EQUILIBRIUM REACTION OF HUMAN METHEMOGLOBIN A

G.B. OGUNMOLA[1], J.T. BAMGBOSE[2,+], and A.A. DEMEHIN[1]
1. Department of Chemistry, University of Ibadan, Ibadan, Nigeria
2. Department of Chemistry, University of Agriculture, Abeokuta, Nigeria

(*Received*: July, 2006; *Accepted*: October, 2007)

Abstract

A study has been carried out on the sulfhydryl group reactivities of human methemoglobin A and its azide complex with 2,2'-dithiobispyridine (2-DTP) in the presence and absence of inositol hexaphosphate (IHP) in order to evaluate electrostatic effects on reactivity and the relationship between conformational states and reactivity. The reaction was monitored spectrophotometrically by the formation of 2-thiopyridone (the product of the reaction) with an absorption peak at 343 nm at 20 °C.

The binding of IHP to methemoglobin A (met-HbA) diminishes the reactivity of β-93 sulfhydryl group towards 2DTP. This effect was used to determine the pH profile of the binding constants of IHP to the surface of the protein while changing the complex at the heme iron. Work was also done on the equilibrium reaction of azide ion with IHP- and 2DTP- chemically modified met-Hb A as a function of pH, and an equilibrium relationship was developed for reactions both at the heme iron and at the surface of the methemoglobin molecule. The result shows that binding at the heme by ligands such as azide ion affects the orientation of charge groups on the surface of the met-Hb molecule as well as binding of organic phosphate.

1. Introduction

Interactions among the sub-units of biological macromolecules have long been recognized to be of key importance in the mechanisms relating physical and chemical properties to biological activities (Grassetti and Murray, 1967; Taketa and Morell, 1969). Such interactions are generally assumed to be mediated in part through changes in the three dimensional structure of these molecules. The cooperative interactions, changes in linked functions and the conformation rearrangement associated with binding of lingands to hemoglobin have made it a popular model for studies of such properties (Erve *et al.*, 1996; Kehinde *et al.*, 1996).

Ligand binding reactions under different conditions of pH, ionic strength and temperature have also provided useful information for accounting for the behaviour of important molecules of biological interest like hemoglobin (Shaanan, 1983; Heindner *et* al., 1976). Hemoglobin has been widely used for the study of the relationship among physical structure, chemistry and physiological properties of protein. Investigation of hemoglobin have included not only measurement of many physical parameters under a variety of conditions but also theoretical considerations of possible mechanisms through which changes in these parameters might be inter-related (Okonjo and Aboluwoye, 1992).

Methemoglobin is a protein formed continuously (in small amounts) in normal red blood. It is formed as a result of the oxidation of iron (II) of oxyhemoglobin to iron (III) with mild oxidizing agents. The sixty coordination position of the iron previously occupied by oxygen in the hemoglobin is now being occupied by a water molecule (Perutz *et al.*, 1974), which is capable of ionizing.

Organic phosphates have a pronounced effect on oxygen equilibrium curve of hemoglobin (Benesch and Benesch, 1967). The shift in the equilibrium curve to the right causes greater oxygen pressure to be required in achieving the desired percentage oxygenation. The decrease in oxygen affinity upon organic binding produces abnormal tertiary structure in methemoglobin S and hence sickling (Benesch and Benesch, 1967).

Organic phosphates regulate the oxygen binding properties of hemoglobin by binding at the entrance to the molecular dyad axis in deoxyhemoglobin (Gottfried *et al.*, 1997).

The reaction of ligands with methemoglobin involves conformational changes of which characteristic pH is the manifestation (Beetlestone *et al.*, 1976, Ogunmola *et al.*, 1999). This information has been used to investigate the ligand binding behaviour of IHP and 2-DTP-chemically modified human

+ *corresponding author* (e-mail: titibam2003@yahoo.com)

methemoglobin A with azide ions in order to determine an equilibrium relationship between complex formation with hemoglobin and reaction with ligands. We have in this study monitored binding at the heme iron and the considerable changes at the tertiary structure arising from changes of charged groups on the surface of the molecules.

