THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 8, Issue of April 25, pp. 3274–3277, 1980
Printed in U.S.A.

Effect of Organic Phosphates on the Sulfhydryl Reactivities of Oxyhemoglobins A and S*

(Received for publication, August 22, 1979)

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The β^{93} sulfhydryl groups of oxyhemoglobins A and S display a difference in reactivity with 5,5′-dithiobis-2-nitrobenzoic acid. It is concluded that this difference arises from differences in tertiary structure in the vicinity of the β^{93} site.

Organic phosphates decrease the β^{93} sulfhydryl reactivity. We have used this effect to measure the organic phosphate binding constants. Hemoglobin S binds organic phosphates very weakly compared to hemoglobin A. This result indicates that the structure at the organic phosphate binding site is different in the two oxyhemoglobins and may be the result of differences in the structure of the NH_2-terminal ends of the β chains.

Sickle cell hemoglobin (HbS[1]) differs from normal hemoglobin (HbA) by the replacement of a glutamic acid residue in HbA by a relatively large valine residue at position β^6 (A3) (1). The clinical consequences of this replacement of a polar residue by a nonpolar residue at the surface of the molecule are well known and have been extensively studied with respect to the behavior and properties of deoxy-HbS (2–4). However, apart from the difference in electrophoretic mobility between HbS and HbA noted by Pauling *et al.* (5), it had been tacitly assumed until recently that in the liganded state both hemoglobins have similar, if not identical, structures. An extension of this assumption would imply that the structure at the organic phosphate binding site would be the same in both molecules.

Organic phosphates regulate the oxygen binding properties of hemoglobin by binding at the entrance to the molecular dyad axis in deoxyhemoglobin (6, 7). However, organic phosphates are also known to bind to oxyhemoglobin (8, 9) in a 1:1 ratio (10, 11), and there is strong experimental evidence (11, 12) suggesting that the same binding site is involved in the oxy as in the deoxy structure.

In this paper we report on the binding of organic phosphates to HbA and HbS. We have determined organic phosphate binding constants by monitoring the reduction in the reactivity of the β^{93} (F9) sulfhydryl groups caused by organic phosphates. Our results indicate that the structures at the organic phosphate binding site and in the vicinity of the β^{93} sulfhydryls are different in the two oxyhemoglobins as well as in the corresponding methemoglobins.

MATERIALS AND METHODS

5,5′-Dithiobis-2-nitrobenzoic acid and 2,3-diphosphoglycerate as the pentacyclohexylammonium salt were purchased from Sigma, St. Louis and were used without further treatment. Inositol hexaphosphate was obtained from British Drug Houses. Analytical grade reagents were used. Freshly drawn human blood from a normal donor and from a sickle cell patient was obtained from the Hematology Clinic, University College Hospital, Ibadan.

The concentration of DTNB was determined by dissolving a known weight in 0.05 M Tris-HCl buffer, pH 7.6, and was checked spectrophotometrically by measuring the absorption at 412 nm after reaction with excess mercaptoethanol. A molar extinction coefficient of 13,600 cm^{-1} was assumed for TNB (13). No significant difference was observed between both methods of determination. Solutions of organic phosphates were pretitrated to pH 7.6 and suitably diluted with 0.05 M Tris-HCl buffer, pH 7.6. Hemoglobin was prepared as described previously (14) and stripped of phosphates by passing through long columns of Sephadex G-25 equilibrated with 0.05 M Tris-HCl buffer, pH 7.6.

The reaction of DTNB with hemoglobin was monitored at 412 nm on a Zeiss PMQ II spectrophotometer. Signals were recorded on an X-t chart recorder. A 10-cm^3 aliquot of hemoglobin was pipetted into a spectrophotometric cell of 20 mm path length. The cell was placed in the thermostated cell compartment and allowed to attain temperature equilibrium. The reaction was started by adding DTNB to the sample cell with a Finn pipette. Stirring of the mixture was ensured with a magnetic stirrer fitted to the base of the cell holder. Constant cell temperature was maintained with a Lauda Table cryostat model TUK 30D. The ionic strength of all solutions was maintained at 0.1 M by addition of suitable amounts of sodium chloride. The pH of all solutions was measured with a Radiometer PHM 4d pH meter. No significant change of pH was observed between solutions.

