The Allosteric Effect of Inositol Hexasulfate on Oxygen Binding by Hemoglobin†

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ABSTRACT: Myoinositol hexasulfate (IHS) is a powerful allosteric effector of oxygen binding by hemoglobin. It binds to deoxyhemoglobin at the same site as 2,3-diphosphoglycerate (DPG) and inositol hexaphosphate (IHP) with an affinity which is intermediate between that of the two phosphate esters. The binding constant calculated from the displacement of the oxygenation curve in the presence of low concentrations of IHS is 0.9×10^{-6} M. The value obtained directly from the number of protons bound as a function of IHS concentration is 1.0×10^{-6} M. The agreement between these two independent measurements provides an experimental verification of the empirical equation, relating the oxygen affinity to the binding constants, proposed previously (Benesch, R. E., Benesch, R., Renthal, R., and Gratzer, W. B. (1971), *Nature (London), New Biol. 234,* 174).

The allosteric site formed by the terminal regions of the β chains in the deoxy conformation of hemoglobin binds a variety of phosphate esters. This results in a stabilization of the deoxy conformation and a corresponding decrease in the oxygen affinity (Benesch and Benesch, 1974). 2,3-Diphosphoglycerate (DPG)[1] and adenosine triphosphate (ATP) in human erythrocytes (Benesch and Benesch, 1967; Chanutin and Curnish, 1967) and inositol pentaphosphate (IPP) in avian red cells (Johnson and Tate, 1969) are naturally occurring cofactors which facilitate oxygen release from hemoglobin to the tissues. The most powerful of all the allosteric polyphosphates is myoinositol hexaphosphate (IHP) (Benesch et al., 1968; Bunn and Guidotti, 1971). It therefore seemed of interest to investigate its sulfate analogue, i.e., myoinositol hexasulfate (IHS).

Experimental Section

Materials and Methods. IHS was first prepared by Takahashi and Egami (1959). For the experiments reported here it was purchased from Terra-Marine Bioresearch, La Jolla, California, where it was synthesized by the method of Fatiadi (1970) as the hexapotassium salt. The myo configuration was confirmed by conversion to the hexacetate (Fatiadi, 1970).

IHP was obtained from Sigma Chemical Co., St. Louis, Missouri, as sodium phytate type V.

DPG was purchased from Calbiochem, La Jolla, California, as the cyclohexylammonium salt and converted to the free acid as described previously (Benesch et al., 1969). The solutions of the phosphate esters were standardized by total phosphate analysis (Ames and Dubin, 1960) and solutions were freshly prepared at frequent intervals. Normal hemoglobin was prepared from human blood as described previously (Benesch et al., 1972). Blood containing Hb Providence was obtained through the courtesy of Dr. P. McCurdy. The mutant component $\beta 82^{lys \rightarrow asp}$ (Charache et al., 1975) was isolated by chromatography on carboxymethyl-Sephadex C-50 using a 0–0.1 M NaCl gradient in 0.05 M phosphate buffer (pH 6.7). The preparations of the two chemically modified hemoglobins, i.e., one which is covalently bridged across the polyphosphate binding site and another in which the two N-terminal valines of the β chains are substituted with pyridoxal phosphate, have been described previously (Benesch, R., et al., 1975; Benesch, R. E., et al., 1972).

Oxygen equilibrium curves were determined in 0.05 M Bistris buffers at 20 °C as described before (Benesch R., et al., 1965; Benesch, R. E., et al., 1973).

For the direct determination of the binding constant of IHS to deoxyhemoglobin, 75 ml of a 1×10^{-5} M solution of hemoglobin in 0.1 M NaCl was deoxygenated by bubbling argon through the solution after the addition of a drop of octyl alcohol and the pH was adjusted to 7.30 using a Radiometer PHM-64 pH meter. The solution was titrated with 0.01 M IHS and, after the addition of each increment, the amount of 0.01 M HCl necessary to restore the original pH (x) was recorded. The titration was continued until further additions of IHS did not alter the pH of 7.30. The maximum amount of HCl added per mole of Hb (a) is equivalent to the protons associated when 1 mol of IHS is bound assuming a stoichiometry of one IHS per hemoglobin tetramer. The fraction bound after each addition of IHS is then given by x/a from which its concentration can be calculated by $C_{bound} = (x/a)(C_{Hb})$ and the free concentration of IHS is $C_{free} = C_{total} - C_{bound}$.

