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INTERACTION OF INOSITOL HEXAPHOSPHATE WITH HUMAN METHEMOGLOBINS A AND S

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ABSTRACT

To correlate reactivity with conformational changes, the reduction in sulfhydryl reactivity rate was employed in this study to determine the binding constants of Inositol hexaphosphate (IHP) to human methemoglobins A and S at the surface of the molecules while changing the complex at the heme-iron. Our study was carried out in the pH range of 6.0-8.0 at three different temperatures (15°C, 20°C and 25°C). The binding of IHP to methemoglobins A and S and their azide derivatives diminishes the rate of reaction of β93 sulfhydryl groups towards 2,2-dithiobispyridine (2-DTP). Differences in reactivity are explained not only on the basis of a single mutation at the A3(6) β position in the two hemoglobin molecules but also in terms of other physical properties. Our result shows that in the absence of IHP, the CysF9 (93) β sulfhydryl group of aquomethemoglobin A and S are more reactive with 2-DTP than their corresponding azidomet derivatives. There was also a progressive decrease in the binding of IHP to all the methemoglobin types with increasing pH. Changes in the pH and consequently ionization of charged groups on the methemoglobin molecules produce different conformational changes in these molecules, resulting in changes in the structures at the IHP binding site and in the vicinity of the β93 sulfhydryl residue of the aquo and azido methemoglobins. The dissociation constants obtained in this study ranged from 82 x 10^{-6} to 167 x 10^{-6} for aquomet HbA and 43 x 10^{-6} to 137 x 10^{-6} for AquometHbS. The corresponding values for azidomet HbA and S were much higher than these values.

Keywords: methemoglobin, conformation, sulfhydryl, allosteric, heme-iron

INTRODUCTION

Chemical studies have shown that human hemoglobins A and S differ from normal hemoglobin A in a single amino acid residue. These hemoglobins have identical α subunits but differ from one another in one out of 146 amino acid residues on each of their two β subunits.

In hemoglobin A, the 6th position in its β subunit is occupied by negatively charge glutamic acid residue. In hemoglobin S, this position is occupied by a neutral valine residue (Ingram, 1959) which means that the two hemoglobin molecules differ by one charge unit. The mutation occurs at the surface of the hemoglobin molecule. In spite of this relatively minor differences in amino acid composition, significant clinical consequences arise for people who are homozygous for hemoglobin S and these have been exustively studied (Ingram, 1959, Pauling, et. al. 1949). Despite the known difference between homoglobin A and S reported by Pauling, et. al. (1949), it has been assumed until recently that in the liganded state both hemoglobin have similar if not identical structure. This suggests that the organic phosphate binding site would be the same in these molecules (Okonjo, et. al. 1996).The role of organic phosphates as allosteric effectors in the regulation of oxygen affinity of human hemoglobin has been widely investigated (Mozzarell, et. al. 1997). In the presence of high salt concentration, the effect of organic phosphate tends to disappear (Konisherg, et. al. 1961) suggesting that the interaction of these phosphates with hemoglobin is electrostatic. It is well documented that both oxy and deoxyhemoglobin bind organic phosphates (Benesch and Benesch 1967). The controversy of the number of binding sites for oganic phosphate per tetramer has been resolved by x-ray studies of Arnone (1974) and Gottfried, et. al. (1997) who showed that one molecule of 2,3,diphosphoglycerate (DPG) binds to one molecule of deoxyhemoglobin on the β-chains and that it takes up a stereochemically complementary position on the two fold symmetry axis and fits into the entrance to the central cavity. Reports from Perutz and Arnone (1974) indicated that the binding site of 2,3-DPG is the same for IHP.

Inositol hexaphosphate has been reported to be the most effective polyphosphate which reduces sulfhydryl reactivity and hence, enhances sickling (Garby, et. al. 1969, De Bruin 1974) consequent on the very stable complex it forms with hemoglobin.

Therefore, it has been generally assumed that the afinity of IHP for hemoglobin is too high to permit measurement of the equilibrium constant for the reaction directly (Gray and Gibson, 1971, Baldwin, 1975).

In the present study, the reduction in sulfhydryl reactivity rate is employed to determine the binding constants of IHP to human methemoglobins A and S

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at the surface of the molecules while changing the complex at the heme iron according to Scrutton-Utter's (1965) equation with the aim of correlating reactivity with conformational changes in these methemoglobin molecules. Assuming that the rate of binding of IHP to the methemoglobin is faster than that of 2-DTP, it is possible to calculate from the reduction of the rate of reaction of 2-DTP by IHP, and write an expression for the equilibrium constants for the binding of IHP in terms of the reduction in the rate constants for the binding of IHP to methemoglobin.

