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EFFECT OF A3[6]B$^{GLU \rightarrow LYS}$ MUTATION ON REACTIVITY OF THE CYSF9[93]B SULPHYDRYL GROUP OF HUMAN HAEMOGLOBIN C

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ABSTRACT

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 the oxy and carbonmonoxy derivatives of stripped haemoglobin C are complex while that of aquomet haemoglobins resembles the titration curve of a diprotic acid. However, in the presence of inositol hexakisphosphate, the profiles of oxy and carbomonoxy derivatives become simple while that of aquomet becomes bowl shaped. Increased ionic strength also simplified the complex profile. The pQs of the ionizable groups linked to the reactivity of DTNB with CysF9[93]β sulphydryl group range between 5.6 and 8.7 in stripped haemoglobin. The presence of inositol-P_6 decreases the pQ values to the range of 4.3 and 8.4. When compared with haemoglobins A and S, the reaction rate of heamoglobin C is lower than haemoglobin A but faster than haemoglobin S, implying that the net charge on the molecule has no direct relationship with the reaction rates.

Keywords: Haemoglobin C, Sulphyldryl group, Ionizable groups; Inositol-P_6; Ionic strength.

INTRODUCTION

Human haemoglobins A and C have identical α subunits, but differ from each other by one out of 146 amino acid residues on each of the β subunits. In haemoglobin A, the position A3[6]β is occupied by a negatively charged glutamic acid residue, while it is occupied by a positively charged lysine (an amino acid with relatively long side chain) residue in haemoglobin C. This mutation on the surface of haemoglobin molecule seems to be minor but it is of significant clinical consequence in individual with homozygous haemoglobin C (Hirsch *et al*., 1985).

The reactivity of CysF9[93]β sulphydryl group of hemoglobin has been employed as an indicator of both the tertiary and quaternary structural changes in relation to its immediate neighbourhood (Guidotti, 1965; Antonini and Brunori, 1969; Hensley *et al*., 1975; Okonjo *et al*., 2010). 5,5□-dithiobis(2-nitrobenzoate) (DTNB) is the most used sulphydryl reagents for this purpose because of its sensitivity to the electrostatic environment of haemoglobin and its stability at room temperature (Okonjo *et al*., 1996).

The reactivity DTNB with haemoglobin sulphydryl group has been used in determining tertiary transitions within haemoglobin (Okonjo *et al.,* 2008, 2010), tetramer dimer dissociation constants (Babalola *et al.*, 2005), Bohr effect (Babalola *et al.*, 2005) and the state of salt bridges within haemoglobin molecule (Okonjo and Nwozo, 1997; Babalola and Nwozo, 2002). Okonjo *et al.* (1995, 1996) has employed the sensitivity of DTNB to monitor the electrostatic environment of CysF9[93]β sulphydryl of human haemoglobins A and S. At 50 mmoldm^3 ionic strength, the pH dependence profiles of the second order rate constant are complex. The addition of inositolhexakisphosphate (inositol-P_6) simplified the profiles, reduced the second order rate constants and increased the pK_as of the ionizable groups linked to the reactivity of CysF9[93]β sulphydryl group. Increased ionic strength also simplified the profiles (Okonjo *et al.,* 1995, 1996).

Okonjo *et al.* (1996) had shown the effect of A3[6]β$^{glu \rightarrow val}$ mutation on the reactivity of CysF9[93]β sulphydryl group of human haemoglobin S compared to that of human haemoglobin A. The reactivity of CysF9[93]β sulphydryl group of human haemoglobin A is faster than that of haemoglobin S. This is contrary to expectation because the net charge on human haemoglobin S is more positive than that of haemoglobin A. Haemoglobin S was therefore expected to be more reactive towards the negatively charged DTNB. It would therefore be interesting to know what the reaction of negatively charged DTNB will be towards haemoglobin C which is known to have a greater net positive charge than both haemoglobins S and A. This is also special because the lysine which replaces valine in haemoglobin S or

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glutamic acid in haemoglobin A has a long side chain with a positively charged amino group end. Therefore, this study is aimed at comparing the effect of A3[6]$\beta^{glu \rightarrow lys}$ mutation on the reactivity of CysF9[93]β sulphydryl of human haemoglobin C with DTNB and those of human haemoglobins A and S.

