Communication

Deoxygenated Sickle Hemoglobin

MODULATION OF ITS SOLUBILITY BY 2,3-DIPHOSPHOGLYCERATE AND OTHER ALLOSTERIC POLYANIONS*

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William N. Poillon, Mark D. Robinson, and Bak C. Kim

From the Center for Sickle Cell Disease and Department of Pediatrics and Child Health, Howard University, Washington, D. C. 20059

The effects of 2,3-diphosphoglycerate (DPG) and other allosteric polyanions of the phosphate or sulfate ester class (inositol hexaphosphate (IHP), ATP, pyridoxamine-5'-phosphate (PMP), and inositol hexasulfate (IHS)) on the solubility of deoxyhemoglobin S and the oxygen affinity of Hb A were evaluated. Their effects on the saturation concentration (c_sat) indicated promotion of gelation in each case, according to the following order of molar effectiveness: IHP > Hb S DPG > ATP > PMP. Four polybasic carboxylic acids (benzenetricarboxylate (trimesic acid), benzenetetracarboxylate (BTC), benzenepentacarboxylate (BPC), and benzenehexacarboxylate (BHC)) were evaluated as well. Their order of molar effectiveness was: BHC > BTC > trimesic acid. Both classes of polyanions influenced oxygen affinity in the same order as solubility. Overall, a good correlation existed between the negative charges of these nine allosteric polyanions at neutral pH and their effects on solubility and oxygen affinity. Because of its possible role in the pathophysiology of sickle cell disease, the effect of DPG on c_sat was examined over the pH range 6.5-7.6. While a decrease in c_sat was observed for DPG-saturated deoxyhemoglobin S throughout this range, the decrement observed in the physiological pH range (1.8 g/dl) was somewhat lower than that below neutral pH (3.0 g/dl); in either case the sickling tendency of SS red cells would be enhanced. Inasmuch as the intracellular concentration of DPG in sickle cell anemia may be elevated as much as 2-fold, maneuvers aimed at its reduction could be therapeutically beneficial.

The in vitro correlate of the intracellular polymerization of sickle hemoglobin (Hb S), which underlies the sickling phenomenon, is the gelation that occurs when concentrated solutions of Hb S are deoxygenated. The two-phase polymerization equilibrium (nHb dss (Hb_dss)) represented by this gelation provides the basis for determining the equilibrium solubility or saturation concentration, c_sat, of deoxy-Hb S (1, 2). This equilibrium may be shifted in either direction by suitable perturbants: those which shift it to the left (c_sat increased) are inhibitors, while those which shift it to the right (c_sat decreased) are promoters of gelation.

The red cell metabolite DPG regulates the oxygen affinity of adult hemoglobin (Hb A) by binding preferentially to the deoxy (T) conformer of the allosteric equilibrium, R dss T. Because only the T-state is incorporated into the deoxy-Hb S polymer (3), the presence of DPG should facilitate polymerization under the hypoxic conditions of the microcirculation. The results of in vitro studies examining this point have been conflicting, however. Some workers (4-8) claim that DPG promotes gelation; others (9-13), that it has no effect. It has, however, been firmly established (14-17) that the organic polyphosphate IHP drastically diminishes the solubility of deoxy-Hb S. We have reexamined this issue with the intention of establishing definitively whether or not DPG affects the solubility of deoxy-Hb S under well-defined conditions of pH, ionic strength, and temperature. It is important to do this because the DPG level can be elevated as much as 2-fold in SS erythrocytes (10, 18, 19) and may, therefore, have a bearing on the pathophysiology of this disease. We also studied seven other organic polyanions (ATP, PMP, IHS, BHC, BPC, BTC, and trimesic acid), all of which are heterotropic allosteric effectors of the oxygen affinity of human hemoglobin (20, 21).

MATERIALS AND METHODS

Sickle Hemoglobin—Packed SS erythrocytes were obtained from units of whole blood from sickle cell anemia patients who required exchange transfusions. Preparation of hemolysates and purification of Hb S by ion exchange chromatography are described elsewhere (22). The latter effectively removes all organic phosphates.

Allosteric Regulators of Oxygen Affinity—Most of the polyvalent anions examined, either the free acid or the sodium salt, were obtained from Sigma. These included ATP, DPG, IHP, IHS, PMP, trimesic acid, and BTC. BHC was purchased from Aldrich, and benzenepentacarboxylic acid (BPC) from Pfaltz and Bauer. All other chemicals were of the best commercial grade available.