The decision to study sulfhydryl group reactivity with 2-DTP in the presence and absence of organic phosphate calls for the use of a buffer that does not bind to methemoglobin (Arnone, 1974). However, there is evidence from Benesch (1967) work that Bis-tris and Tris/HCl do not bind to hemoglobin. Bis-Tris and Tris/HCl therefore appear to satisfy the requirement for this study.

All the methemoglobin employed in this study were purified and had only one pair of reactive sulfhydryl groups per hemoglobin molecule at the Cys F9(93) β position.

In this paper we report the structural characteristics of the binding of IHP to human aquo and azidomethemoglobins A and S. Previous work on the determination of binding constants of IHP with hemoglobins have been carried out at particular pH and temperature (Darby, et. al. 1969, Okonjo, 1980). In none of these studies have more detailed investigation into the pH and temperature profiles of the binding constants of IHP been reported.

This study carried out in order to evaluate the basic mechanism of the factors responsible for the effect of changes of pH and temperature on the reduced reactivity of the β93 sulfhydryl groups of these methemoglobins by IHP and to determine if the suggestions concerning relationships between conformational and reactivity are reflected in these methemo[illegible]s.

MATERIALS AND METHODS

2,2'-dithiobispyridine and inositol hexaphosphate obtained as the sodium salt from Sigma St. Louis were used without further purification. Normal human blood from Blood Bank and freshly drawn blood from sickle cell patient was obtained from Haematology Department, University College Hospital, Ibadan. Hemoglobin was prepared according to normal laboratory procedures. Azido methemoglobin was prepared according to the method of Okonjo, et. al. (1996). A cation exchanger, Carboxymethyl cellulose (microgranular, pre-swollen, cm 32 Whatman) was used to purify the hemoglobins. Purification procedure that separate the hemoglobins by means of a gradient of pH that was produced by Bistris buffer was adopted.

The kinetics of the reaction of 2-DTP with aquo and azidomethemoglobins A and S were carried out in the presence and absence of IHP at three different temperatures: 15°C, 20°C and 25°C. Bis-tris and Tris/HCL buffer solutions at ionic strength of 0.1M were used throughout this work for all measurements on SH reactivities from pH 6.0 to pH 7.2. At higher pH up to 8.0 Tris/HCl buffers were used. The 2-DTP reactivity with these methemoglobins was monitored on Cary

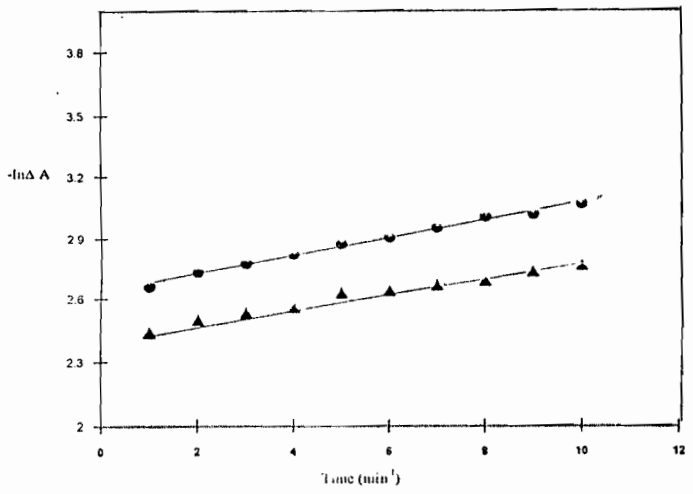

Figure 1a Pseudo first rate order plots for the reaction of human Aquomethemoglobin A with 2-DTP and for comparison, similar plots in the presence of IHP. Conditions of experiment as in figure 1b
● Circle, without IHP, ▲triangle, plus 130μM IHP

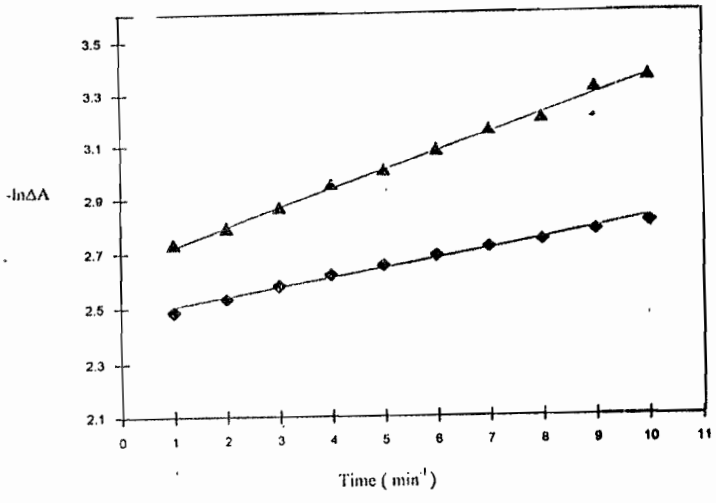

Figure 1b Pseudo-first order rate plots for the reaction of human Aquomethemoglobin S with 2-DTP and for comparison similar plots in the presence of IHP.
Triangle, without IHP, squares, + 90μM IHP, pH 7.6 20°C. [2-DTP] =97μM, [methemoglobin] = 4μM, I = 0.1M