We have also determined organic phosphate binding constants by monitoring the reduction in the reactivity of the β-93 cys groups caused by inositol hexaphosphate.

2. Materials and Methods

Blood from normal human donors was obtained from the Blood Bank, University College Hospital, Ibadan. The 2-DTP used is a product of Sigma St. Louis, USA. Inositol hexaphosphate (IHP) was obtained from British Drug Houses. Hemoglobin A was prepared according to normal laboratory procedure. The methemoglobin was prepared as described by Ogunmola, *et al.* (2001). IHP-metHb complex was formed by adding a calculated volume of stock solution (10 μM IHP) to make 100 ml of metHb solution. This calculation was done using the knowledge of the stoichiometry of the binding HIP to metHb molecule which is of ratio 1:1 (one mole of IHP molecule binds to one mole of hemoglobin tetramer) (Gottfried *et al.*, 1997). The concentration of the metHb used was 4 μM. The 2-DTP-metHb complex was formed according to the method of Ogunmola *et al.* (1999). Bis-Tris and Tris/HCl buffer solution of ionic strength 0.1 M were used throughout this work for all measurements. From pH 6.0 to pH 7.2, Bis Tris buffer was used. At higher pH up to 8.0 Tris/HCl buffer solutions were used. The ionic strength of these solutions was maintained by the addition of suitable amounts of sodium chloride. The pH values of all solutions were checked with Crimson Micro-pH 2000 Instrument.

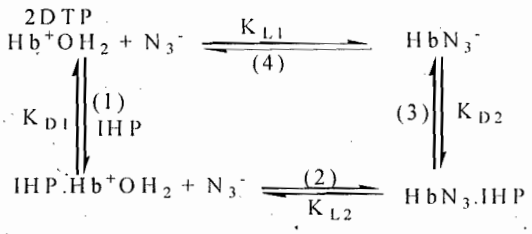
The Kinetics of the reaction of 2-DTP with the metHbA were monitored at 343 nm on a Perkin Elmer Lambda 38 UV/visible spectrophotometer according to Ogunmola *et al.* (1999). All reactions were carried out in Triplicate at 20 °C. The hemoglobin concentration was 4 μM per tetramer. The extinction coefficient of 2-thiopyridone, the product of the 2-DTP reaction was assumed to be 9730 M^{-1} CM^{-1} and it is independent of pH (Grassetti and Murray, 1967; Hallaway *et al.*, 1980).

The azido methemoglobin was formed according to the method of Okonjo and Aboluwoye (1992). The equilibrium reaction of azide ion with chemically modified human metHb A was monitored according to the method of Beetlestone *et al.* (1976).

3. Results and Discussion

The thermodynamic cycle that describes the dependence of human methemoglobiin reactions with 2-DTP, IHP and azide ion is presented in Scheme 1 below:

SCHEME 1



Owing to the cyclic relationship between the four equilibria that describe:

(1) Binding of IHP to $metHb^{+}OH_2$ (Aquomethemologlobin)

(2) Equilibrium of azide complex with IHP - methemoglobin

(3) Binding of IHP to azide – methemoglobin

(4) Equilibrium of azide complex with 2DTP-methemoglobin

It is assumed that:

$$K_{L1} - K_{L2} = K_{D1} - D_{D2} \qquad (1)$$

Taking log, equation (1) becomes:

$$LogK_{L1} - LogK_{L2} = Log\ K_{D1} - LogK_{D2}$$

or

$$LogK_{L1} = LogK_{L2} + Log\frac{K_{D1}}{K_{D2}} \qquad (2)$$

It is necessary that:

$$G_1 - G_3 = G_4 - G_2 \qquad (3)$$

Where G_1 and G_3 are the free energies of IHP binding to aquo-and azido-metHbA, G_2 and G_4 the binding of azide to IHP-metHbA and 2DTP –metHbA respectively.