RESULTS AND DISCUSSION

In Fig. 1 we compare the rates of reaction with DTNB of stripped HbS and HbS in the presence of IHP and DPG. It is apparent that although IHP diminishes the reactivity of the sulfhydryl groups, DPG does not have a similar effect. On the other hand, both organic phosphates diminish the sulfhydryl reactivity of HbA (Fig. 2). Similar results were obtained for methemoglobin: IHP and DPG diminish the sulfhydryl reactivity of MetHbA but have no detectable effect on MetHbS. Decreased reactivity of the β^{93} sulfhydryl groups of oxyhemoglobin on IHP binding has been reported previously (15). The implication of our result is that both IHP and DPG bind to HbA, but only IHP binds strongly enough to HbS to produce a detectable change in sulfhydryl reactivity. Similarly, none of the organic phosphates binds strongly to MetHbS.

We have used the organic phosphate effect on sulfhydryl reactivity to determine the organic phosphate binding constants by the method of Marsh *et al.* (16). Such determinations were only possible for IHP plus HbA, DPG plus HbA, IHP

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[1] The abbreviations used are: HbA, oxyhemoglobin A; HbS, oxyhemoglobin S; MetHbA, methemoglobin A; MetHbS, methemoglobin S; Hb_4, hemoglobin tetramer; IHP, inositol hexaphosphate; DPG, 2,3-diphosphoglycerate; DTNB, 5,5′-dithiobis-2-nitrobenzoate; TNB, 5-thio-2-nitrobenzoate.

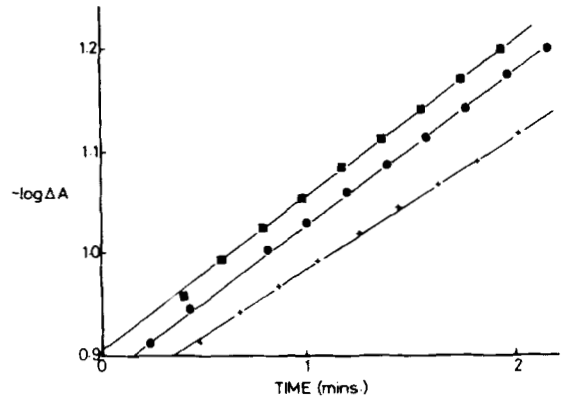

FIG. 1. **Pseudo-first order rate plots for the reaction of stripped oxyhemoglobin S with DTNB and, for comparison, similar plots in the presence of IHP and DPG.** *Circles*, stripped HbS; *squares*, HbS plus 386.4 μM DPG; *crosses*, HbS plus 193.2 μM IHP. Conditions: 0.05 M Tris-HCl buffer, pH 7.6, ionic strength 0.1 M (added salt NaCl), 27°C; DTNB, 144 μM; Hb_4, 3.83 μM. For clarity, the plot for DPG has been displaced upwards along the ordinate axis by 0.01.

plus HbS, IHP plus MetHbA, and DPG plus MetHbA, the systems where decreases in sulfhydryl reactivity were observed.

Under our experimental conditions, a binding stoichiometry of 1 organic phosphate molecule per hemoglobin tetramer is expected (*cf.* Refs. 10 and 11). The following scheme therefore applies to our system:

$$Hb_4 + X \overset{K_d}{\rightleftarrows} Hb_4 \cdot X$$

$$Hb_4 + 2DTNB \xrightarrow{k_0} (TNB)_2 \cdot Hb_4 + 2TNB$$

$$Hb_4 \cdot X + 2DTNB \xrightarrow{k_x} (TNB)_2 \cdot Hb_4 \cdot X + 2TNB$$

where X represents organic phosphate and K_d is the organic phosphate dissociation constant. We may assume complete kinetic decoupling between the DTNB reaction and the equilibration of X with Hb_4 (14). The concentrations of both DTNB and organic phosphates are large compared to the hemoglobin concentration (see figure legends); the equation of Scrutton and Utter (17) is therefore applicable, *i.e.*

$$V_x/V_0 = k_x/k_0 + K_d \cdot \frac{1 - V_x/V_0}{[x]} \qquad (1)$$

where V_x and V_0, the pseudo-first order rates of the DTNB reaction in the presence and absence of organic phosphate, respectively, are obtained from the least squares slopes of plots such as those in Figs. 1 and 2.

