INTERACTION OF HAEMOGLOBIN WITH IONS BINDING OF INOSITOL HEXAPHOSPHATE TO HUMAN HAEMOGLOBIN A

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1. Introduction

Since the first communication by Benesch and Benesch [1] that 2,3-diphosphoglycerate (2,3-DPG) is bound to human haemoglobin (DeoxyHb) decreasing the oxygen affinity, it has been described that the binding is not limited to organic phosphates [2–4] but it can be observed also for inorganic polyvalent anions [5]. Moreover, it has been shown that HbO_2 and methaemoglobin (MetHb) are also able to bind anions [6–9]. Finally, the binding of polyvalent anions is not restricted to human Hb. Other species also are influence by anions: for instance 2,3-DPG and ATP decrease the oxygen affinity of avian Hb [10] as well as inositol hexaphosphate (IHP) is effective in lowering the affinity of human Hb [3, 5]. By means of equilibrium dialysis Chanutin and Hermann [6] have shown the existence of two classes of binding sites with association constants in the order of 10^5 M^{-1} and 10^4-10^3 M^{-1} for DeoxyHb, HbO_2, and MetHb.

In order to correlate the functional changes in the presence of anions with a definite association constant we have studied the interaction of IHP with DeoxyHb and HbO_2. IHP is the most effective anion so far investigated in lowering the oxygen affinity [5], however, its association constant is not known.

2. Materials and methods

Haemolysates prepared from preserved (stored for 3 weeks in ACD stabilizator) were concentrated by ultrafiltration (Diaflo membrane PM 30; under oxygen) at 4°. Subsequently the Hb solution was diluted to 400 ml with a solution of 0.13 M KCl + 0.02 M NaCl, pH 7.2, and again concentrated by ultrafiltration under oxygen. This procedure was repeated six times for the removal of organic phosphates [11]. The concentration of total phosphate determined from trichloroacetic acid supernatants after ashing by the ascorbic acid method [12] was 0.02 mole phosphorus per mole Hb. Samples of such solution containing about 3 mM Hb were stored in liquid nitrogen until used. Hb was determined as methaemoglobin cyanide using a millimolar extinction coefficient $\epsilon = 44.0$ cm^{-1} mM^{-1} per tetrameric Hb [13].

Oxygen binding curves were measured as described earlier [5] on 2.5×10^{-5} M Hb concentrations in 0.15 M Tris-acetic acid buffer [14] at 23 ± 1.5°.

Calcium salt of IHP (Koch-Light) was converted to the free acid by percolation through a column of Dowex 50 W-X8 and then adjusted to pH 7.0 with NaOH. Concentrations of IHP solutions were determined by analysis of total and inorganic phosphate by the ascorbic acid method [12].

The binding of IHP to HbO_2 was investigated by the Sephadex equilibrium gelfiltration method (see fig. 1) according to Hummel and Dreyer [15] and by equilibrium dialysis technique. The two-cavity dialysis cells were equipped with Nephrophane membranes (ORWO Wolfen). Each cavity of a dialysis cell held 4 ml of solution. All dialysis experiments (Hb concentration 0.155 mM) were carried out in 0.15 M Tris-acetic acid buffer at 23 ± 1.5°. Dialysis equilibrium was reached in approximately 24 hr with shaking. A phosphate analysis showed that no IHP was degraded during the experiment.

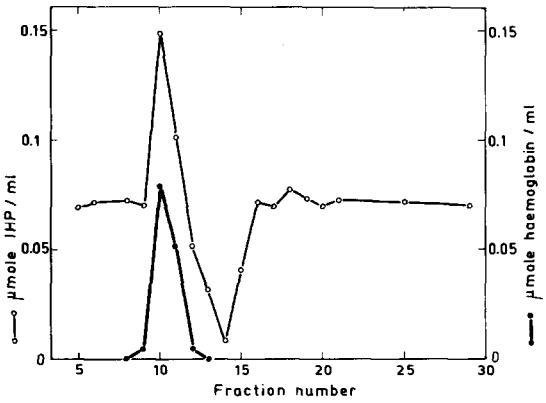

Fig. 1. Binding of IHP to HbO_2. A column 0.5 × 100 cm was packed with Sephadex G-25 and equilibrated at 25° with 0.072 mM IHP in 0.13 M KCl + 0.02 M NaCl, pH 7.0. A solution (0.8 ml) containing 0.151 mM HbO_2 and 0.72 mM IHP in 0.13 M KCl + 0.02 NaCl, pH 7.0, was loaded on the column which was then eluted with the equilibration solution, at a rate of 10 ml/hr. Fractions of 0.83 ml were collected and assayed for Hb and phosphate.