Results and Discussion

The effect of IHS on the oxygenation curve of hemoglobin is compared with that of DPG and IHP in Figure 1. It is clear that IHS causes a decrease in oxygen affinity which is intermediate between that produced by the two phosphate esters.

Since 1 mol of DPG and 1 mol of IHP are bound to the same site in the tetramer of deoxyhemoglobin (Benesch and Benesch, 1969; Arnone, 1972; Arnone and Perutz, 1974), it must be assumed that the hexasulfate is likewise bound at this locus with the same stoichiometry. This is supported by the observations that like IHP, IHS has no effect on a hemoglobin in which this binding site is obstructed either by a cross-link (Benesch et al., 1975) or by pyridoxylation of both N-terminal amino groups (Benesch et al., 1972) (Figure 2). Hb Providence, where one of the crucial residues in the binding site has been replaced (Charache et al., 1975), shows only a minimal response to both cofactors (Figure 2).

The magnitude of the shift in oxygen affinity expressed as $\log p_{50}$ is shown as a function of the effector concentration for all three compounds in Figure 3. The lines in this figure were calculated from the equation (Benesch et al., 1971)

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[1] Abbreviations used are: IHS, myoinositol hexasulfate; DPG, 2,3-diphosphoglycerate; PLP, pyridoxal 5′-phosphate; ATP, adenosine triphosphate; IHP, myoinositol hexaphosphate; IPP, myoinositol pentaphosphate; Hb, hemoglobin; Bistris, *N*,*N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

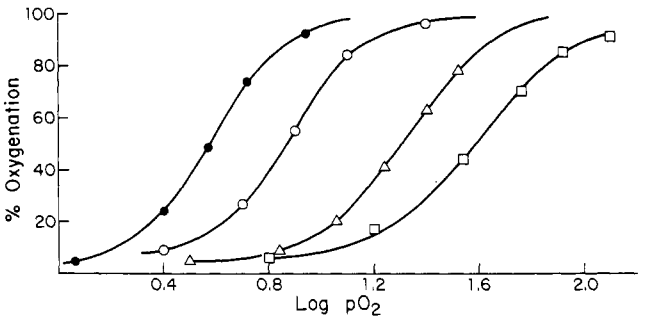

FIGURE 1: Effect of DPG, IHS, and IHP on the oxygenation curve of normal hemoglobin. (●—●) Stripped hemoglobin; (○—○) stripped hemoglobin + 2.5 × 10^{-4} M DPG; (△—△) stripped hemoglobin + 2.5 × 10^{-4} M IHS; (□—□) stripped hemoglobin + 2.5 × 10^{-4} M IHP. Hemoglobin concentration, 5 × 10^{-5} M, in 0.05 M Bistris buffer (pH 7.3). Total [Cl^-], 0.1 M. Temperature, 20°C.

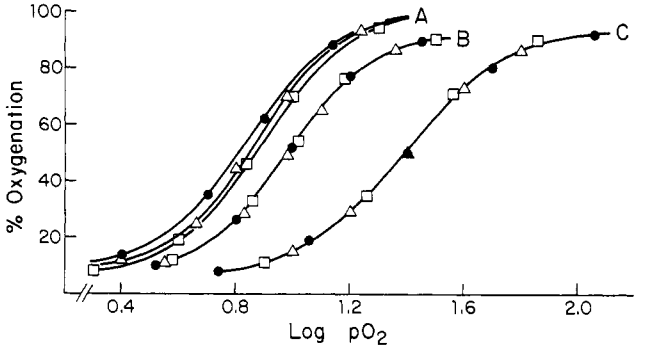

FIGURE 2: Effect of IHS and IHP on the oxygenation curve of some modified hemoglobins. (A) Hb Providence; (B) Di PLP Hb ($\alpha_2{}^A\beta_2{}^{PLP}$); (C) cross-linked Hb ($\alpha^A\overline{\beta\beta}\alpha^A$); (●—●) Hb alone; (△—△) Hb + 2.5 × 10^{-4} M IHS; (□—□) Hb + 2.5 × 10^{-4} M IHP. Hemoglobin concentration, 5 × 10^{-5} M, in 0.05 M Bistris buffer (pH 7.3). Total [Cl^-]. 0.1 M. Temperature, 20 °C.

$$\log p_{50} = \text{constant} + \frac{1}{n}\log\frac{1 + C/K_D}{1 + C/K'} \qquad (1)$$