Solubility Measurements—The method for determining the equilibrium solubility, c_sat, of deoxy-Hb S by ultracentrifugation and its modification to a microscale are described elsewhere (1, 23). Samples of 250-µl total volume were centrifuged in plastic tubes (8 x 41 mm) for 1 h at 242,000 x g in an SW 50.1 rotor at 30 °C. After separation of the gel into its two component phases, the concentration of the supernatant (monomeric Hb S) was determined after conversion to cyanmet Hb with Drabkin’s solution. The pH was measured in the usual manner (1) and was always between 6.5 and 6.8, i.e. within the range in which we have found c_sat to be invariant (6.5-7.0; see Fig. 1). Conversion of oxy- to deoxy-Hb S was achieved with a 3-fold molar excess of sodium dithionite; the initial concentration of Hb S was 22-25 g/dl. Because of the variable dilution incurred by the addition of the effectors evaluated, the final concentration of Batrias buffer ranged from 64 to 72 mm. Solubility profiles (linear plots of c_sat versus effector/Hb molar ratio) were constructed for each allosteric effector examined. The magnitude of the slopes of these plots, obtained by linear regression analysis, is proportional to molar effectiveness.

Effects of DPG as a Function of pH—Measurements of c_sat were performed over the pH range 6.5 to 7.6 in the absence and presence of an equimolar amount of DPG at 30 °C. In the pH range 6.5-7.0, c_sat...
RESULTS AND DISCUSSION

Baseline Solubility—Overall, the effects of nine different allosteric polyanions were examined. Control values of solubility in the absence of effector \( c_s^{\infty} \) were extracted from the solubility profiles displayed for each species in Figs. 2 and 3 and subjected to statistical analysis. The value of \( c_s^{\infty} = 17.5 \) g/dl ± 0.6 (S.D.) \( (n = 13) \) obtained represents the solubility of "stripped" deoxy-Hb S (i.e., free of any bound polyanion).

Effects of pH on the Solubility of DPG-saturated Deoxy-Hb S—Solubility data in the absence and presence of a 1:1 molar ratio of DPG, are presented in the lower part of Fig. 1. It is evident that throughout the pH range examined (6.5–7.6), the data points for the solubility in the presence of DPG are consistently lower than those in its absence. Between pH 6.5 and 7.0, the solubility of deoxy-Hb S is relatively invariant for both Hb and Hb-DPG, while above neutral pH solubility increases steeply up to pH 7.6 (1, 6, 24, 25).

In the upper part of Fig. 1, the decrement in solubility, \( \Delta c_s^{\infty} \), evoked by DPG as a function of pH for each matched pair is shown. It can be seen that \( \Delta c_s^{\infty} \) values are larger below pH 7.05 than above it. Accordingly, the mean values of \( \Delta c_s^{\infty} \) (and its standard deviation) below and above this pH were compared. In the pH range 6.50–7.05, \( \Delta c_s^{\infty} = -3.0 \) g/dl ± 0.5 \( (n = 9) \), while in the pH range 7.05–7.60, \( \Delta c_s^{\infty} = -1.8 \) g/dl ± 0.6 \( (n = 15) \). The lesser decrement in solubility which occurs above pH 7.05 may, in some way, reflect the known pH dependence of DPC binding to Hb A (26, 27). In any case, the magnitude of the decrement \( (-1.8 \text{ g/dl}) \) in the pH range of physiological relevance (7.0–7.4) is too large to be ascribed to experimental error. Rather, the binding of DPG to deoxy-Hb S evokes a significant reduction in its solubility.

There has been considerable controversy regarding this point (4–13); indeed, a paper coauthored by one of us (12) drew the opposite conclusion. We can offer no reason for this.

The error associated with replicate determinations of \( c_s^{\infty} \) within the same run is of the order 1–2% (S.D.); between runs it is 3–4% (see baseline solubility data).

FIG. 1. Effect of DPG on the solubility of deoxy-Hb S as a function of pH. Two buffer systems (0.1 M Bistris, triangles; 0.1 M Tris, circles) were used to encompass the pH range 6.5–7.6. At any particular pH, equilibrium solubilities, \( c_s^{\infty} \), were determined for two samples: stripped Hb S and Hb S plus an equimolar amount of DPG. Bottom, plots of \( c_s^{\infty} \) versus pH for deoxy-Hb S in the absence (filled symbols) and presence (open symbols) of an equimolar amount of DPG. Top, decrement in solubility (\( \Delta c_s^{\infty} \)) of deoxy-Hb S evoked by DPG for each closely matched pair as a function of pH.

FIG. 2. Solubility profiles for allosteric polyanions of the phosphate and sulfate ester class. The equilibrium solubility, \( c_s^{\infty} \), is plotted against the effector/Hb molar ratio. Hb S samples with varying Hb/DPG molar ratios were equilibrated for 1 h at 30 °C in 0.1 M Bistris buffer. After deoxygenation by a 3-fold molar excess of sodium dithionite, the effective pH was in the range 6.5–6.8. Phase separation was achieved by centrifugation at 240,000 × g for 1 h at 30 °C. The concentration of monomeric Hb S in the supernatant corresponds to \( c_s^{\infty} \). Individual slopes of the linear plots (in the effector/Hb molar ratio range of 0–1) are given in the inset, in units of g/dl.