Thus a difference in the IHP binding to azido and to aquo-methemoglobin must be reflected in a comparable difference in the binding between reactant and product, especially a change in the binding of some ligands when protein is modified. The equilibrium relationship between complex formation with this protein and reaction with a ligand, as can be deduced from equation 3 is:

$$\Delta G^{\circ}_4 - \Delta G^{\circ}_4 = RT\ln\frac{K_1}{K_3} \qquad (4)$$

where:

ΔG°_4 = Standard free energy change accompanying the binding of azide to 2DTP–metHb

ΔG°_2 = Standard free energy change accompanying the binding of azide to IHP-metHb

K_1 and K_3 = The measures of the IHP binding to aquometHb and azidometHb respectively.

Fig. 1: pH dependence of binding constants K_D of IHP to human AquometHbA and Azido derivative at 20 °C [AquomethbA] = 4 x10^{-6} M, [AzidometHbA] = 4 x 10^{-6} M, I = 0.1 M, [2DTP] = 97 x 10^{-6} M

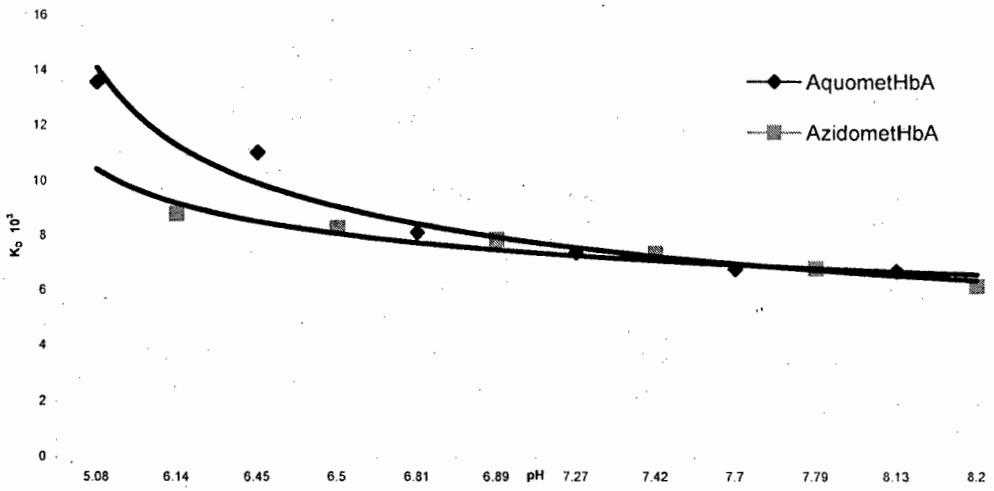
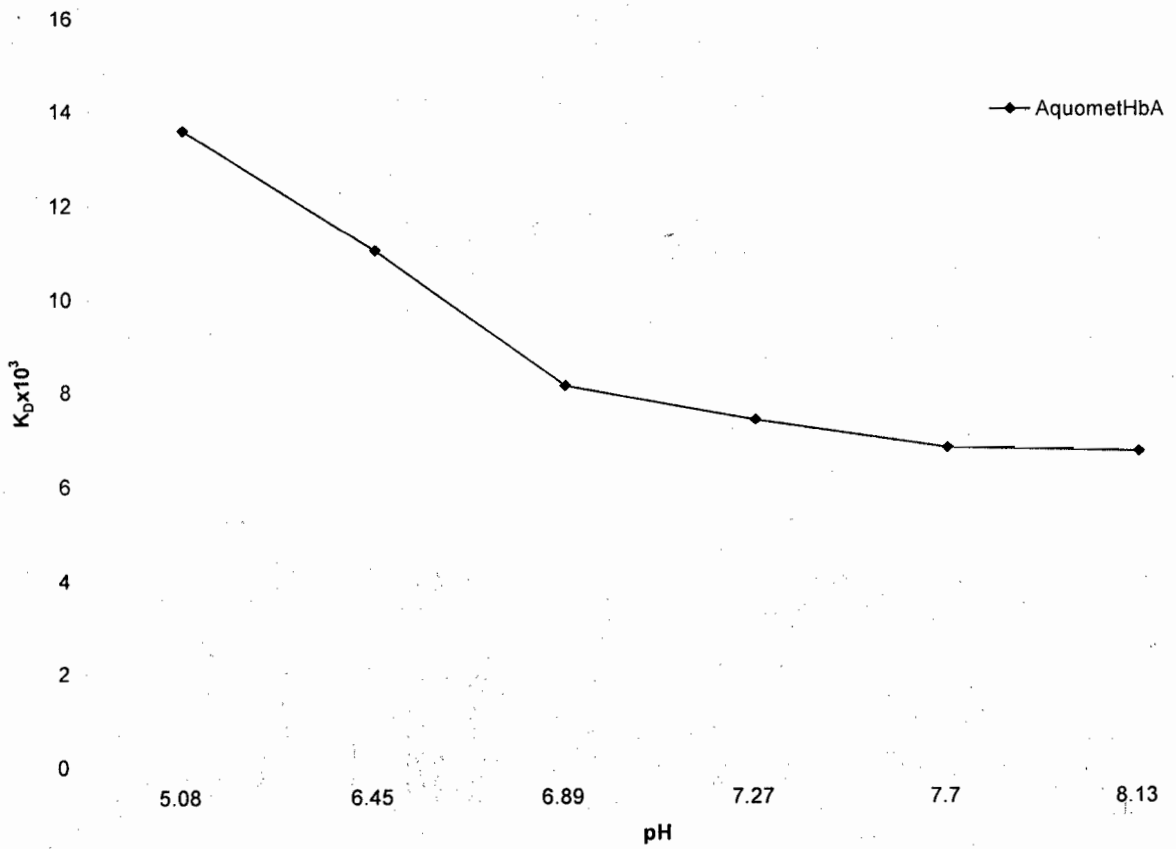