219 spectrophotometer at 343nm. The absorbance at one minute interval was recorded with a W.G. PYE and Co. Ltd. Cambridge stop-clock. Constant cell temperature was maintained with low temperature bath made by Wilkens Anderson Company, USA. The ionic strength of all buffer solutions was maintained at 0.1M by addition of suitable amount of sodium chloride. The pH of all solutions was measured with a Crimson micro pH 2000 instrument. Each kinetic run was repeated thrice under identical experimental conditions. The 2-DTP concentration was 96.7μm, while the methemoglobin concentration was 4μm in sulfhydryl groups.

RESULTS AND DISCUSSION

Tables 1a and b show the result of the effect of IHP on the sulfhydryl reactivities of aquomethemoglobins A and S. A comparison of the values of pseudo-first order rate constants of the 2-DTP reaction with aquomet HbA and S in the presence of IHP shows that IHP diminishes the reactivity of the Cys F9 (93) β sulfhydryl groups of these methemoglobins (Table 1 and Fig. 1a, & b). It is clear from Figure 1 that IHP

Table 1(a): Temperature and pH dependence of mean pseudo-first order rate constant (K_{obs}) of 2-DTP reaction with human Aquomethemoglobin A and for comparison in the presence of various concentrations of IHP. [AquometHb.A] = 4μM tetramer, I=0.1M, [2-DTP]=97μM

pH	Plus 2-DTP, without IHP K_{obs} X 10^{-2} (min^{-1})	[IHP]=90μM K_{obs} X 10^{-2} (min^{-1})	[IHP]=110μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=130μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=150μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=180μM K_{obs} x 10^{-2} (min^{-1})
			15°C			
6.13	3.79 ± 0.01	2.26 ± 0.01	2.11 ± 0.03	1.84 ± 0.01	1.78 ± 0.03	1.78 ± 0.01
6.53	4.80 ± 0.03	2.70 ± 0.03	2.42 ± 0.02	1.29 ± 0.01	1.09 ± 0.06	1.05 ± 0.01
6.94	4.94 ± 0.01	2.69 ± 0.01	2.21 ± 0.03	1.94 ± 0.02	1.71 ± 0.01	1.48 ± 0.04
7.33	5.30 ± 0.02	2.79 ± 0.03	2.41 ± 0.01	2.24 ± 0.01	1.99 ± 0.04	1.59 ± 0.01
7.73	5.47 ± 0.03	3.99 ± 0.01	3.89 ± 0.02	3.69 ± 0.02	3.58 ± 0.03	3.26 ± 0.05
8.14	6.36 ± 0.01	4.93 ± 0.01	4.78 ± 0.05	4.63 ± 0.03	4.51 ± 0.01	4.18 ± 0.03
			20°C			
pH	Plus 2-DTP, without IHP K_{obs} X 10^{-2} (min^{-1})	[IHP]=90μM K_{obs} X 10^{-2} (min^{-1})	[IHP]=110μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=130μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=150μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=180μM K_{obs} x 10^{-2} (min^{-1})
6.08	4.01 ± 0.06	2.90 ± 0.01	0.71 ± 0.03	0.44 ± 0.01	0.02 ± 0.01	0.04 ± 0.03
6.45	5.72 ± 0.01	2.80 ± 0.04	1.13 ± 0.02	1.90 ± 0.04	0.60 ± 0.03	0.39 ± 0.01
6.89	6.12 ± 0.06	3.00 ± 0.01	1.84 ± 0.01	3.61 ± 0.01	3.11 ± 0.01	3.14 ± 0.03
7.27	6.30 ± 0.01	3.11 ± 0.04	3.01 ± 0.03	2.81 ± 0.04	2.61 ± 0.02	2.01 ± 0.02
7.75	6.58 ± 0.02	3.18 ± 0.06	3.24 ± 0.01	2.67 ± 0.06	2.49 ± 0.04	2.10 ± 0.01
8.13	6.91 ± 0.03	4.69 ± 0.01	4.21 ± 0.02	3.94 ± 0.01	3.71 ± 0.05	3.48 ± 0.01
			25°C			
pH	Plus 2-DTP, without IHP K_{obs} X 10^{-2} (min^{-1})	[IHP]=90μM K_{obs} X 10^{-2} (min^{-1})	[IHP] 110μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=130μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=150μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=180μM K_{obs} x 10^{-2} (min^{-1})
6.03	4.88 ± 0.03	2.61 ± 0.01	2.47 ± 0.02	2.30 ± 0.02	2.22 ± 0.02	1.54 ± 0.01
6.44	6.68 ± 0.01	2.03 ± 0.04	1.71 ± 0.03	1.50 ± 0.01	1.25 ± 0.01	1.07 ± 0.02
6.81	7.42 ± 0.04	3.98 ± 0.01	3.85 ± 0.05	3.66 ± 0.01	3.49 ± 0.04	2.59 ± 0.01
7.23	7.56 ± 0.01	3.10 ± 0.02	2.60 ± 0.01	2.42 ± 0.06	2.18 ± 0.02	1.44 ± 0.01
6.65	7.63 ± 0.02	3.50 ± 0.01	3.03 ± 0.04	2.82 ± 0.02	2.58 ± 0.01	1.84 ± 0.01
8.05	7.88 ± 0.01	4.21 ± 0.05	3.45 ± 0.01	2.34 ± 0.03	2.30 ± 0.01	1.93 ± 0.02

