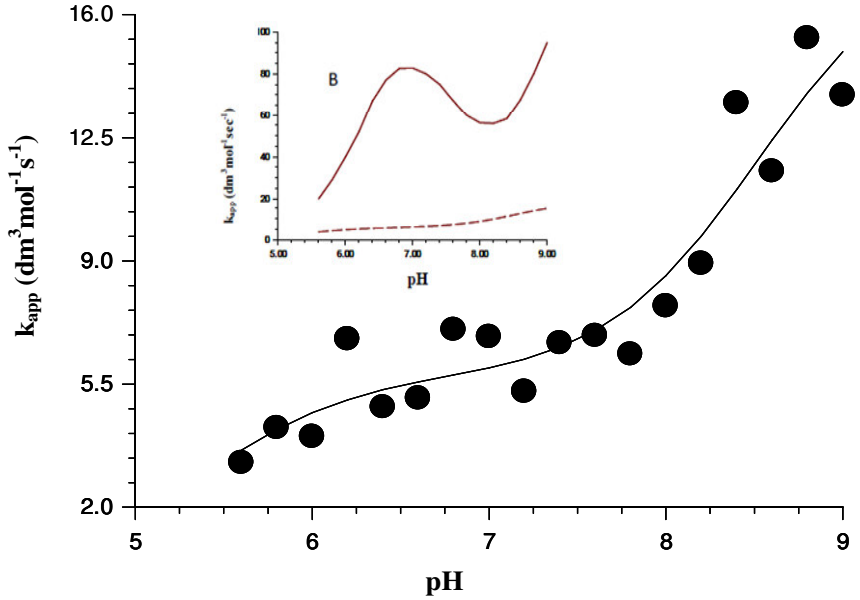

Figure 2. Dependence of K_{app} on pH for the reaction of DTNB with CysF9[93]β sulphydryl group of des-HisHC3[146]β carbonmonoxyhaemoglobin A at 50 mmoldm^{-3} ionic strength. Inset is the comparison of the theoretical best fit line for normal human carbonmonoxyhaemoglobin A to that of des-HisHC3[146]β carbonmonoxyhaemoglobin A. Conditions: 10 µmol dm^{-3} haemoglobin concentration, (haem) (5 µmol dm^{-3} in reactive sulphydryl groups); 100 µmol dm^{-3} DTNB concentration; 5.6 ≥ pH ≥ 8.0 phosphate buffers; pH≥8 borate buffers; ionic strength made up with NaCl; temperature of 25°C.