MATERIALS AND METHODS

Haemoglobin Preparation

Fresh blood containing homozygous haemoglobin C was obtained from the Haematology Clinic of the University College Hospital, Ibadan into heparinized tubes. Haemoglobin was prepared from these blood samples using standard methods as previously described by Okonjo *et al.* (2008). Low molecular weight impurities contained in the haemoglobin were removed by dialysis using polyvinyl chloride dialysis tubing and subsequently deionized by passage through a Dintzis ion-exchange column (Dintzis, 1952).

Kinetic Experiment

The kinetics of the reaction of DTNB with haemoglobin C was monitored at 412nm on a Zeiss PMQ II UV-VIS spectrophotometer thermostatted with a Lauda 30D table Kryostat. The reactions were carried out in triplicate at 20^{o}C in phosphate (pH 5.6 to 8.0) and borate (pH 8.2 to 9.0) buffers. 10 cm^3 of haemoglobin with concentration 10μM haem (5μM in reactive sulphydryl group) was pipetted into the 2cm path length cuvette, the reaction was initiated by adding a predetermined volume of DTNB with stirring. The same conditions were used in the presence of inositol-P_6 except that 10 μmoldm^3 of inositol-P_6 was added. To determine the influence of high ionic strength, NaCl was added to the buffer to adjust the ionic strength to 200 mmoldm^3. The second-order rate constants were calculated from its rate equation after converting the transmittance reading to absorbance. The kinetic data were fitted using micromath scientist software package (Salt Lake City, Utah, USA) using the equation derived from the scheme of the reaction.

RESULTS AND DISCUSSION

The reaction of human haemoglobin C with DTNB showed that only CysF9[93]β is reactive just like we have haemoglobins A and S (Okonjo *et al.*, 1996) the change in absorbance with time is exponential across the pH ranges of the experiments.

Nature of the pH dependent profile of DTNB reaction with CysF9[93]β sulphydryl group of haemoglobin C

Ionic strength of 50 mmoldm^{-3}: Figures 1a, 1b, and 1c show the pH dependence profiles of the second order rate constants for reaction of DTNB with CysF9[93]β sulphydryl group of oxy, carbonmonoxy and aquomet derivatives of haemoglobin C respectively at ionic strength of 50 mM. The profiles for oxy (Fig. 1a) and carbonmoxy (Fig. 1b) have a peak each at around pH 7.2. The rate of reaction of oxy haemoglobin A and C are similar between pH 5 and 8 after which haemoglobin A reacts faster. The reaction of oxyhaemoglobin C is faster than that of S throughout the pH range of the experiment. In the case of the reaction of DTNB with carbonmonoxy haemoglobins the profiles are similar but with different rates. Haemoglobin A is at least about three times faster than haemoglobin C while that of haemoglobin S is lower. The profile of pH dependence of second order rate constant for aquomet derivative of haemoglobin C gave a simple profile resembling the titration curve of a diprotic acid (Fig. 1c). This is completely different from the profiles earlier obtained for haemoglobins A and S (Okonjo *et al.,* 1995, 1996). This is an indication that the electrostatic environment of CysF9[93]β is completely different in aquomet haemoglobin C compared to haemoglobins A and S. It can therefore be assumed that there is interaction between the iron III on the haem, LysA3[6]β and CysF9[93]β of haemoglobin C.