Fig. 2: pH dependence of the binding constant(K_D) of inositol hevaphosphate(IHP) to human AquometHbA at 20 °C [AquometHbA] = 4 x 10^6 M, I = 0.1 M, [2DTP] = 97 x 10^6 M



When Ks are replaced by the binding, equation (4) becomes

$$\Delta G_1 - \Delta G_1 = RT \ln \frac{\text{IHP binding to aquometHb}}{\text{IHP binding to azidometHb}} \quad (5)$$

With ΔG^{o}_{1}, ΔG^{o}_{2}, replaced by K_{L1} and K_{L2} equation (4) becomes:

$$\text{Log}K_{L1} = \text{Log}K_{L2} + \text{Log}\frac{K_{D1}}{K_{D2}} \quad (6)$$

which is similar to equation (2)

Reactivity of Aquomethemoglobin and its Azido Derivatives:

Decreased reactivity of the β-93 sulfhydryl groups of aquomet HbA on IHP binding has been reported previously (Ogunmola *et al.*, 2001; Okonjo, 1980). Our results have shown that IHP binds to metHbA. We have used the organic phosphate effect on sulfhydryl reactivity to determine the IHP binding constant by the method of Ogunmola *et al.* (2001).

In Fig. 1, we present the dependence of IHP binding to aquometHbA and its azido derivative. The binding of IHP to both aquo-and azido-metHbA decreases with increasing pH (Table 1, Fig. 2) for a given pH, the IHP binding constant of the aquometHbA is higher than that obtained for the azido derivative (Table 1). The lower values of IHP binding to metHb types at higher pH shows very weak IHP binding at higher pH where the methemoglobin would carry a net negative charge.

The addition of azide ion to metHb not only decreases its net charge by one but also gives rise to a decrease in the pK_a of ionized group on the metHb and as a result protons are taken up (Ogunmola *et al.*, 2001; Aboluwoye, 2003). The change in the pH and consequently ionization of charged groups on the hemoglobin molecule produces different conformational changes in these molecules which result in changes in the structure at the IHP binding site and at the structure near the β-93 sulfhydryl group of these molecules.