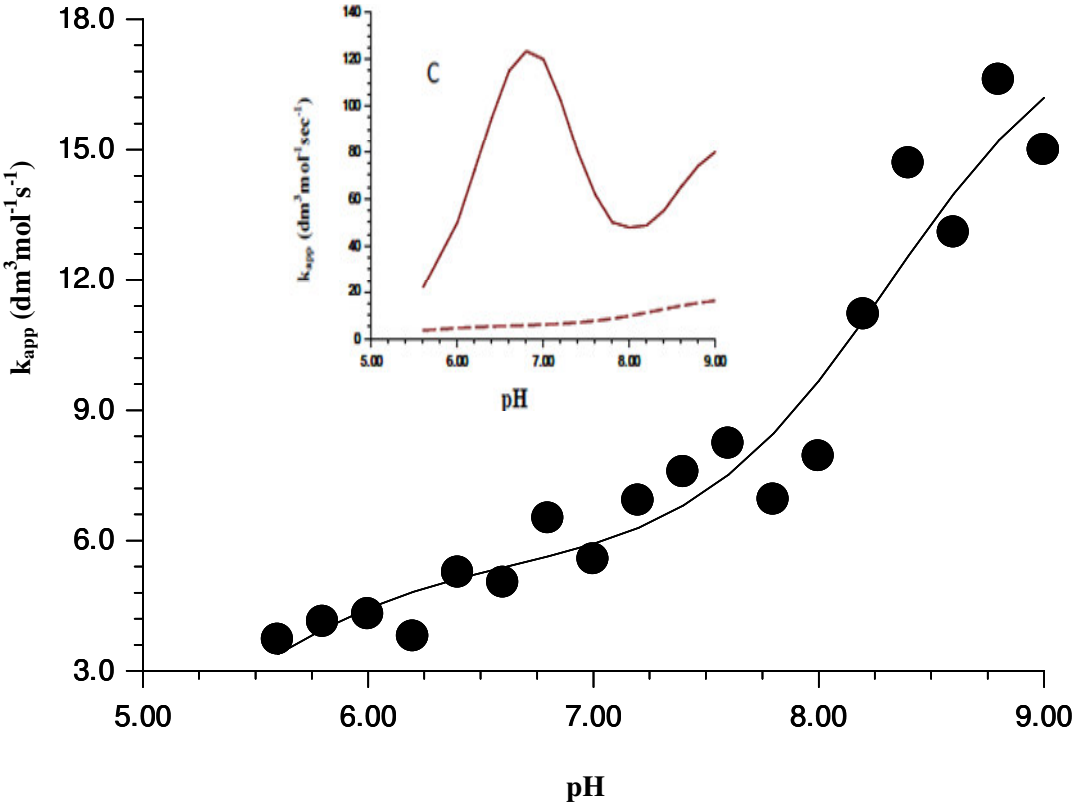

Figure 3. Dependence of k_{app} on pH for the reaction of DTNB with CysF9[93]β sulphydryl group of des-HisHC3[146]β aquomethaemoglobin A at 50 mmol dm^{-3} ionic strength. Inset is the comparison of the theoretical best fit line for normal human aquomethaemoglobin A to that of des-HisHC3[146]β aquomethaemoglobin A. Conditions: 10 µmol dm^{-3} haemoglobin concentration, (haem) (5 µmol dm^{-3} in reactive sulphydryl groups); 100 µmol dm^{-3} DTNB concentration; 5.6 ≥ pH ≥ 8.0 phosphate buffers; pH≥8 borate buffers; ionic strength made up with NaCl; temperature of 25°C.

ionizable groups on the protein, as well as the pK of the sulphydryl group. On the basis of these facts, Equation 1 can be tentatively considered for the analysis of the second order kinetic profile. Similarly, simple profiles like those in the Figures had been previously analysed using Equation 1.

$$k_{app} = k_1 \frac{Q_1}{Q_1 + [H^+]} + k_2 \frac{Q_2}{Q_2 + [H^+]} \quad (1)$$

In this Equation, k_1 is the limiting apparent second-order rate constant at high pH for the DTNB reaction when the reactivity of the CysF9(93)β sulphydryl group is linked to the ionization of HisH21(143)β, with ionization constant Q_1; k_2 is the limiting apparent second order rate constant at high pH when the sulphydryl reactivity is linked to the ionization of CysF9(93)β, with ionization constant Q_2. The analyses of the simple profiles in the Figure 1 with Equation (1) gave the best-fit parameters reported in Table 1. The mean pQ_1 and pQ_2 values are 5.52 ± 0.01 and 8.29 ± 0.1 respectively, which are lower than the mean values of 6.6 and 8.8 obtained for pQ_1 and pQ_2 for intact haemoglobin A (Okonjo et al., 1995). However the values can still be attributed to the same ionizable residues electrostatically linked to the reactivity of CysF9[93]β sulphydryl group.

The effect of removing C-terminal of the β-chains on pH dependence profile

Salt bridges play important roles in protein structure; these include oligomerization, molecular recognition and allosteric regulation (Perutz, 1970; Fersht, 1972). Contrary to the expectation that removal of salt bridge will increase the accessibility to sulphydryl reagent and subsequently an increased rate of reaction, it was observed that native HbA reacted faster than the des-HisHC3(146)β haemoglobin. The expectation is based on the fact that the salt bridge between HisHC3[146]β and AspFG1[94]β imposes strain on CysF9[93]β which is just next to AspFG1[94]β. The removal of the strain was expected to give CysF9[93]β freedom to react faster. However, at the removal of the ion pair between HisHC3[146]β and AspFG1[94]β, the pH dependence of

Table 1. Reaction of DTNB with CysF9[93]β sulphydryl group of des-HisHC3[146]β human haemoglobin A.

Parameter	k_1 ($dm^3mol^{-1}s^{-1}$)	k_2 ($dm^3mol^{-1}s^{-1}$)	pQ_1	pQ_2
Oxy	5.56	6.76	5.55	8.06
Carbon monoxy	5.95	11.71	5.57	8.51
Aquomet	5.61	12.61	5.53	8.31
Mean			5.52 ± 0.01	8.29 ± 0.1

K_{app} of the reaction of haemoglobin A with DTNB became simplified and the rate of reaction decreased in all the pH range by more than half in all the haemoglobin derivatives. The implication is that the strain on CysF9[93]β was actually responsible for its exposure to sulphydryl reagents and the breakage of the salt bridge occluded it. The change in conformation of CysF9[93]β made it to lose the electrostatic environment that gave it a complex pH dependence profile. By implication, half of the Bohr effect of haemoglobin is related to complex profile and the high rate of reaction of CysF9[93]β. This has further confirmed the fact that CysF9[93]β is an indicator of tertiary and quaternary changes in haemoglobin

Conclusion

The removal of HisHC3[146]β - AspFG1[94] ion pairs of haemoglobin A is of structural consequence. CysF9[93]β sulphydryl group, rather than been exposed to the sulphydryl reagent, recoiled inward thereby reducing the accessibility of the reagent. The resultant effect is obvious in the reduced rate of reaction and simple profile obtained for the pH dependence of the apparent second order rate constant.

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