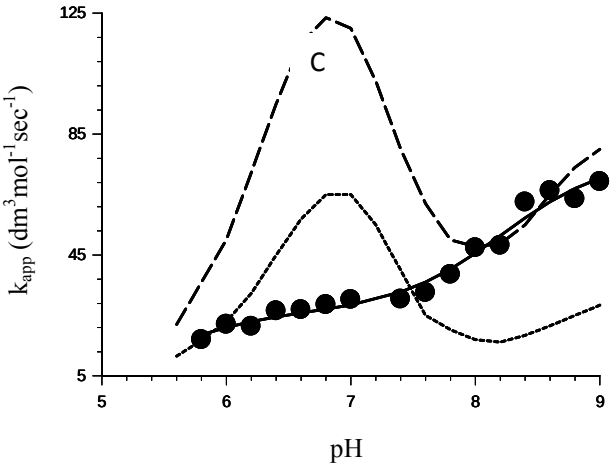
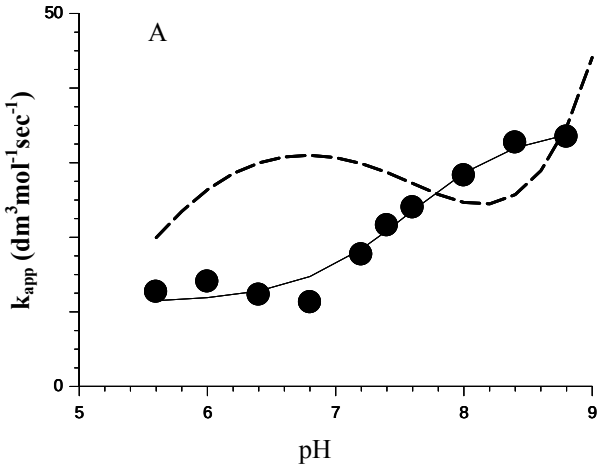


Fig. 1. Dependence of k_{app} on pH for the reaction of DTNB with CysF9[93]β sulphydryl group of haemoglobin C stripped of organic phosphate (filled circle). (a) oxy hemoglobin (b) carbonmonoxy hemoglobin (c) aquomet hemoglobin. The lines through the data points are the theoretical best-fit lines calculated with the parameters given in table 1. For comparison with the theoretical best fit lines for hemoglobin A (long dashes-lines) and S (short dashed-lines) drawn in full lines.

Naturally, the expectation would be that the reaction of DTNB with various haemoglobin would be in this order Haemoglobins C > S > A (according to their net positive charge. However this is not so except for aquomet haemoglobin between pHs 5.8 and 7.5. Going by the result obtained by Okonjo *et al.* (1996) that shows that haemoglobin A reacts faster than haemoglobin S with DTNB, one would expect that haemoglobin S would be faster than haemoglobin C but this is not so. This is an

indication that the net charge on the haemoglobin does not play a specific role on the overall rate of reaction of DTNB. The charges that would be probably important would be those of the amino acid residue that are electrostatically linked with CysF9[93]β sulphydryl group.

Ionic strength of 50 mmoldm^{-3} plus inositol –P$_6$: In the presence of organic phosphate, inositol-P_6, the pH dependence profile of k_{app} for haemoglobin C changes drastically in all the derivatives. The complex profile of oxy and carbonmonoxy haemoglobin C in the absence of organic phosphate became simple on addition of organic phosphate in figures 2a and 2b, respectively. The simple profiles resemble the titration curve of a diprotic acid, this result is similar to what was obtained when organic phosphate was added to haemoglobins A and S. This confirms that the binding of organic phosphate to ValNA1[1]β, HisNA2[2]β LysEF6[82]β and HisH21[143]β changes the conformation of CysF9[93]β, thereby changing its electrostatic environment and making it occluded below pH 7.8 for oxy haemoglobin C and between pH 6.4 and 8.2 for carbonmonoxy haemoglobin C. The most striking result in the reaction of DTNB with aquomet haemoglobin C in the presence of inositol-P_6 is the bowl shaped profile obtained for the pH dependence of $k_{app.}$ Such a shape has not been reported for CysF9[93]β sulphydryl group to the best of our knowledge. The presence of inositol-P_6 increases the rate of reaction in aquomet derivative in the pH range of this study. This is an indication the binding of organic phosphate to ValNA1[1]β, HisNA2[2]β LysEF6[82]β and HisH21[143]β change the conformation of the molecule. In most cases organic phosphate lowered the rate of reaction of DTNB with CysF9[93]β sulphydryl group (Okonjo *et al.,* 1995, 1996), the increased rates observed here could be attributed to the interaction of the side chain of LysA3[6]β, the haem and CysF9[93]β sulphydryl group.