Table I shows the values of V_0, the rate of reaction of stripped hemoglobin with DTNB. A comparison of the values for HbA and MetHbA shows that the sulfhydryls of MetHbA are 1.4 times more reactive than those of HbA. Similarly, the sulfhydryls of MetHbS are 1.8 times more reactive than those of HbS. A similar difference in sulfhydryl reactivity was observed by Perutz *et al.* (15) for carbonmonoxyhemoglobin and MetHbA, the latter having the more reactive sulfhydryls. A structural explanation for this result is found in the x-ray data of Heidner *et al.* (18). These authors have shown that carbonmonoxyhemoglobin and MetHbA have different tertiary structures near the β^{93} site, the sulfhydryl groups of MetHbA being more exposed than those of carbonmonoxyhemoglobin. Since carbonmonoxy- and oxyhemoglobin have similar structures (18), it may be inferred from our result that MetHbA and HbA differ in structure near the β^{93} site. A similar conclusion may be drawn for MetHbS and HbS. A comparison of the rates for HbA and HbS (Table I) shows that HbS sulfhydryls react 1.2 times as fast as HbA sulfhydryls. From the above argument, we conclude that HbA and HbS have different tertiary structures near the β^{93} site and that the sulfhydryls of HbS are more exposed than those of HbA. Similarly, the sulfhydryls of MetHbS are more exposed than those of MetHbA.

Figs. 3, 4, and 5 show the plots of the data according to Equation 1 for HbA, HbS, and MetHbA, respectively. As expected, straight line plots are obtained.

Table II gives the values of the least squares slopes, K_d, of these plots as well as the ordinate intercepts, k_x/k_0, which give a measure of the maximum protection of the sulfhydryl groups against reaction with DTNB (16).

The values of the dissociation constants reported in Table II have been obtained by an indirect method, by observing the reduction in sulfhydryl reactivity in the presence of organic phosphate, assuming (14) that a pre-equilibrium between organic phosphate and hemoglobin tetramer is already in existence before the DTNB reaction. A comparison of our value for IHP binding to HbA with the value obtained by the direct method of Gray and Gibson (10) shows that our value is a reasonable one to expect under our experimental condi-

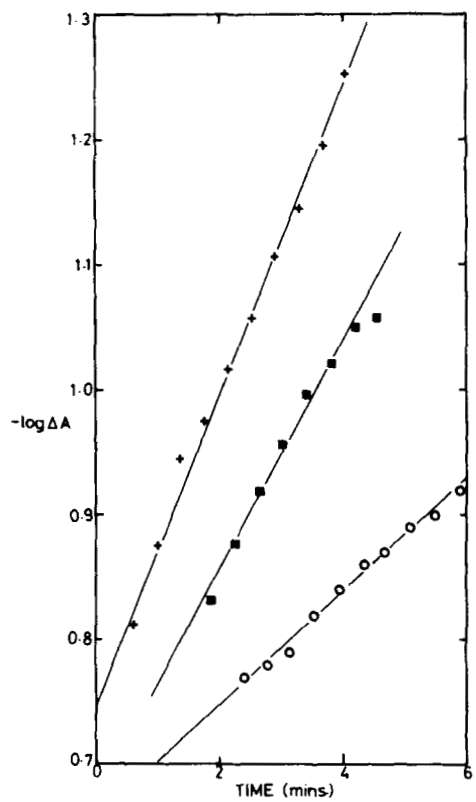

FIG. 2. **Pseudo-first order rate plots for the reaction of stripped oxyhemoglobin A with DTNB and, for comparison, similar plots in the presence of IHP and DPG.** *Crosses*, stripped HbA; *squares*, HbA plus 193.2 μM DPG; *circles*, HbA plus 193.2 μM IHP. Conditions as in Fig. 1.

TABLE I

Rates of reaction of stripped hemoglobins with DTNB

Conditions were as in Fig. 1. Values of V_0 are subject to a standard error of ±0.002 min^{-1}.

