THE REACTION OF INOSITOL HEXAPHOSPHATE WITH HEMOGLOBIN

Quentin H. Gibson and Robert D. Gray

Section of Biochemistry and Molecular Biology

Wing Hall, Cornell University

Ithaca, New York 14850

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Summary: The rate of oxygen dissociation from the fully liganded hemoglobin tetramer ($Hb_4(O_2)_4$) is increased by the presence of inositol hexaphosphate from approximately 50/sec to 180/sec at 22°. It follows from this observation that inositol hexaphosphate is bound by oxygen-saturated human hemoglobin. This result is in marked contrast to absence of an effect of 2,3-diphosphoglycerate or pyridoxal phosphate on the rate of oxygen dissociation from $Hb_4(O_2)_4$. Inositol hexaphosphate does not, however, modify the rate of CO dissociation from $Hb_4(CO)_4$ but it does decrease the rate of CO binding to $Hb_4(CO)_3$ by about seven-fold compared to the phosphate-free system.

The remarkable effect of inositol hexaphosphate (IHP) on the affinity of human hemoglobin for O_2 was noted by Benesch *et al.* (1). They discovered that IHP equivalent to the concentration of tetrameric hemoglobin present increased the partial pressure of O_2 necessary for half saturation from 0.23 to 22 mm Hg at 10°.

This communication describes the kinetic basis of the IHP-induced decrease in the O_2 affinity of human hemoglobin. As in the case of 2,3-diphosphoglycerate (DPG) (2,3), ATP (3), and pyridoxal phosphate (3), the IHP effect results from its ability to bind to deoxyhemoglobin, as well as to partially saturated intermediates, and thereby increase the magnitude of the respective dissociation constants of the ligand-bound O_2. Under the conditions of the previous work (2,3) neither DPG, ATP, nor pyridoxal phosphate affected the rate of dissociation of the fourth ligand molecule from fully saturated oxyhemoglobin. However, IHP apparently interacts with $Hb_4(O_2)_4$ since in its presence the value of k_4 for the reaction $Hb_4(O_2)_4 \xrightarrow{k_4} Hb_4(O_2)_3 + O_2$ is augmented from 50/sec to about 180/sec at 22°.

Stripped hemoglobin (1) was prepared and stopped flow experiments were conducted in 0.05 M 2,2-bis(hydroxymethyl)-2,2',2"-nitrilotriethanol (bis-Tris), pH 7.0, exactly as described by Gibson (2). Flash photolysis of carbon monoxide hemoglobin utilized an apparatus capable of dissipating 800 joules with a time constant of approximately 50 μsec. Optical spectra were recorded on a Beckman DK-2A spectrophotometer made available by Dr. D.C. Wharton. IHP, NO, bis-Tris, and sodium dithionite were obtained from Sigma, Matheson, Aldrich, and Hardman & Holden, Ltd., respectively. The principles for the determination of the various rate constants have been described by Gibson (4); the symbolism used to denote these constants is also that of Gibson (2) for the simplest set of four reversible reactions:

$$Hb_4(O_2)_{n-1} + O_2 \underset{k_n}{\overset{k'_n}{\rightleftarrows}} Hb_4(O_2)_n \quad \cdots\cdots \quad n=1,4$$