3. Results and discussion

Fig. 1 represents the binding of IHP to HbO_2 at pH 7.0 as studied by equilibrium gelfiltration on a Sephadex column according to Hummel and Dreyer [15]. The amount of IHP bound per 0.12 μmole HbO_2 is found to be 0.13 μmole, i.e. IHP forms a 1:1 complex with HbO_2. The association constant of this complex was determined by equilibrium dialysis meas-

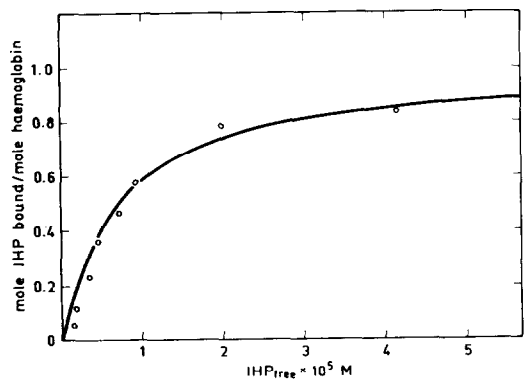

Fig. 2. Binding of IHP to HbO_2 as a function of the free IHP concentration at pH 6.5. 0.155 mM HbO_2; 0.15 M Tris-acetic acid buffer; temperature 23 ± 1.5°. The curve is calculated assuming 1:1 complex formation with an association constant of 1.4×10^5 M^{-1}.

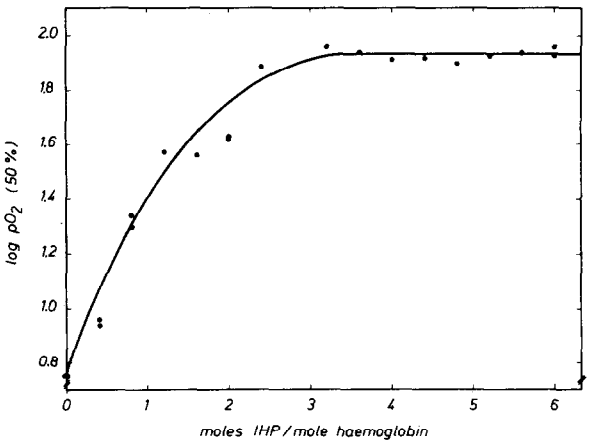

Fig. 3. Influence of increasing IHP concentrations on the oxygen affinity (log pO_2 (50%)) at pH 7.0. 0.025 mM Hb; 0.15 M Tris-acetic acid buffer; temperature 23 ± 1.5°.

urements. The binding of IHP to HbO_2 in dependence on the IHP concentration is shown in fig. 2. With increasing IHP concentrations up to 2×10^{-4} M the binding ratio increases reaching a saturation level. The lower part of the binding curve shows a slight cooperative behaviour. From the binding ratios, as well as from the Scatchard plot [16] of the data, it is clear that 1 mole IHP is bound per molecule (tetrameric) HbO_2. The association constant was calculated from

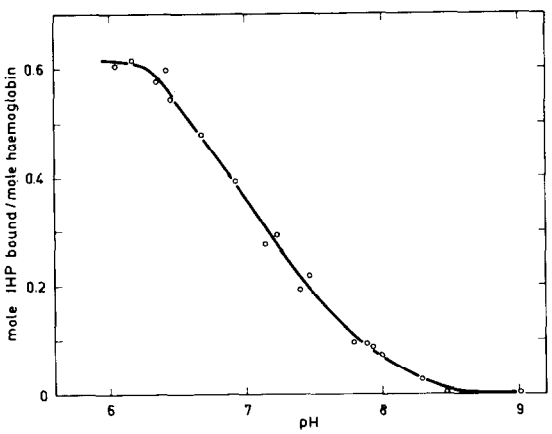

Fig. 4. Binding of IHP to HbO_2 as a function of pH. 0.155 mM HbO_2; 0.1 mM IHP; 0.15 M Tris-acetic acid buffer. Increasing amounts of NaCl were added to obtain a constant ionic strength of $\Gamma/2 = 0.15$ at all pH values. Temperature 23 ± 1.5°.

the Scatchard plot to be 1.4×10^5 M^{-1} at pH 6.5 and a temperature of 24°. Recently Gibson and Gray have given a kinetic evidence for the binding of IHP to HbO_2 [17].