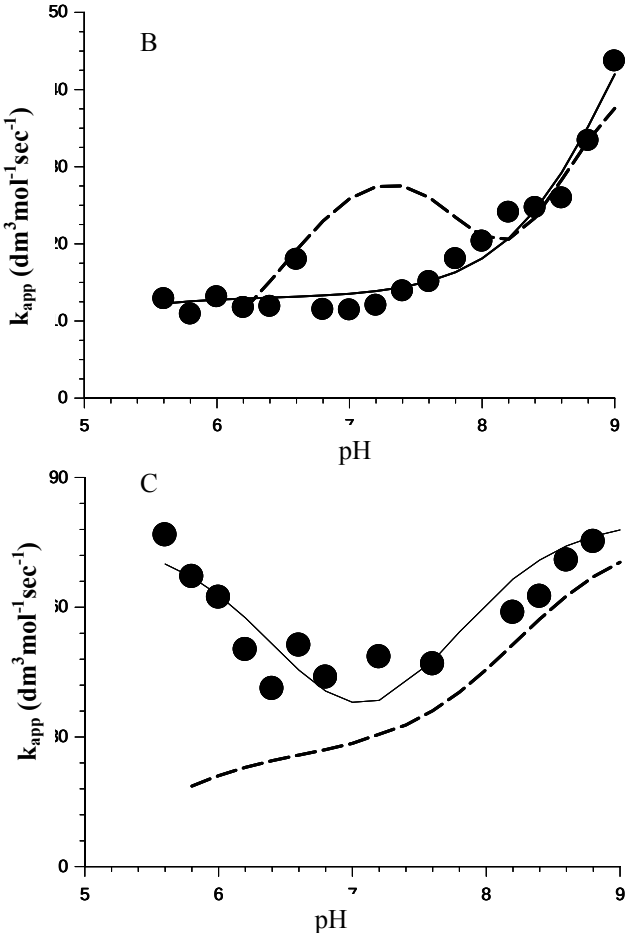


Fig. 2. Dependence of k_{app} on pH for the reaction of DTNB with CysF9[93]β sulphydryl group of haemoglobin C in the presence of organic phosphate (solid line). (a) oxy hemoglobin (b) carbonmonoxy hemoglobin (c) aquomet hemoglobin. For comparison with the theoretical best fit lines for stripped hemoglobin C (long dashed-lines).

Ionic strength of 200 mmoldm^{-3}: Haemoglobin tetramer dissociates to dimers under the influence of increasing ionic strength (Okonjo *et al.*, 1996), to this end a more stable derivative of haemoglobin (carbonmonoxy haemoglobin) was used to investigate the effect of increased ionic strength. The complex profile obtained at 50 moldm^{-3} NaCl become simplified when the ionic strength was increased to 200 moldm^{-3} (Fig. 3), this is in agreement with the earlier observations made for haemoglobins A and S (Okonjo *et al.*, 1996). The simplification is due to the fact that the added salt screened off the electrostatic environment and exposed CysF9[93]β sulphydryl group. When compared, the rates of reactions in the presence of 200 moldm^{-3} NaCl were higher than in the presence of organic phosphate, as expected this is because the increased ionic strength increased the proportion of the dimers which are known to react faster (Babalola *et al.,* 2005).