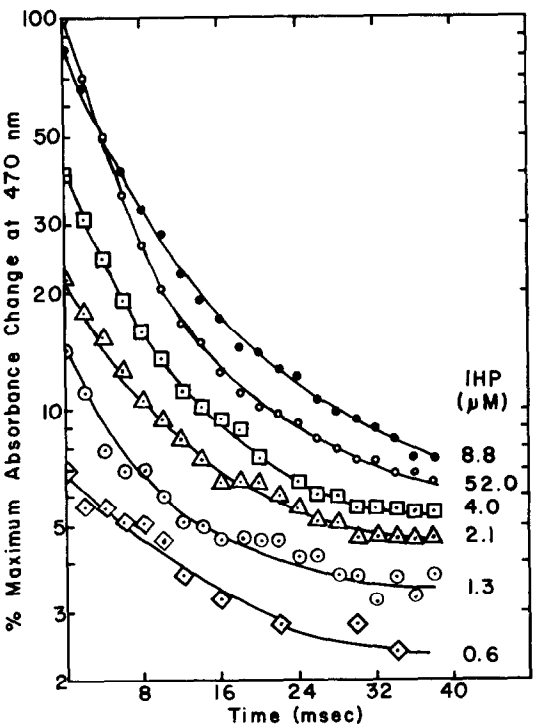

Fig. 1 Reaction of stripped oxyhemoglobin with IHP in 0.05 M bis-Tris, pH 7.0 at 22°. Conditions: $[Hb]$ = 32 μM, $[O_2]$ = 42 μM (after mixing). IHP concentrations are given beside the corresponding reaction record.

The corresponding rate constants for CO are l'_n and l_n.

An experiment which demonstrates the kinetics of the IHP effect on the O_2 affinity of hemoglobin is illustrated in Fig. 1. When stripped oxyhemoglobin was mixed with IHP in deoxygenated buffer a biphasic release of O_2 ensued which proceeded further and at an increasingly rapid rate as the concentration of added IHP was raised. The maximum initial rate of deoxygenation at the highest concentration of IHP used was about 180/sec. Since this experiment was carried out under conditions such that at the time of mixing the hemoglobin was >97% saturated with oxygen, the observed initial rate at saturating levels of IHP represents a minimal estimate of k_4 for the complex between IHP and oxyhemoglobin.

When an identical experiment was carried out at 0.75 μM oxyhemoglobin where a substantial portion of the protein exists as αβ-dimers (5), the release of O_2 was slower (approximately 80/sec initially) than when the hemoglobin was present in the tetrameric state. This observation suggests that either IHP does not interact with liganded hemoglobin dimers, or that it does not alter the kinetics of O_2 release by the αβ-dimer.

Fig. 2 shows how IHP changes the kinetics of deoxygenation of oxyhemoglobin by sodium dithionite. The salient feature of this series of experiments is the effect of increasing IHP concentration on the initial lag in hemoglobin formation which is apparent when IHP is absent. This lag period has been attributed to the competition between dithionite and partially saturated hemoglobin species for unbound O_2 as well as to the finite time period necessary for dithionite to destroy the free O_2 (4). The observed elimination of the lag period can be explained if IHP increases k_4, as is required by the data of Fig. 1. A maximum rate of about 60/sec was observed for the deoxygenation reaction in the presence of dithionite and approximately 7 μM IHP was required for half the maximum rate effect when the total heme concentration was 25 μM. It should also be noted that the half time for the reaction of IHP with $Hb_4(O_2)_4$ is considerably less

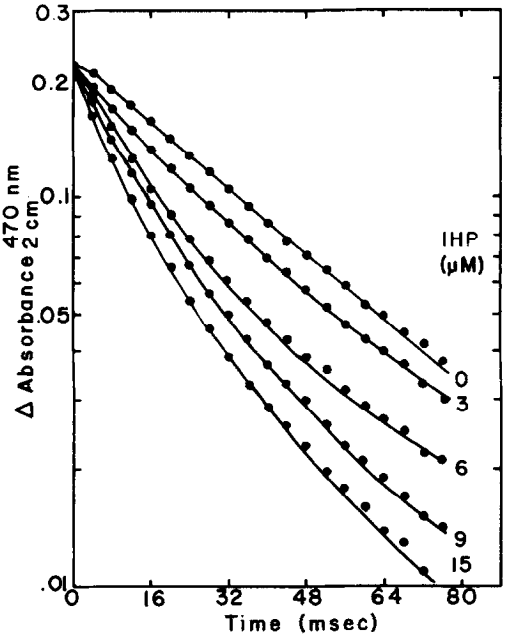

Fig. 2 Reaction of stripped oxyhemoglobin with approximately 0.2% sodium dithionite in 0.05 M bis-Tris, pH 7.0, 22°. Conditions: [Hb] = 25 μM (after mixing). IHP concentrations are given next to the corresponding reaction record.