FIG. 3. Solubility profiles for allosteric polyanions of the benzenepolycarboxylic acid class. The equilibrium solubility, \( c_s^{\infty} \), is plotted against the effector/Hb molar ratio. Other experimental conditions are given in the legend to Fig. 2.
Table I

Influence of phosphoryl and sulfuryl polyanions on the solubility of deoxy-Hb S and the oxygen affinity of Hb A

<table>
<thead>
<tr>
<th>Polyanion</th>
<th>No. of negative charges at pH 7</th>
<th>Slope*</th>
<th>ΔpH*</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMP</td>
<td>2</td>
<td>-0.7</td>
<td>1</td>
<td>3.0</td>
</tr>
<tr>
<td>ATP</td>
<td>4</td>
<td>-2.9</td>
<td>4</td>
<td>2.9</td>
</tr>
<tr>
<td>DPG</td>
<td>5*</td>
<td>-3.7</td>
<td>6</td>
<td>2.9</td>
</tr>
<tr>
<td>IHS</td>
<td>6</td>
<td>-6.4</td>
<td>35</td>
<td>2.3</td>
</tr>
<tr>
<td>IHP</td>
<td>8</td>
<td>-10.0</td>
<td>67</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*The slope of the solubility profile (c_90 versus effector/Hb molar ratio) in the range 0–1 (see Fig. 2); the negative slopes observed indicate promotion of gelation in this region.

**These values were derived from oxygen equilibrium curves measured at 37°C in 0.05 M Bistris buffer, 0.05 M NaCl, 0.1 mM EDTA, pH 7.0; concentrations of Hb and polyanion were 12 and 0.2 mM, respectively. The P50 in the absence of effector was 11 torr.

**Hill coefficients were derived from oxygen equilibrium curves in the range 20–80% oxygenation.

**While the change of DPG alone would be near -4, the decrease in pK_a of the second ionization of the two phosphates evoked by the binding of DPG to Hb brings it closer to the value indicated (38).

**Slope in the molar ratio region 1–3, where IHP acts as an inhibitor of gelation.

Drim, studies for each effector, are compiled in the last two columns of Table I. Values of ΔP50, a measure of relative effectiveness in decreasing oxygen affinity, followed the same order as those for diminishing solubility.

The solubility profiles for the four benzene polycarboxylic acids examined are shown in Fig. 3, with the slopes in the effector/Hb molar ratio range 0–1 given in the inset. Other relevant data are compiled in Table II. Like the polyanion phosphate and sulfate anions, the magnitude of the slope for each polycarboxylic acid was related to its negative charge at neutral pH: BHC > BTC > BPC > trimesic acid. However, because of the limited water solubility of these compounds, it was not possible to ascertain whether the solubility profiles plateau above any effector/Hb molar ratio of 1. The oxygenation parameters ΔP50 and nH, derived from the oxygen equilibrium curves, are compiled in the last two columns of Table II. Like the other multivalent anions described, the relative effectiveness of the polycarboxylic acids in decreasing oxygen affinity followed the same order as that for diminishing solubility. Overall, the two classes of compounds (polyposphates and polysulfates or polycarboxylic acids), the number of negative charges on the polyanion determines the magnitude of its effect on both the solubility of deoxy-Hb S and the oxygen affinity of Hb A.

Molecular Basis of Differential Response of Deoxy-Hb S to Allosteric Polyanions—A good starting point in attempting to account for the modulation of the solubility of deoxy-Hb S by various allosteric polyanions is the determination of the allosteric parameters for each effector. While these values were derived from oxygen equilibrium curves in the range 20–80% oxygenation, the negative slopes observed indicate promotion of gelation in this region. Nevertheless, we feel that the data presented here provide compelling evidence that DPG does, in fact, exert a significant effect on the monomer-polymer equilibrium under conditions close to physiological.

This being the case, DPG may play a greater role in intracellular polymerization in SS red cells than has hitherto been appreciated. In light of the well-documented elevation of DPG levels inside SS red cells (10, 18, 19) and the enhanced response of the O2 affinity of SS red cells to DPG (28), as well as the oxygen affinity independent action of DPG on sickling (18, 29), it should now be considered whether maneuvers aimed at reduction of intracellular DPG might not be therapeutically beneficial in sickle cell disease.

It is evident from Fig. 1 (lower part) that the effects of DPG and pH on the solubility of deoxy-Hb S are distinct phenomena and that each would tend to enhance sickling in the biological situation. Thus, for SS cells whose intracellular pH does, in fact, exert a significant effect on the monomer-polymer equilibrium under conditions close to physiological.