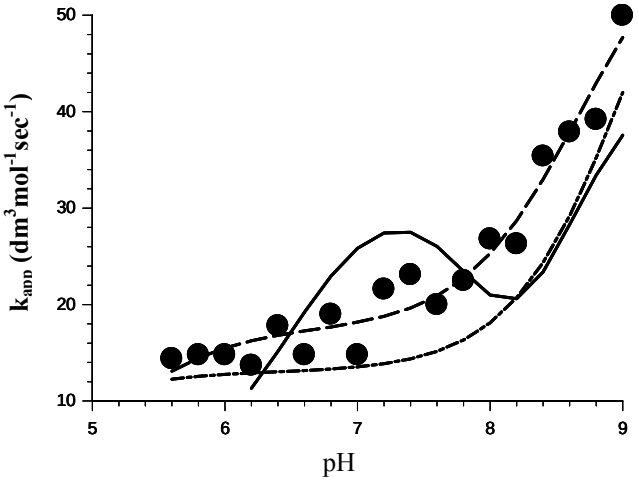


Fig. 3. Dependence of k_{app} on pH for the reaction of DTNB with CysF9[93]β sulphydryl group of carbonmonoxy haemoglobin C at 200 mM ionic strength. Full-line represents the best fit line for similar reaction with stripped haemoglobin at 50 mM while dotted-line represents the best fit line for the reaction in the presence of 10 μmoldm^{-3} inositol hexakisphosphate.

Analysis of pH dependence profiles

It has been suggested that the reactivity of sulphydryl group depends on its conformation and its electrostatic environment, particularly the number and nature of ionizable groups electrostatically linked to it (Okonjo *et al*., 1996). These factors determine the nature and shape of the pH dependence of $k_{app.}$

Simple profile:

It is known that in haemoglobin only the thiol anion reacts with DTNB (Robyt *et al.,* 1971; Hallaway *et al.,* 1980) for this reason we previously accounted for the profile similar to those reported for haemoglobin C in Figures 1c, 2a, 2b and 3 in term of the fraction of thiol anion form of sulphydryl (Okonjo *et al.,* 1995, 1996, 1997). Moreover, the salt bridge is formed in R state haemoglobin between HisHC3[146]β and AspFG1[94]β and this salt bridge hinders access to CysF9[93]β sulphydryl group. These considerations give rise to the two term equation 1 below (Okonjo and Aboluwoye, 1992):

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

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 HisHC3(146)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 this study with equation (1) gave the best-fit parameters for oxy and carbonmonoxy in Table 1 and aquomet haemoglobin in Table 2 (the profile that resembles the titration curve of a diprotic acid). The mean pQ_1 and pQ_2 values are 4.33 ± 0.3 and 8.36 ± 0.7 respectively, these values are lower than the mean values of 6.6 and 8.8 obtained for pQ_1 and pQ_2 for both haemoglobins A and S (Okonjo *et al.*, 1995, 1996). However the values can still be attributed to the same ionizable residues electrostatically linked to the reactivity of CysF9[93]β sulphydryl group i.e. the pQ_1 of 4.33 is assigned to the HisHC3[143]β. The value pQ_1 value for Histidine should be around 6.0, but the presence of the positively charged LysA3[6]β (pQ_1 around 10.8) caused a repulsion that reduced the pQ_1 value of HisHC3[143]β. The 8.36 value obtained for pQ_2 is assigned to CysF9[93]β which normally has a pQ value of 8.3. The pQ_1 and pQ_2 values of 5.6 and 8.2 obtained for aquomet derivatives could not be compared with those obtained for haemoglobins A and S because of the difference in their profiles, however the value of pQ_2 can be assigned to the CysF9[93]β while the pQ_1 value of 5.6 is assigned to HisHC3[143]β. The value of pQ_2 is similar to what is expected for cysteine indicating that in aquomet haemoglobin in the absence of organic phosphate the LysA3[6]β do not affect its pQ_2 value while it increased that of pQ_1, this implies that the orientation of the positively charged lysine is towards the histidine group rather than the cysteine or the haem.