Hemoglobin	V_0 min^{-1}
HbA	0.127
MetHbA	0.178
HbS	0.151
MetHbS	0.275

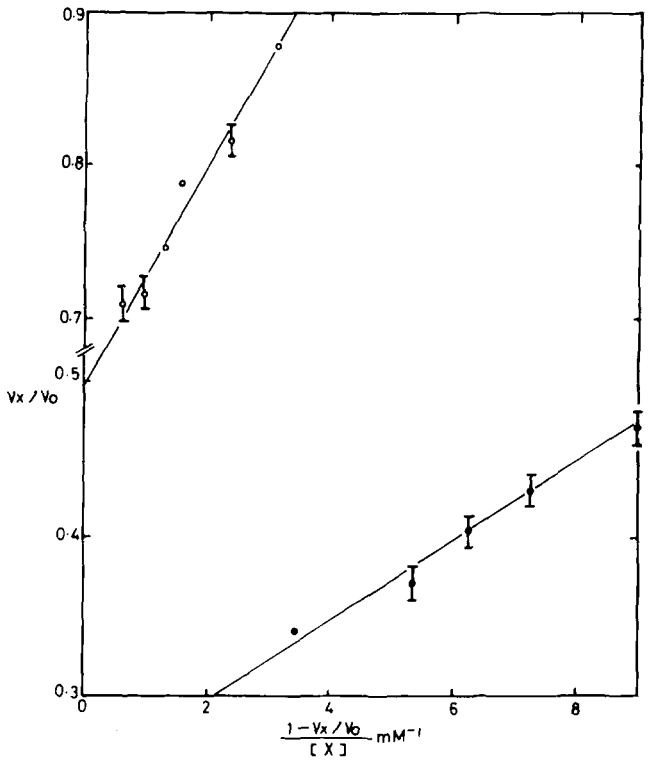

FIG. 3. **Scrutton-Utter plots of the kinetic data for DTNB reacting with HbA in the presence of IHP and DPG at various concentrations (*cf.* Equation 1 of text).** Conditions as in Fig. 1. *Closed circles*, IHP; *open circles*, DPG.

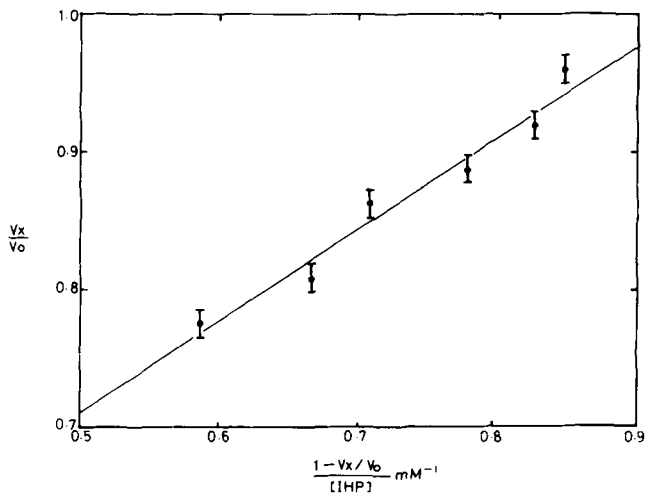

FIG. 4. **Scrutton-Utter plot of the kinetic data for DTNB reacting with HbS in the presence of IHP at various concentrations (*cf.* Equation 1 of text).** Conditions as in Fig. 1.

tions. Furthermore, as may be expected if the values in Table II are true binding constants, IHP binds 2.8 times as strongly to HbA as DPG. This is the result to be expected purely on the basis of electrostatic interactions between the organic phosphates and the basic groups at the binding site. We conclude that the values reported in Table II are true binding constants.

A very interesting feature of our results is seen from a comparison of the dissociation constants of IHP from HbA and HbS. Table II shows that IHP binds 27 times as strongly to HbA as it does to HbS. It is also remarkable that DPG binds 10 times as strongly to HbA as IHP does to HbS. The lack of a DPG effect on the sulfhydryl reactivity of HbS (see Fig. 1) also indicates very weak DPG binding. There is no indication that either organic phosphate binds to MetHbS whereas they bind to MetHbA. These results are rather surprising in view of the known higher net positive charge of HbS (1) and the known differential electrophoretic mobilities of HbA and HbS (5). If the organic phosphate binding site is the same in the oxy as in the deoxy structure (11, 12), these results would imply that the geometrical arrangement of the basic groups at the organic phosphate binding site must be considerably different in HbA and HbS, and must be such in HbS that organic phosphate binding is not favored.

A number of interesting differences between HbA and HbS have been reported before: HbS is unstable compared to HbA (19); NMR measurements show that the resonance of some surface residues of HbS are altered compared to HbA (20); differences in the polarization of the resonance Raman lines have been observed (21); there are differences in optical activity in the Soret region between the cyanoferric forms of the β chains (22) and between the carbonmonoxy derivatives of the intact tetramers (23); differences in solubility have been observed in concentrated phosphate buffers (24); finally, Curd *et al.* (25) have purified antibody fractions specific for HbS and their result suggests a possible modification of the NH_2-terminal region of the β chain of HbS compared to HbA.