In the case of DPG it was shown previously that K_D calculated from this equation agrees very well with the dissociation constant of the deoxyhemoglobin DPG complex obtained from direct binding measurements under identical conditions (Renthal, 1972). K' represents an average value for the binding of DPG to partially oxygenated intermediates with an effect on p_{50} opposite to that due to the binding expressed by K_D and was found to be 3 × 10^{-4} M. It is evident from eq 1 that, for a tightly bound cofactor such as IHS and IHP, log p_{50} reaches a limiting value at high concentrations when $C \gg K' \gg K_D$, i.e.

$$\log p_{50} = \text{constant} + \frac{1}{n}\log\frac{K'}{K_D} \qquad (1a)$$

It can therefore be concluded from the results in Figure 3 that the ratio of the two constants, K'/K_D, which is about 20 for DPG (Benesch et al., 1971), increases about sixfold for IHS and about eightfold for IHP (Table I).

On the other hand, at concentrations where C is small compared with K', eq 1 reduces to:

$$\log p_{50} = \text{constant} + \frac{1}{n}\log(1 + C/K_D) \qquad (1b)$$

Application of this equation requires oxygenation data at low cofactor concentrations and even lower hemoglobin concentration, so that the free cofactor concentration can be regarded as constant. The results are shown in Table I.

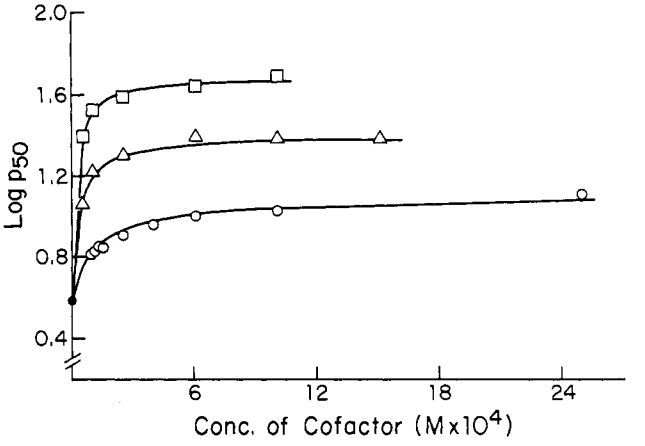

FIGURE 3: Relation between oxygen affinity and cofactor concentration. The lines are drawn from the equation, $\log p_{50} = 0.58 + (1/n)\log[(1 + C/K_D)/(1 + C/K')]$, using the values of K_D and K' in Table I. p_{50} is the oxygen pressure at 50% oxygenation. n is the exponent in Hill's equation; $Y/(1 - Y) = K(p_{O_2})^n$, where Y is the fractional saturation with oxygen. C is the concentration of cofactor. (○—○) DPG; (△—△) IHS; (□—□) IHP. Hemoglobin concentration, 5 × 10^{-5} M, in 0.05 M Bistris buffer (pH 7.3). Total [Cl^-], 0.1 M. Temperature, 20 °C.

TABLE I.

Compd	$K_D{}^a$ (M × 10^6)	K'/K_D	K' (M × 10^4)
DPG	14.3	20	2.9
IHS	0.92	128	1.2
IHP	0.5	166	0.83

Compd[b]	log p_{50}	n
None	0.51	3.0
2.5 × 10^{-5} M DPG	0.65	2.9
5 × 10^{-5} M DPG	0.73	2.9
2.5 × 10^{-5} M IHS	1.09	2.5
5 × 10^{-5} M IHS	1.18	2.5
2.5 × 10^{-5} M IHP	1.40	2.0
5 × 10^{-5} M IHP	1.47	2.0

[a] These constants were calculated from eq 1b and the p_{50} values from the lower portion of this table. [b] Hemoglobin concentration 5 × 10^{-6} M in 0.05 M Bistris (pH 7.3). Total [Cl^-] was 0.1 M, temperature 20 °C.

It should be noted that, while K_D decreases substantially for the series DPG → IHS → IHP, the corresponding decrease in K' is not as great. This might reflect the greater difficulty which the more complex inositol esters have in fitting into a partially distorted binding site.

Direct measurement of K_D by methods involving membranes such as equilibrium dialysis or ultrafiltration is difficult for DPG and becomes impossible for IHP. An alternative method, i.e., the measurement of protons associated with the binding of the polyanion, cannot be used in the case of the phosphate esters because of the strong buffering of these groups at neutral pH. IHS binding, on the other hand, can be measured very accurately since about two protons are associated per mole bound at pH 7.3 and the six sulfate residues are completely ionized at this pH. This approach was used earlier by Bucci (1974) for measuring the binding of benzenepentacarboxylate to deoxyhemoglobin.