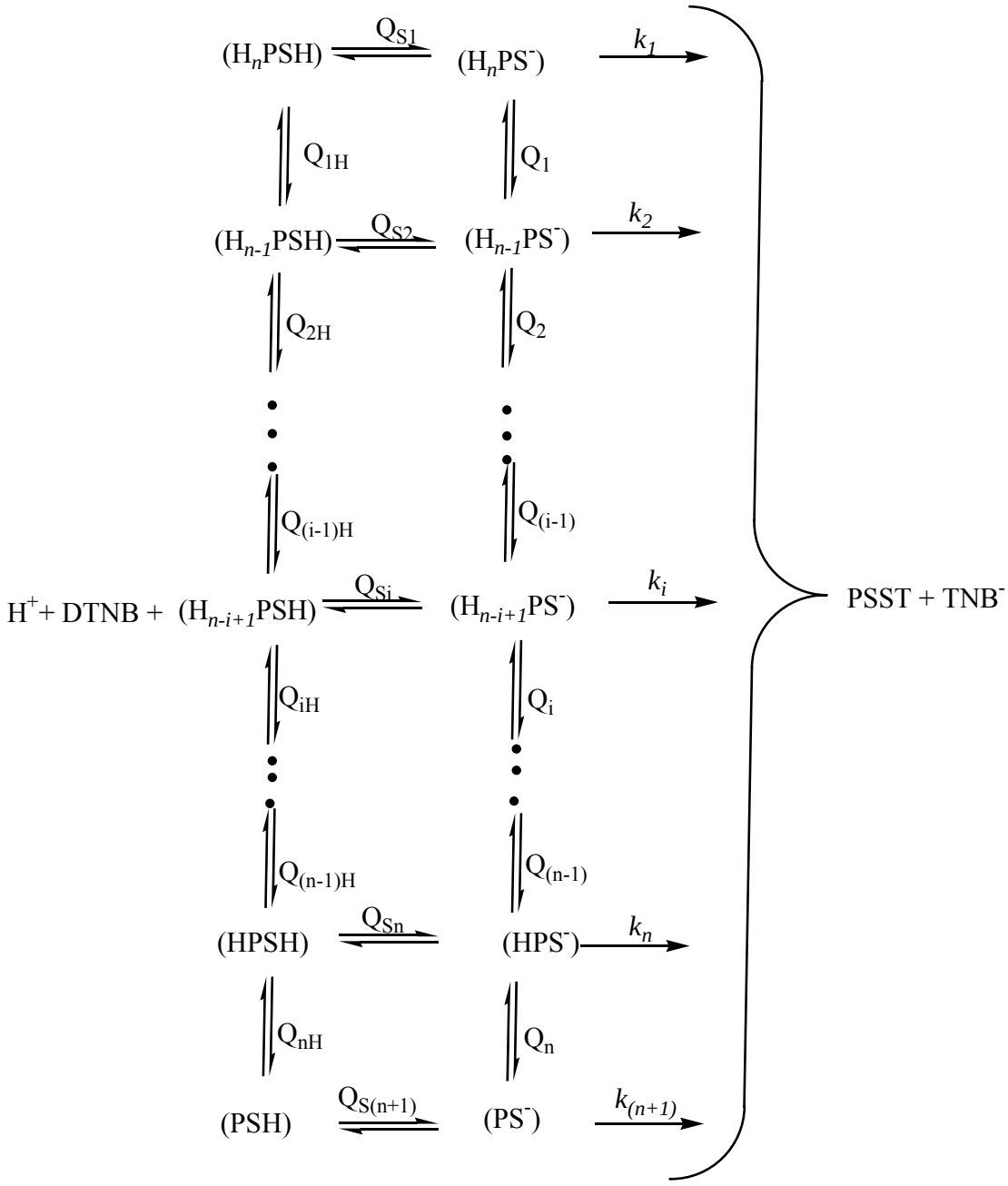
Bowl shaped profile:

Bowl shaped profile can be theoretically accounted for by assuming that there is an ionizable cationic group close to the sulphydryl group. The implication is that at low pH the cationic group is positively charged and the reaction with the negatively charged DTNB is fast. Bowl shaped profiles were previously analysed with equation 2 below:

$$k_{app} = k_1 \frac{[H^+]}{Q_1 + [H^+]} + k_2 \frac{Q_2}{Q_2 + [H^+]} \qquad 2$$