Despite the marked change in the oxygen affinity of Hb A, however, calculation of the changes in free energy of polymerization for these two classes of compounds (polyposphates and polysulfates or polycarboxylic acids), the number of negative charges on the polyanion determines the magnitude of its effect on both the solubility of deoxy-Hb S and the oxygen affinity of Hb A.

Table II

Influence of polybasic carboxylic acids on the solubility of deoxy-Hb S and the oxygen affinity of Hb A

<table>
<thead>
<tr>
<th>Polyanion</th>
<th>Position of COOH groups</th>
<th>No. of negative charges at pH 7</th>
<th>Slope*</th>
<th>ΔpH*</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimesic acid</td>
<td>1, 3, 5</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>2.5</td>
</tr>
<tr>
<td>BTC</td>
<td>1, 2, 4, 5</td>
<td>4</td>
<td>-2.6</td>
<td>10</td>
<td>2.6</td>
</tr>
<tr>
<td>BPC</td>
<td>1, 2, 3, 4, 5</td>
<td>5</td>
<td>-4.4</td>
<td>32</td>
<td>2.9</td>
</tr>
<tr>
<td>BHC</td>
<td>1, 2, 3, 4, 5, 6</td>
<td>6</td>
<td>-7.7</td>
<td>67</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*Estimated from values of acid dissociation constants given in Ref. 39.

**The slope of the solubility profile (c_90 versus effector/Hb molar ratio) in the range 0–1 (see Fig. 3).

Values for this parameter were derived as described in Footnote a, Table I, except that the polyanion concentration was 200 μM.

Derived as described in Footnote d, Table I.
ences in the stereochemistry of binding of DPG and IHP: 1) because the binding of DPG is symmetry-averaged, its anionic groups form only seven salt bridges with the eight available cationic groups in the central cavity, while those of IHP form eight; 2) while there is some distortion in the position of Lys 82 in Hb-DPG, there is none for Hb-IHP; and 3) the amino group of Aan 138, situated just above Lys 82, is in a position to form hydrogen bonds with phosphates 1 and 2 of IHP, while no such interaction occurs for DPG. Determination of whether such subtle structural differences can account for the profoundly different effects on solubility and oxygen affinity (i.e. IHP is nearly three times more effective in decreasing the solubility and 10 times more effective in lowering the oxygen affinity than DPG; see Table I) awaits crystalographic studies of higher resolution. Nevertheless, one can account for the effects observed if the species with the greater negative charge (IHP) evokes greater constraints in the tertiary structure of the β chains than does DPG.

Such a mechanism could account for the differential response of solubility to the binding of other polyanions as well (see Tables I and II). As a rule, modifications which pull the A helix toward the central cavity (binding of DPG or IHP and cross-linking of the β chains by 2-nor-2-formylpyridoxal-5'-phosphate) decrease cₘₐᵦ, while those which push it away (carbamylation of Val 1) increase cₘₐᵦ (for further details, see Ref. 34). It appears, therefore, that the spatial disposition of the dodecapeptide at the N terminus of the β chain plays a pivotal role in determining the solubility of deoxy-Hb S: the inward displacement of Val 6 somehow distorts its complementary fit with residues Phe 85 and Leu 88 in the opposite eight; 2) while there is some distortion in the position of Lys 82 in Hb-DPG, there is none for Hb-IHP; and 3) IHP is roughly equipotent with BTC, DPG with BPC, and IHS with BHC. That two structures as widely dissimilar as IHS and 82 in Hb-DPG, there is none for Hb-IHP; and 3) IHP is roughly equipotent with BTC, DPG with BPC, and IHS with BHC. That two Structures as widely dissimilar as IHS and

Allosteric Modulators of Deoxy-Hb S Solubility

While ionic strength effects may exist as background phenomena, they are overshadowed by what we feel is a specific interaction of IHP with the Hb S molecule. There is evidence for a second polyanion binding site in human hemoglobin: Horiiuchi and Asai (36) have shown that β-Nap₂, binds to deoxy-Hb A in the molar ratio 2:1 and that only one of the two β-Nap₂ molecules is competitive with DPG; the second molecule is released by the addition of IHP. While these authors were unable to assign unequivocally the second, non-competitive binding site for β-Nap₂, they inferred from other data that it was probably between the 8 chains. Attachment of IHP at this second binding site could cause an additional perturbation to the quaternary structure of deoxy-Hb S which inhibits, rather than promotes, polymer formation. This could account for the reversal of the solubility profile above molar ratio 1 (see Fig. 2). Work in progress seeks to characterize further this putative second binding site for IHP.

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REFERENCES