A binding curve of IHS to deoxyhemoglobin, at a concentration of 1 × 10^{-5} M and the same pH, salt concentration, and temperature as those used for the oxygenation curves in Figures

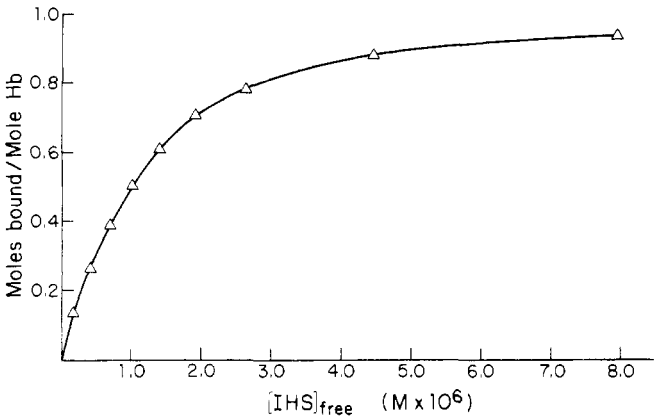

FIGURE 4: Binding of IHS to deoxyhemoglobin at 20 °C. The measurements and calculations are described in the text. The dissociation constant measured from this curve is 1.0×10^{-6} M.

1 and 3, is shown in Figure 4. The dissociation constant, 1×10^{-6} M, is in very good agreement with the value of K_D of 0.9×10^{-6} M derived from the oxygenation data (Table I). The strength of binding of IHS is similar to that reported for benzenepentacarboxylic acid by Bucci (1974), although the data are not strictly comparable because they were obtained at different salt concentrations.

The close agreement between the independently measured dissociation constant for IHS (1.0×10^{-6} M) and that estimated from the oxygenation data (0.92×10^{-6} M) supports the validity of eq 1. It should be noted that the value of n used in this equation is the Hill coefficient found experimentally, i.e., 2.5 (Table I). By contrast, if n is taken to be 4 (Baldwin, 1975), the calculated value of K_D (1.0×10^{-7} M) becomes ten times lower.

The p_{50} values of hemoglobin as a function of IHP concentration Table (I) were used to calculate K_D for the hexaphosphate with the result shown in Table I.[2] The value of 5×10^{-7} M is, of course, considerably higher than previous estimates, i.e., $\sim 10^{-8}$ (Gray and Gibson, 1971) and $\sim 10^{-10}$ (Baldwin, 1975). Both of these were based on a measured binding constant to oxyhemoglobin estimated to lie between 0.1 and 10×10^{-6} M (Gray and Gibson, 1971). In any case, the parameter needed to derive K_D from oxygenation data is not the binding constant for fully oxygenated hemoglobin, but should include that for partially oxygenated intermediates, i.e., K' in eq 1. In addition, the very low estimate of Baldwin (1975) again involves the use of a Hill coefficient of 4, i.e., the assumption that the concentration of partially oxygenated intermediates is zero. This is not in agreement with the observed value of 2 (Table I) which also fits the relationship between n and p_{50} compiled by Bunn and Guidotti (1971) very well. It should, of course, be noted that such a 2-fold change in n will result in about a 100-fold change in K_D (eq 1).

The results presented here show that the magnitude of the helcotropic effect (Benesch and Benesch, 1974) of the polyanionic cofactors is directly proportional to the number of charges which, at pH 7.3, amounts to about 3.5 for DPG, 6 for IHS, and 8 for IHP. The same conclusion was reached by Shimizu and Bucci (1974) from their study of a series of aliphatic and aromatic polycarboxylic acids.

[2] In spite of the wide use of IHP, no data on the effect of different concentrations of this cofactor on the oxygen affintiy seem to have appeared in the literature.

In summary, IHS has a number of special advantages for the quantitative interpretation of the allosteric regulation of oxygen release by polyanions. Its effect on the oxygen affinity is large, owing to the tight binding at the allosteric site in the deoxy conformation. At the same time the complete ionization of the sulfate side chains permits a direct and accurate determination of K_D by measuring the protons bound along with the hexasulfate. Combining this K_D with the $\Delta \log p_{50}$ at low cofactor concentration has permitted an experimental verification of eq 1 and has shown that the coefficient n in this equation should be 2.5 and not 4 to account for the observed $\log p_{50}$ and K_D values (Benesch et al., 1971; Baldwin, 1975).

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