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than the dead time of the stopped flow apparatus (< 2 msec) because the total IHP effect was realized regardless of whether the organic phosphate was added initially to the hemoglobin or to the dithionite containing syringe.

It is interesting to compare the effects of IHP and DPG on the rates of oxygen release by oxyhemoglobin in the presence of dithionite. Salhany et al. (6) observed an increasing rate of O_2 release between 85% and 40% saturation with increasing DPG : hemoglobin ratios; DPG did not, however, alter the length of the lag period preceding the apparent onset of O_2 dissociation. This observation is also consistent with the interpretation that IHP, and not DPG, alters the kinetic properties of $Hb_4(O_2)_4$.

Evidence that a stoichiometric reaction between IHP and deoxyhemoglobin occurs was obtained by mixing hemoglobin in dithionite solution containing added IHP with a solution containing an equal concentration of stripped oxyhemoglobin. The IHP effect on the kinetics of O_2 release was not

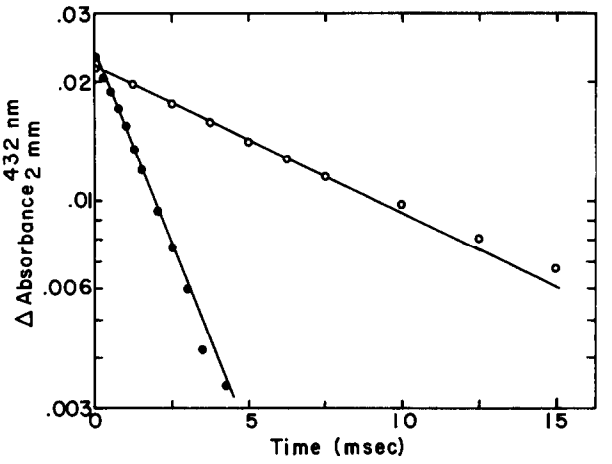

Fig. 3 Partial photolysis of stripped HbCO (●) and stripped HbCO + 15 μM IHP (o) at 25° in 0.05 M bis-Tris, pH 7.0. Conditions: [Hb] = 36 μM, [CO] = 100 μM (equilibrium), flash energy = 200 joules.

observed until a stoichiometric quantity (12 μM) had been added to the deoxyhemoglobin solution (50 μM heme before mixing).

IHP does not, however, alter the rate of dissociation of CO from $Hb_4(CO)_4$ because of our observation that it does not change the rate of CO replacement by NO even at ratios of IHP to $Hb_4(CO)_4$ in excess of 50. In addition, IHP had no observable effect on the distribution of O_2 and CO bound to hemoglobin under conditions in which the concentration of ligand-free heme groups was negligible (i.e. 30 μM heme, 40 μM CO, 1250 μM O_2). At constant pCO and pO_2, this distribution coefficient depends only on the ratio $l_4' \cdot k_4 / l_4 \cdot k_4'$ (7). Therefore, in order that IHP not change the relative affinity for O_2 and CO in view of the increase in k_4 demonstrated here, a compensating change in k_4', l_4', or both, must occur. Flash photolysis experiments (Fig. 3) demonstrated that l_4' is indeed decreased when IHP is present. When a small proportion of the CO was removed from stripped HbCO by photolysis, the recombination rate was 7.3 $\mu M^{-1} sec^{-1}$, whereas in the case of partial photolysis of the same HbCO solution in the presence of IHP, l_4' dropped to 1.4 $\mu M^{-1} sec^{-1}$ (25°). Thus the increase in k_4 is offset, not by a symmetrical increase in l_4, but predominately by a decrease in l_4'.

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