Table 1b: Temperature and pH dependence of mean pseudo-first order rte constants (K_{obs}) of 2-DTP reaction with human Aquomethemoglobin S and for comparison in the presence of various concentrations of IHP, [AquometHb.S]=4μM tetramer, I=0.1M, [2DTP]=97μM

pH	Plus 2-DTP, without IHP K_{obs} X 10^{-2} (min^{-1})	[IHP]=90μM K_{obs} X 10^{-2} (min^{-1})	[IHP]=110μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=130μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=150μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=180μM K_{obs} x 10^{-2} (min^{-1})
			15°C			
6.14	6.00 ± 0.01	2.81 ± 0.02	2.73 ± 0.06	2.64 ± 0.01	2.13 ± 0.03	2.01 ± 0.02
6.52	6.01 ± 0.01	2.93 ± 0.03	2.72 ± 0.03	2.41 ± 0.01	2.23 ± 0.01	2.04 ± 0.01
6.95	6.41 ± 0.01	3.17 ± 0.07	3.69 ± 0.05	2.49 ± 0.01	1.15 ± 0.05	2.58 ± 0.06
7.33	6.67 ± 0.04	3.85 ± 0.02	3.78 ± 0.02	3.54 ± 0.02	3.10 ± 0.01	2.57 ± 0.03
7.71	7.62 ± 0.03	3.71 ± 0.01	3.15 ± 0.01	2.60 ± 0.04	2.39 ± 0.04	2.08 ± 0.01
8.10	7.70 ± 0.01	3.41 ± 0.01	3.12 ± 0.01	2.80 ± 0.04	2.13 ± 0.01	1.91 ± 0.01
			20°C			
pH	Plus 2-DTP, without IHP K_{obs} X 10^{-2} (min^{-1})	[IHP]=90μM K_{obs} X 10^{-2} (min^{-1})	[IHP]=110μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=130μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=150μM K_{obs} x 10^{-2} (min^{-1})	[IHP]=180μM K_{obs} x 10^{-2} (min^{-1})
6.08	6.12 ± 0.01	3.25 ± 0.05	3.16 ± 0.04	3.07 ± 0.05	2.83 ± 0.01	2.80 ± 0.03
6.50	6.62 ± 0.01	2.38 ± 0.01	2.31 ± 0.06	2.52 ± 0.01	2.47 ± 0.05	2.18 ± 0.04
6.88	6.67 ± 0.01	3.25 ± 0.01	2.98 ± 0.01	2.72 ± 0.06	2.30 ± 0.01	2.27 ± 0.01
7.36	6.67 ± 0.01	3.95 ± 0.06	3.37 ± 0.02	3.19 ± 0.01	2.86 ± 0.06	2.82 ± 0.01
7.75	6.99 ± 0.02	3.64 ± 0.01	3.24 ± 0.02	3.07 ± 0.02	2.81 ± 0.03	2.24 ± 0.01
8.15	7.55 ± 0.02	5.60 ± 0.01	5.53 ± 0.02	5.39 ± 0.01	5.09 ± 0.01	4.58 ± 0.05
			25°C			
PH	Plus 2-DTP, without IHP K_{obs} X 10^{-2} (min^{-1})	[IHP]=90μM K_{obs} X 10^{-2} (min^{-1})	[IHP] 110μM K_{obs} x 10^{-2} (min^{-1})	[IHP] 130μM K_{obs} x 10^{-2} (min^{-1})	[IHP] 150μM K_{obs} x 10^{-2} (min^{-1})	[IHP] 180μM K_{obs} x 10^{-2} (min^{-1})
6.05	6.50 ± 0.02	2.21 ± 0.01	1.53 ± 0.01	1.[illegible]	1.72 ± 0.04	1.11 ± 0.03
6.43	6.77 ± 0.02	3.26 ± 0.02	2.96 ± 0.01	2.62 ± 0.06	2.66 ± 0.04	2.53 ± 0.06
6.85	7.84 ± 0.01	3.62 ± 0.03	3.14 ± 0.02	2.85 ± 0.03	2.68 ± 0.01	2.62 ± 0.04
7.24	8.95 ± 0.03	3.77 ± 0.01	3.24 ± 0.01	3.50 ± 0.05	3.70 ± 0.01	3.03 ± 0.01
7.61	9.77 ± 0.02	4.15 ± 0.03	4.06 ± 0.01	3.63 ± 0.06	3.97 ± 0.04	2.60 ± 0.01
8.08	10.41 ± 0.02	6.17 ± 0.01	5.69 ± 0.05	5.49 ± 0.05	5.15 ± 0.01	4.58 ± 0.04