The binding of IHP to DeoxyHb was measured indirectly from the shift of the oxygenation curves in presence of increasing IHP concentrations. As can be seen from fig. 3, there is a progressive decrease of the oxygen affinity expressed as log pO_2 at 50% saturation as the IHP level is raised. At pH 7.0 the oxygen affinity decreases from 5.6 mm Hg without IHP to 87 mm Hg on addition of 3.6 mole IHP per mole DeoxyHb at which a saturation level is reached. The maximum effect of IHP is considerably higher than that of 2,3-DPG and ATP [4, 10, 18] and is achieved at lower concentrations. One can assume that the higher "intrinsic affinity" of IHP is caused by the higher number of negative charged groups involving electrostatic interactions with cationic groups of the protein. There are in the IHP molecule more than 6 negative charges [19] compared to about 3 in 2,3-DPG [20] at pH 6.5.

According to the binding of IHP to one binding site on HbO_2 we made the simplifying assumption of one binding site for IHP on DeoxyHb. Then, from the log pO_2 (50%) values shown in fig. 3 an association constant can be estimated to be about 1×10^5 M^{-1}. The constants for IHP binding on HbO_2 and DeoxyHb reported here are in the same order of magnitude with those reported on high-affinity binding of 2,3-DPG and ATP to both species by Chanutin and Hermann [6] using about the same Hb concentration.

As Garby et al. [7] have shown the binding of 2,3-DPG and ATP to HbO_2 and DeoxyHb is pH dependent and increases with increasing H^+ concentration. The pH dependence of anion binding [7, 9], the binding enthalpy for ATP of about −6.8 kcal per mole [8], and the pH dependence of the oxygen dissociation kinetics in presence of 2,3-DPG [21] would indicate the participation of imidazole residues in anion binding of Hb. In order to ascertain whether or not IHP binds to the same residues on HbO_2 as 2,3-DPG and ATP, the pH dependence of IHP binding was measured. It can be seen from fig. 4 that the binding is pH independent below pH 6.2. Above pH 6.2 the binding decreases with increasing pH, and it is near 0 at pH 8.5. This steep decline in the pH range 6–8 would indicate the participation of imidazole groups in the IHP binding of HbO_2. The contributions of other groups for instance of the α-amino groups of the β-chains are not excluded. According to Perutz [22] the following residues are involved in the binding of 2,3-DPG: valine β 1, lysine β 82, and histidine β 143. The pH dependence is in good agreement with those reported by Garby et al. [7] and Jung [9] for other polyvalent anions.

Our data indicating a 1:1 complex formation between IHP and Hb suggest the same binding site on Hb for anions such as 2,3-DPG and IHP regarding the high-affinity class of binding sites. However, there are considerable differences in the structure and in the spatial arrangement of the phosphate groups in both compounds. We have, therefore, considered the distances between the phosphate groups based on correctly proportioned molecular models made from Courtauld atomic models. The distance between the phosphate groups is 9.7 Å in the 2,3-DPG molecule. In the IHP molecule which contains 5 phosphate groups in equatorial (*e*) positions and one in an axial (*a*) position the distances of neighbouring groups are 9.0 and 9.7 Å for *ee* and *ea* groups, respectively. These distances of phosphate groups are in excellent agreement with that in 2,3-DPG. Thus we might assume the same binding site on the Hb molecule for 2,3-DPG and IHP in spite of the different structures of the two molecules.

The association constant for the binding of IHP to DeoxyHb, estimated from the shift of the oxygenation curves to be about 1×10^5 M^{-1} at pH 7.0, indicates that this high-affinity class of the binding sites is probably involved in lowering the affinity of Hb for oxygen.

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