The terms in the equation 2 are similar except that the first fractional term is the fractional population of the cationic form of this group. The analyses of the bowl shaped profiles for aquomet haemoglobin C in the presence of inositol-P_6 figure 2c with equation (2) gave the best-fit parameters reported in table 1. This clearly explained that the binding of inositol-P_6 brings the

LysA3[6] closer to the sulphydryl as provided by the equation and the observed increase rate. The pQ_1 and pQ_2 values of 6.6 and 7.6 obtained for aquomet derivatives could not be compared with those obtained for haemoglobins A and S because of the difference in their profiles, however the value of pQ_1 can be assigned to the HisHC3[143]β while the pQ_2 value of 7.6 is assigned to CysF9[93]β. The value of pQ_1 is similar to what is



Scheme I

expected for Histidine indicating that in aquomet haemoglobin in the presence of organic phosphate the lysA3[6]β do not affect its pQ_1 while it reduce that of pQ_2 this implies that the orientation of the positively charged lysine is towards the sulphydryl group rather than the histidine or the haem and this clearly explain why the electrostatic environment of CysF9[93]β is affected and the profile is bowl shaped unlike those of oxy and carbonmonoxy.

Complex profile

However, the complex profiles in the figures 1a and b were analysed using equation 3 derived from scheme I.

Table 1. Reaction of DTNB with CysF9[93]β sulphydryl group of haemoglobin C in the presence of inositol-P_6

	$k_1(dm^3mol^{-1}s^{-1})$	$k_2(dm^3mol^{-1}s^{-1})$	pQ_1	pQ_2
Oxy	11.70	23.97	3.98	7.64
Carbon monoxy	14.50 (17.46)*	55.52 (44.29)*	4.67 (5.13)*	9.07 (8.67)*
Mean			4.33 ± 0.3	8.36 ± 0.7
Aquomet	76.13	80.62	6.60	7.60

*the values in the bracket are the parameters used for fitting the kinetic data for stripped carbonmonoxy derivatives in the presence of 200 $mmoldm^{-3}$ NaCl and were not used to find the mean values.

Table 2. Reaction of DTNB with CysF9[93]β sulphydryl group of Stripped Haemoglobin C.

	k_1 $(dm^3\,mol^{-1}s^{-1})$	k_2 $(dm^3\,mol^{-1}s^{-1})$	k_3 $(dm^3\,mol^{-1}s^{-1})$	pQ_1	pQ_2	pQ_{S3}	pQ_{1H}	pQ_{2H}
Oxy	38.29	2059.04	253.96	8.47	9.00	9.86	6.38	6.87
Carbon monoxy	43.12	2.90	52.66	7.93	8.48	8.08	8.00	6.98
Mean				8.20 ± 0.3	8.74 ± 0.3	8.97 ± 0.9	7.19 ± 0.8	6.93 ± 0.1
Aquomet	27.23	50.15	-	5.57	8.23	-	-	-

This was previously explained based on the assumption that the complex profile is a result of the electrostatic interaction of the ionizable groups with CysF9[93]β sulphydryl group in its reaction with DTNB. The best-fit parameters for the complex profiles for figures 1a and 1b are presented in table 2.

$$K_{app} = \frac{k_{n+1} + \sum_{i=1}^{n} k_i (H^+)^{n+1-i} \left(\prod_{j=i}^{n} Q_j \right)^{-1}}{\left\{ 1 + \sum_{i=1}^{n} k_i (H^+)^{n+1-i} \left(\prod_{j=i}^{n} Q_j \right)^{-1} + \frac{[H^+]}{Q_{s(n+1)}} \left[\sum_{i=1}^{n} k_i (H^+)^{n+1-i} \left(\prod_{j=i}^{n} Q_{jH} \right)^{-1} \right] \right\}} \quad 3$$

The mean pQ_1 and pQ_2 values for complex profiles are 8.2 ± 0.3 and 8.7 ± 0.3 respectively for stripped haemoglobin C, these two values are higher than those obtained for haemoglobins A and S (Okonjo *et al.,* 1995, 1996).