considerably reduced reactivity of the aquomethemoglobin A and S sulfhydryl groups. Similar results were obtained for the corresponding azidomethemoglobin A and S derivatives at all temperatures and pH. A similar decrease in reactivity of β93 sulfhydryl groups of oxyhemoglobin A and methemoglobin A by IHP binding have been reported by Perutz, et. al. (1974) and Okonjo (1980). We have used this reduction in the reactivity rate caused by IHP to determine the binding constants of IHP to both azido and aquomethemoglobins A and S by the method of Marsh,et. al. (1975).

In this study, the dissociation constants of the complex between human metHb and IHP has been deduced and the binding constant calculated using Scrutton-Utter (1965) equation. A binding stoichiometry of one IHP molecule per hemoglobin tetramer was also assumed for this work.

The interaction of metHb with 2-DTP in the presence and absence of IHP may be described by the following scheme:

$$MetHb_4.IHP \rightleftharpoons MetHb_4 + IHP$$

$$MetHb_4 + 2DTP \text{ ---------- } (TP)_2Hb_4 + 2TP$$

$$MetHb_4.IHP + 2DTP \text{ ---------- } (TP)_2Hb_4.IHP + 2TP$$

Where K_D is the dissociation constant of metHb$_4$IHP complex. Assuming the equilibrium between metHb, IHP and metHB.IHP is reached before the reaction with 2-DTP is initiated and the concentration of IHP and 2-DTP are large compared with that of metHb, the following equation derived by Scrutton and Utter (1965) holds.

$$\frac{k}{k_o} = \frac{k_2}{k_1} + K_D \frac{1-k/k_o}{(IHP)} \quad (1)$$

Where k and k_o represent the pseudo first order rate constants of sulfhydryl reaction with methemoglobin towards 2-DTP in the presence and absence of IHP respectively.

The k and k_o were obtained from the values of least square slopes of plots such as shown in Figures 1a and b.

In Figures 2a and b, we present the plots of our data according to equation (1) for the methemoglobin molecules. Straight line plots were obtained. The values of the least square slopes K_D of these plots are presented in Table 2. The values of the dissociation constants reported in this table were obtained by an indirect method by observing the reduction in sulfhydryl reactivity of the methemoglobin molecules in the presence of IHP (Taiwo, et. al. 1979) assuming that a pre-equilibrium between organic phosphate and hemoglobin tetramer is already in existence before the 2-DTP reaction was initiated. The dissociation constants obtained in these studies range from 82 x 10^{-6} to 167 x 10^{-6} for aquomet Hb A and 43 x 10^{-6} to 137 x 10^{-6} for aquomet Hb S. The corresponding values for azidomet Hb A and S are much higher than these. For the present study, the values in Table 2 are adopted as true binding constants. These values show that IHP binds more strongly to methemoglobin S than to methemoglobin A. The analysis of our results show that the value sof the dissociation constants reported in Table 2 are much higher than those that have been assumed by previous investigators (Baldwin, 1975). The dissociation constant of 6 x 10^{-8}M reported by Edalji, et. al. (1976) for deoxyhemoglobin IHP complex in their proton uptake associated with binding studies is particularly lower than the data obtained by the present method. However, it is closer to that obtained by the kinetic studies of Gray and Gibson (1971) and that of Benesch and Benesch (1970) who found that at pH 7.0, 20°C, one mole of DPG binds to one mole of hemoglobin tetramer with a dissociation constant of 21 x 10^{-6}M.