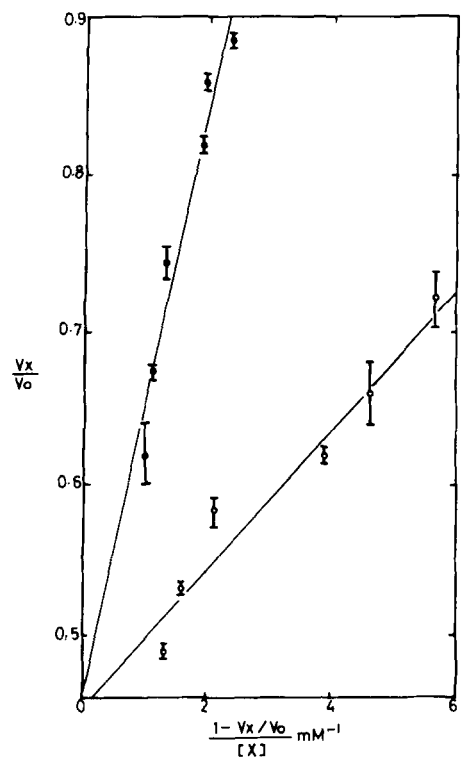

FIG. 5. **Scrutton-Utter plots of the kinetic data for DTNB reacting with MetHbA in the presence of IHP and DPG at various concentrations.** Conditions as in Fig. 1. *Closed circles*, IHP; *open circles*, DPG.

TABLE II

Values of least squares slopes, K_d, and ordinate intercepts, k_x/k_0 of the plots in Figs. 3, 4, and 5

Compare these values with Equation 1 of text.

System	K_d	k_x/k_0
	μM	
IHP plus HbA	24.4 ± 1	0.25 ± 0.004
IHP plus HbS	670.4 ± 4	0.38 ± 0.002
DPG plus HbA	67.6 ± 12	0.66 ± 0.04
IHP plus MetHbA	191.5 ± 13	0.46 ± 0.04
DPG plus MetHbA	46.4 ± 9	0.45 ± 0.01

Our results show that HbS binds organic phosphates very weakly compared to HbA. Since the organic phosphate binding site is close to the NH_2-terminal region of the β chains, we may infer that the structure in this region of the β chains is different compared to HbA. The explanation usually given for filamentous aggregation in deoxy-HbS is that a nonpolar side chain is exposed at the molecular surface. That being the case, it may safely be assumed that this nonpolar side chain is not exposed in the oxy conformation since HbS does not aggregate. There is therefore of necessity a conformational difference between HbS and HbA in the vicinity of the β^6 (A3) residue. For one thing, the hydrogen bond between Glu β^6 (A3) of HbA and the nitrogen of Leu β^3 (NA3) would be absent in HbS; so would the electrostatic interaction between Glu β^6 and Lys β^{132} (H10). The absence of these interactions would mean that the tertiary structure near what is presumably the organic phosphate binding site is different in HbS from what it is in HbA. Such a difference in structure could account for the fact that HbA binds IHP 27 times as strongly as HbS, and also for the fact that even DPG binds 10 times as strongly to HbA as IHP does to HbS, in spite of the charge difference between the two organic phosphates.

The finding that HbS binds organic phosphates far less strongly than HbA may be relevant to an understanding of the results of Bunn and Briehl (26). These authors find that DPG decreases the oxygen affinity of hemoglobin A more than that of hemoglobin S. This result may be rationalised if it is assumed that deoxyHbA binds DPG more strongly than deoxyHbS. The result for hemoglobin C (26) is in line with this explanation. In this hemoglobin, a lysine residue replaces the glutamic acid residue in hemoglobin A at the β^6 position. The extra positive charge at a point not too distant from the organic phosphate binding site should result in a greater decrease by DPG of the oxygen affinity of hemoglobin C compared to hemoglobin A.

The result that DPG binds more strongly to MetHbA than IHP (Table II) (in contrast to HbA) suggests that in the ferric state steric considerations may be more important than considerations of charge, and it is more difficult to bind the bulky IHP molecule than the small DPG molecule. No conclusion can be drawn from the values of k_x/k_0 (Table II) except that these values do not appear to be related to the strength of binding of organic phosphates. Similar results were obtained by Marsh *et al.* (16).

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