CONCLUSION

The replacement of glutamic acid at A3[6]β position of hemoglobin A with lysine in hemoglobin C is of structural consequence. Contrary to expectation, the rate of reaction of haemoglobin C with DTNB is lower than that of haemoglobin A despite increase net positive charge on haemoglobin C. Surprisingly, the aquomet derivative (both stripped and in the presence of organic phosphate) displayed some characteristic profiles that bring to the fore the effect of replacement of glutamic acid at A3[6]β position with lysine.

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REFERENCES

Antonini, E. and Brunori, M. 1969. On the rate of conformation change associated with ligand binding in hemoglobin. Journal of Biological Chemistry. 244:3909-3912.

Babalola, JO. and Nwozo, S. 2002. A new structure in the vicinity of HisHC3[146]β of pigeon haemoglobin induced by inositol hexakisphosphate. Scientia Iranica. 9:139-147.

Babalola, JO., Babarinde, NA. and Akingbola, TS. 2005. Varying apparent rate constant: determination of uptake and release of protons during tetramer-dimer dissociation in human hemoglobin A. Italian Journal of Biochemistry. 54:61-68.

Bettati, S., Viappiani, C. and Mozzarelli, A. 2009. Hemoglobin, an "evergreen" red protein. Biochimica et Biophysica Acta. 1794:1317-1324.

Dintzis, HM. 1952. Dielectric properties of human mercaptalbumin. Ph.D thesis, Harvard University, Cambridge, Mass.

Guidotti, G. 1965. The rates of reaction of the sulphydryl group of human hemoglobin. Journal of Biological Chemistry. 240:3924-3927.

Hensley, P., Edelstein, SJ., Wharton, DC. and Gibson, QH. 1975. Conformation and spin state in methemoglobin. Journal of Biological Chemistry. 250:952-960.

Hirsch, RE., Ravetos-Suarez, C., Olison, JA. and Nagel, RL. 1985. Ligand state of intraerythrocytic circulating HbC crystal in homozygote CC patient. Blood. 66:775-777.

Okonjo, KO. 1980. The effect of organic phosphates on the sulphydryl reactivities of oxyhemoglobins, A and S. Journal of Biological Chemistry. 255:3274-3277.

Okonjo, KO., Aken'ova, YA., Aboluwoye, CO., Nwozo, S., Akhigbe, FU., Babalola JO. and Babarinde, NA. 1996. Effect of A3[6]$\beta^{Glu\rightarrow Val}$ mutation on reactivity of CysF9[93]β sulfhydryl group of human hemoglobin S. Journal of the Chemical Society (Faraday Transactions). 92:1739-1746.

Okonjo, KO. and Nwozo, S. 1997. Ligand – dependent reactivity of the CysB5(23)β sulfhydryl group of the major haemoglobin of chicken. Journal of the Chemical Society (Faraday Transaction). 93:1361-1366.

Okonjo, KO., Bello, OS. and Babalola, JO. 2008. Transition of hemoglobin between two tertiary conformations: the transition constant differs significantly for the major and minor hemoglobins of the Japanese quail (*Cortunix cortunix japonica*). Biochimica et Biophysica Acta. 1784:464-471.

Okonjo, KO., Adeogun, IA. and Babalola, JO. 2010. Tertiary conformational transition in sheep hemoglobins induced by reaction with 5,5´-dithiobis(2-nitrobenzoate) and by binding of inositol hexakisphosphate. Journal of Biophysical Chemistry. 146:65-75.

Hallaway, BE., Hedlund, BE. and Benson, ES. 1980. Studies on the effect of reagent and protein charges on reactivity of the β93 sulfhydryl group of human hemoglobin using selected mutations. Archives of Biochemistry and Biophysics. 203:332-342.

Robyt, JF., Ackerman, RJ. and Chittenden, CG. 1971. Reaction of protein disulfide groups with Ellman's reagent: A Case study of the number of sulfhydryl and disulfidegroups in apergillus oryzea α-amylase, papain, and lysozyme. Archives of Biochemistry and Biophysics. 147:262-269.

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