However, the result of our work is in agreement with the work of Bunn and Briehl (1970). It is interesting to note that part of the results presented here are not comparable with the findings of Okonjo (1980) who worked on oxy and metHb A and S at pH 7.6, in phosphate buffer at 20°C. He found no detectable binding of IHP to metHb S. The data he reported are not strictly comparable with ours because they were obtained at different salt concentrations and in phosphate buffer at pH range 6-9.

Our result is not surprising in view of the known higher net-positive charge of hemoglobin S (Ingram, 1959) and the

Table 2a pH and temperature dependence of dissociation constants (K_D) of IHP with human Aquomethemoglobins A, S.

Aquomethemoglobin A

15°C			20°C			25°C		
pH	K_D	μM	pH	K_D	μM	pH	K_D	μM
6.13	82 191	± 2.90	6 08	73.636	± 7.70	6 03	65 322	± 5.81
6.53	99.184	± 7.42	6 45	90.492	± 3.01	6.44	69 833	± 2.73
6.94	140.885	± 2.54	6 89	122.880	± 4.72	6.81	78.703	+ 4.85
7.33	146 310	± 10.13	7.27	135.140	± 2.63	7.23	85.310	± 3.72
7 73	153.271	± 12.09	7.70	146.889	± 4.62	7 65	98 658	± 8.11
8.14	166.899	± 5.80	8.13	148.726	± 1.54	8.05	139.032	± 4.31

Aquomethemoglobin S

15°C			20°C			25°C		
pH	K_D	μM	pH	K_D	μM	pH	K_D	μM
6.14	43.099	± 1 07	6.08	36.333	± 3.13	6.05	32.285	± 1.97
6.52	76 670	± 3.76	6.50	64.863	± 3.52	6.43	48.417	± 2.06
6.95	96 383	± 5 12	6.88	79.157	± 2.54	6.85	56.628	± 3.23
7.33	117.549	± 2 56	7.36	95.109	± 2.71	7 24	63.351	± 1.43
7.71	124.039	± 2.94	7 65	106.575	± 2.43	7 61	68.856	± 4.62
8.19	137.580	± 12 31	8.15	120.288	± 7.11	8.08	96.532	± 3.71

Table 2b pH and temperature dependence of dissociation constants (K_D) of IHP from Azidomet Hb, A, S

Azidomethemoglobin A

15°C			20°C			25°C		
pH	K_D	μM	pH	K_D	μM	pH	K_D	μM
6.18	119 638	± 3.05	6.14	113.501	± 2.69	6.11	111.429	± 2.85
6.57	126.975	± 5.11	6.50	120.288	± 4.63	6.42	104.713	± 6.33
6.99	146.310	± 2.75	6.81	126.975	± 5.32	6.85	111.944	± 4.52
7.37	147.564	± 6 21	7 42	135.568	± 4.65	7.28	121.339	± 3.28
7.82	166.900	± 5.91	7.79	146.311	± 3.90	7.66	137.564	± 3.05
8.17	168.590	± 3.51	8 20	161.708	± 7.64	8.14	146.900	± 6 88

Azidomethemoglobin S

15°C			20°C			25°C		
pH	K_D	μM	pH	K_D	μM	pH	K_D	μM
6.20	65.322	± 2.16	6.23	64.542	± 4.30	6.21	62 873	± 3.52
6.57	67.161	± 5.38	6.60	65.381	± 3.75	6.55	63.833	± 2.21
7 11	95 868	± 2.78	6 98	90.860	± 2.66	6.83	76.777	± 3.25
7.39	119	6 ± 5 23	7 45	96.533	± 2.36	7.36	80.112	± 4.01
7.79	13	2 ± 7.85	7.79	109.598	± 2.57	7.80	91 793	± 3.78
8.24	139.032	± 6.52	8.21	123.507	± 4.01	8.18	103.158	± 5.80