Table 1: pH dependence of the binding constants (K_D) of inositol hexaphosphate (IHP) to human Aquomethemoglobin A and its Azido derivative at various concentrations of IHP at 20 °C. [AquometHbA] = 4 μM, [AzidometHbA) = 4 μM, I = 0.1 M, [2DTP] = 97 μM.

AquometHb A		AzidometHb A	
pH	$K_{D1}(*10^3)$	pH	$K_{D2}(*10^3)$
5.08	13.580	6.14	8.810
6.45	11.051	6.50	8.313
6.89	8.138	6.81	7.876
7.27	7.406	7.42	7.376
7.70	6.808	7.79	6.835
8.13	6.724	8.20	6.184

Table 2: Equilibrium binding reaction of azide ion with human 2DTP-and IHP-modified human metHbA.

2DTP-MET HbA		IHP-MET HbA	
pH	Log K_{L1}	pH	Log K_{L2}
6.20	5.53 ± 0.01	6.14	5.41 ± 0.03
6.50	5.58 ± 0.01	6.27	5.43 ± 0.01
6.83	5.45 ± 0.05	6.59	5.41 ± 0.01
7.41	5.44 ± 0.02	7.30	5.40 ± 0.01
7.85	5.32 ± 0.01	7.72	5.35 ± 0.02
8.19	5.26 ± 0.01	8.04	5.20 ± 0.01

Table 3: Estimated and Experimentally measured equilibrium constant (log K_L) of the binding reaction of azide complex of 2DTP-and IHP-modified human met HbA at 20 °C.

2DTP-metHb A			IHP – met HbA		
pH	Estimated values	Experimental values	pH	Estimated values	Experimental values
6.20	5.60 ± 0.02	5.53 ± 0.01	6.14	5.34 ± -0.02	5.41 ± 0.03
6.50	5.55 ± 0.01	5.58 ± 0.03	6.47	5.45 ± 0.01	5.43 ± 0.01
6.83	5.42 ± 0.02	5.45 ± 0.02	6.59	5.44 ± 0.02	5.41 ± 0.02
7.41	5.40 ± 0.01	5.44 ± 0.01	7.30	5.44 ± 0.01	5.40 ± 0.01
7.85	5.35 ± 0.02	5.32 ± 0.01	7.20	5.32 ± 0.01	5.35 ± 0.01
8.19	5.24 ± 0.01	5.26 ± 0.01	8.04	5.22 ± 0.01	5.20 ± 0.01

Previous studies have shown that the same residues in both aquo-and azido-metHb are involved in the binding of IHP (Perutz *et al.*, 1974; Ogunmola *et al.*, 2001; Edalji *et al.*, 1976).

Reports have shown that some charged groups different from those making up the binding site must be involved (Ogunmola *et al.*, 2001). It can therefore be said that the difference in reactivity of aquometHbA and its derivatives is as a result of the small changes in the tertiary structure of the β-chain that occur as a function of difference in the ligand at the heme presumably arising from changes in the spin state of the heme iron.

The results of the azide reactions of IHP-and 2DTP-metHbA at 20 °C are shown in Table 2. The experimentally-measured values of the equilibrium constants (Log K_1) of the binding reaction of azide complexes of IHP-and 2DTP-metHb A at 20 °C are in very good agreement with the estimated values (Table 3).

The close agreement between experimentally measured values and the estimated log K_1 values supports the validity of equation (6).

REFERENCES

Grassetti, D.R. and Murray, J.F., 1967. Determination of Sulfhydryl group with 2.22 -and 4.42 - dithiobispyridine. Archives of Biochemistry Biophysics. 199. 41-49.

Taketa, F. and Morell, S.A., 1969. Changes in the 4PDS reactive SH group of Hemoglobin Associaited with Binding of Phosphates and Ligands. *Anal. Biochem.*, 32, 169-174.