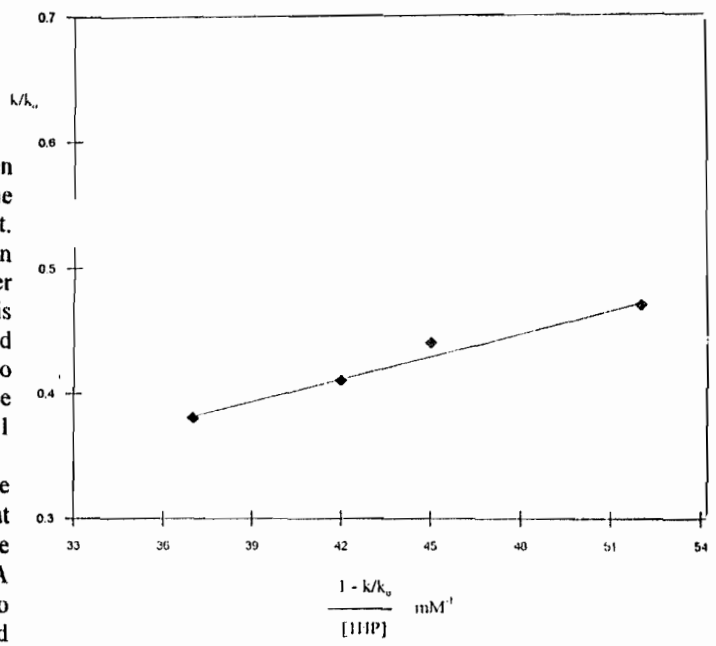

Figure2a Scrutton-Utter plot of the kinetic data for 2-DTP reacting with human aquomethemoglobin A in the presence of IHP at various concentrations. pH 7 2, 15°C. Experimental conditions as in figure 1b

known different electrophoretic mobilities of hemoglobins A and S (Pauling, et. al. 1949). Methemoglobin S carries a neutral valine residue at the β6 of the beta chain from the carboxy terminal which is replaced in methemoglobin A by glutamic acid. If the IHP binding site is the same in these two methemoglobins, our result would imply that the geometrical arrangement of the basic groups at the IHP binding site must be considerably different in methemoglobins A and S.

Numerous experimental evidence exist for a number of differences between hemoglobin A and S; there is a difference in the solubility of the two molecules in concentrated phosphate buffers (Benesch, et. al. 1977) a difference in optical activity has been observed between the carbon monoxy derivatives of the two proteins (Fronticelli, 1978), antibody fractions specific to HbS have been purified, 303 MNR measurements show that there is a difference in the resonance of some surface residues in the two molecules (Benesch and Benesch, 1970). Consequently, we must conclude that these differences in their reactivity or physical properties cannot easily be explained purely on the basis of the single point mutation at the A3(6) β position.

The results for methemoglobins A and S reported in the present study, suggest that there is conformational differences between methemoglobin A and S in the vicinity of the β6 (A3) residue. Strong experimental evidence exist for interactions between Gluβ6(A3) of methemoglobin A and a number of amino acid residues around it. These interactions had been demonstrated to be absent in methemoglobin S (Baldwin, 1975). The absence of these interacting molecules would mean that the tertiary structure near the presumed IHP binding site is different in metHbS from what it is in metheglobin A (Okonjo, et. al. 1996). Such difference in structure would account for the fact that IHP binds more strongly to metHbS than to metHbA. IHP have a pronounced effect on oxygen equilibrium curve of hemoglobin (Benesh and Benesh, 1967; Chanutin, 1967). The shift in the equilibrium curve to the right causes a greater oxygen pressure to be required in achieving the desired percentage oxygenation. The decrease in oxygen affinity upon IHP binding produces abnormal tertiary structure in metHbS and hence sickling.

Figure 3 shows pH dependency of the binding of IHP to aquo and azidomethemoglobin A. The binding of IHP to all the methemoglobins decreased with increasing pH. Comparing the curve of aquometHb A with those of aquometHbS, it is obvious that the binding of IHP to all the methemoglobins decreases with increasing pH. The same is also evident from Figure 4 which shows the pH dependence of IHP binding to their azide derivatives. For a given pH, the IHP binding constant of the human azide methemoglobin is consistently lower than that obtained for the corresponding aquometHbS. The lower values of IHP binding to metHb species at higher pH shows very weak IHP binding at higher pH where the methemoglobins would carry a net negative charge.

The addition of an azide ion to metHb not only decreases its net charge by one but also gives rise to a decrease in the PKa of ionised group on the hemoglobin and as a result, protons are taken up. The change in the pH and consequently ionisation of charged groups on the hemoglobin molecule produces different conformational changes in these molecules. This effect results in changes in the structure at the

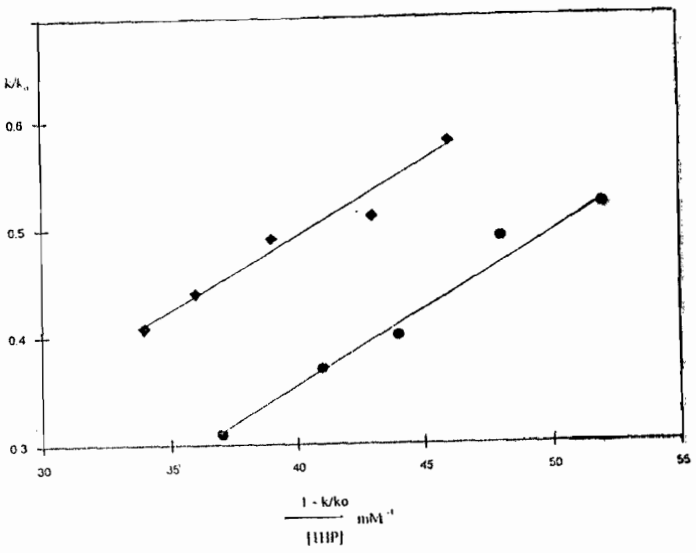

Figure 2b Scrutton- Utter plot of the kinetic data for 2-DTP reacting with human Aquomethemoglobin A and Azidomethomoglobin A at pH 7.6, 20°C. Experimental conditions as in figure 1b

● circle, Aquomethemoglobin A, ◆square Azidomethemoglobin A

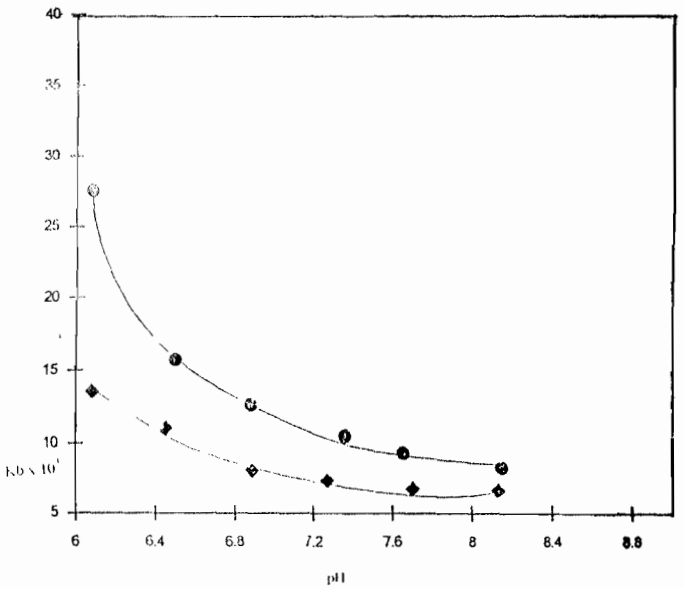

Figure 3 pH dependence of the binding constant (kb) of IHP to human Aquomethemoglobin A, ✗ S , The condition of experiment as in figure 1b

◆ square, aquomethemoglobin A, ● circle, aquomethemoglobin S

IHP binding site and in the vicinity of the β93 sulfhydryl residue of the aquo and azido methemoglobins. In spite of the differences between methemoglobin A and S at the β6 of their beta chains, the two methemoglobins have the same affinity for IHP if they are compared not at the same pH but at pH sufficiently different such that ionization of the other side chains presumably histidine residue compensates for the charge difference.

Previous studies have shown that the same residues in both aquo and azidomethemoglobins are involved in the binding of IHP (Arnone, 1974; Edalji, et. al. 1976; Bunn and Briehl 1970; Perutz, et. al. 1974). In the absence of IHP, these residues

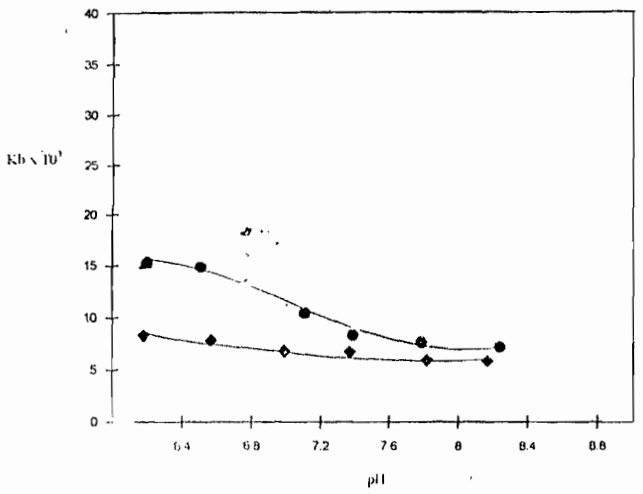

Figure 4 pH variation of binding constant (Kb) of IHP binding to human Azidomethemoglobin A compared with the Azidomethemoglobin S at 15°C as a function of pH.
◆ square, azidomethemoglobin A, ● circle, azidomethemoglobin S,

supposedly have the same PKa value in both the aquo and the azidomethemoglobin structures. If the IHP binding groups contribute to the differential reactivities of the two hemoglobins, they cannot be solely responsible for this. Some charged groups different from those making up the binding site must be involved. It is therefore suggested that IHP binds more strongly to aquomet HbA than to azidomethemoglobin A as a result of the small changes in 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.

We therefore, conclude that, binding reactions of IHP to methemoglobin and invariably the binding of organic phosphate to hemoglobin in general are intimately related to the amino acid composition and conformational states of each hemoglobin. Also, decrease in oxygen affinity upon IHP binding produces abnormal tertiary structure in HbS and hence sickling.

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