Erve, J.C.L., Deinzer, M.L.and Reed, D.J., 1996. Reaction of Human Hemoglobin Toward the Alkylating Agent S-(2-Chloroethyl) Glutathione. *Hourn. Journ. of Toxicology and Environmental Health*. 49, 127-143.

Kehinde, O.K., Aken'ova, Y.A., Aboluwoye, C.O., Nwozo, Akhigbe, F.U., Babalola, J.O. and Babarinde, N.A., 1996. Effect of A3(6) $\beta^{Glu \rightarrow Val}$ Mutation on Reactivity of the Cys F9 (93) β Sulfhydryl group of Human Haemoglobin. *S. Journ. Chem. Soc. Faraday Trans.*, 92(10). 1739-1746.

Shaanan, B., 1983. Structure of Human Oxyhemoglobin at 2. 7. A resolution: Journal of Molecular Biology. 171. 31-59.

Heindner, E.J., Ladner, R.C. and Perutz, M.F., 1976. Structure of Horse Carbonmonoxy Hemoglobin. Journal of Molecular Biology. 104. 707-722.

Okonjo, O.K. and Aboluwoye, C.O., 1992. Ionizable groups linked to the Reaction of 2.2'-dithiobis-pyridine with Hemoglobin. *Biochmica, et. Biophysica Acta.*, 1159. 303-310.

Perutz, M.F., Lander, J.E., Simon, S.R. and Ho Chien, 1974. Influence of Globin Structure on the State of the Heme. I. Human deoxyhemoglobin. Biochemsitry. 13(10). 2163-2173.

Benesch, R. and Benesch, R.E., 1967. The Effect of Organic Phosphate from Human Erythrocyte to Allosteric Properties of Hemoglobin. Biochemical and Biophysical Research Communications. 26. 162-167.

Gottfried, D.S., Juszczak, L.J., Fatalier, N.A., Acharya, A.S., Hirsch, R.E. and Friendman, J.M., 1997. Probing the Hemoglobin Central Cavity by Direct Quantification of Effector Binding using Fluorescence Lifetime Methods. Journal of Biological Chemistry. 272(3). 1571-1578.

Beetlestone, J.G., Adeosun, O.S., Goddard, J.E., Kushima, J.B., Ogunlesi, M.M., Ogunmola, G.B., Okonjo, K.O. and Seamonds, B., 1976. Reactivity Differences between Hemoglobin part XIX. Journal of Chemical Society. Dalton Transaction. 1251-1275.

Ogunmola, G.B., Bamgbose, J.T., and Demehin, A.A., 1999. Reactivity of 2.2'-Dithiobispyridine with Human metHb. A.S and C and their Azide Complexes. *Nig. Journal of Genetics*. 14. 69-77.

Ogunmola, G.B., Bamgbose, J.T. and Dehemhin, A.A., 2001. Interaction of IHP with Human Methemoglobin A and S. Global Journal of Pure and Applied Sciences. 7(2). 257-263.

Hallaway, B.E., Hedlund, B.E. and Benson, E.S., 1980. Studies on the Effect of Reagent and Protein Charges on Reactivity of the β-93 Sulfhydryl group of Human Hemoglobin using Selected Mutations. Archives of Biochemistry and Biophysics. 203. 332-342.

Okonjo, K.O., 1980. Effect of Organic Phosphates on the Sulfhhydryl Reactivities of Oxythemoglobins A and S. Journal of Biological Chemistry. 255. 3274-3277.

Aboluwoye, C.O., 2003. Organic Phosphate Binding groups Elestrostatically Linked to the Reaction of Cyanide ion with Aquomethemoglobin A. *International Journ. of Chem.*, 13(3). 133-138.

Edalji, R., Benesch, R.E. and Benesch, R., 1976. Binding of Inositol Hexaphosphate to Deoxyhemoglobin. Journal of Biological Chemistry. 251. 7720-7721.