The Binding of Phosphorylated Red Cell Metabolites to Human Hemoglobin A*

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SUMMARY

The binding affinity to hemoglobin of 2,3-bisphosphoglycerate, ATP, and some other phosphorylated red cell metabolites has been measured at pH 7.2 in 0.12 M KCl to simulate physiological conditions. The dissociation constant with 2,3-bisphosphoglycerate is independent of hemoglobin concentration in the range 0.06 to 4 mM. There is one binding site per deoxyhemoglobin tetramer. Phosphorylated compounds bind weakly to one site per tetramer of oxyhemoglobin. Dissociation constants determined for 2,3-bisphosphoglycerate are \(4.22 \times 10^{-5}\) M at 22°C and \(1.03 \times 10^{-5}\) M at 37°C with deoxyhemoglobin and \(~2.5 \times 10^{-4}\) M at 22°C and \(4.75 \times 10^{-4}\) M at 37°C with oxyhemoglobin. Dissociation constants for ATP are \(8.54 \times 10^{-4}\) M at 25°C with deoxyhemoglobin and \(2.55 \times 10^{-4}\) M for oxyhemoglobin at 25°C. Binding to ADP, 1,3-bisphosphoglycerate and glucose-1,6-P$_2$ was measured. The dissociation constant for magnesium complexes of 2,3-bisphosphoglycerate to deoxyhemoglobin was too weak to measure (> \(10^{-7}\) M at 37°C); magnesium complexes of ATP have an apparent dissociation constant of \(1.15 \times 10^{-3}\) M at 25°C. The effects of the differential binding of cellular intermediates on red cell glycolysis and 2,3-bisphosphoglycerate metabolism are discussed.

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1 The abbreviations used are: 2,3-DPG, 2,3-bisphosphoglycerate; 1,3-DPG, 1,3-bisphosphoglycerate; bis-tris, 2,2-bis(hydroxymethyl)-2',2'-nitroethanol.

(e.g. see Refs. 8-10) or pathological conditions such as anemia (11). These effects have been attributed to the release of inhibition of diphosphoglycerate mutase (12, 13) caused by the decrease in free 2,3-DPG that would result from its tight binding to deoxyhemoglobin. However, in rats exposed to low oxygen tension, the increase in the 2,3-DPG level did not occur when the pH rise that normally accompanies anaerobiosis was prevented (14). Also, in humans exposed to simulated high altitude conditions, even with increased plasma pH (15), there was no increase in the 2,3-DPG level without mild exercise (16). When red cells were incubated anaerobically in vitro, some workers observed the expected increase in 2,3-DPG level (17), but others did not (14, 18). Garby and deVerdier (19) studied the binding of 2,3-DPG to hemoglobin under simulated physiological conditions of salt concentration, pH, and hemoglobin level. They reported that the binding affinity decreased with increased hemoglobin concentration. They also observed that binding of deoxyhemoglobin was only twice as tight as to oxyhemoglobin.

Recently we determined kinetic constants for diphosphoglycerate mutase applicable to cellular conditions which confirm the dependence of the rate on the levels of the substrate, 1,3-DPG, and the inhibitor, 2,3-DPG (18). Calculation of the rate of the reaction in the cell requires knowledge of the kinetically significant, i.e. noncomplexed, concentrations of the reactants. Therefore, in this study, we have examined the interactions of hemoglobin with phosphorylated compounds under cellular conditions of pH and salt concentration and have considered specifically the effects of hemoglobin concentration, the possible binding to oxyhemoglobin, the binding of a variety of phosphorylated metabolites, and the influence of magnesium ions on binding. A study that considered some of these questions appeared recently (20, 21).

EXPERIMENTAL PROCEDURES

Materials—DEAE-cellulose was from Eastman. 2,2-Bis(hydroxymethyl)-2',2'-nitroethanol (bis-tris) was from Aldrich. Sephadex G-25 was purchased from Pharmacia. Carbowax (flaked polyethylene glycol 20,000) from Union Carbide was purified by dialysis against water containing Chelex (Bio-Rad); it was then lyophilized. 2,3-DPG (cylohexylamine salt) was from Calbiochem. The cylohexylamine was removed by passage through Dowex-50 (H$^+$) and the acid was neutralized with KOH.

[32P]Phosphoric acid (carrier-free) was from New England Nuclear. Diphosphoglycerate mutase from human red cells was purified through the ammonium sulfate step (12). Glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase were from Boehringer-Mannheim.
Radioactivity was determined with a liquid scintillation spectrometer with H2O-toluene-ethanol (1:20:10) mixtures. The toluene contained 0.4% 2,3-diphenylloxazole (PPO), and 0.005% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl POPOP) from Packard.

Analytical Procedures—Total hemoglobin was determined according to Drabkin (22) and methemoglobin by the procedure of Evelyn and Mailey (23). Chloride was determined by the procedure of Schales and Schales (24). Deoxyhemoglobin phosphate was measured colorimetrically according to Gomori (25). The distribution of 2P between organic and inorganic forms was determined by extraction from acid molybdate solution with isobutyl alcohol-benzene (1:1) (26).

Preparation of Hemoglobin Solutions—All operations were carried out at 0-4°C. Human red blood cells were washed three times with 0.9% NaCl, sedimenting after each wash at 4,000 x g for 5 min. White cells were removed with suction. The cells (33 ml) were hemolyzed by the addition of 100 ml of water followed by freezing and thawing. The hemolysate was added to DEAE-cellulose 100-m1 packed volume, equilibrated with sodium phosphate buffer, 50 mm, pH 7.2. The suspension was stirred for 30 min and then centrifuged at 30,000 x g for 30 min. To the liquid phase containing the hemoglobin was added 1.4 x 10^7 cpm per ml of 2,3-[U-32P]DPG. This sample was passed through a column of Sephadex G-25 equilibrated with bis-tris Cl- buffer, 0.02 M, pH 7.2, containing 0.12 M KCl. The hemoglobin fractions containing no radioactivity were combined. The hemoglobin was concentrated in dialysis bags surrounded by purified Carbowax or in an Amicon chamber that holds the protein solution has a dialysis membrane. Hemoglobin, methemoglobin, and chloride levels were determined for each preparation. The methemoglobin content was usually about 5%. The purified hemoglobin was stable when stored as droplets in liquid nitrogen.

Purification of Methemoglobin—All operations were carried out at 0-4°C. A purified sample of methemoglobin was used for most experiments. A purified sample of methemoglobin was used to flow past the dialysis chamber. The deoxygenation of hemoglobin was accomplished by passing an atmosphere enriched with oxygen over the solution stirred or rotated in a 50-ml round bottom flask. Purified argon was passed through the bis-tris Cl- buffer, pH 7.2, containing 0.12 M KCl, which was to be used to flow past the dialysis chamber. The deoxyhemoglobin was transferred anaerobically and added to a reaction mixture containing the component of the diphosphoglycerate mutase assay (22). The deoxygenated hemoglobin was transferred anaerobically with a syringe. Gassing was continued until the spectrum indicated at least 80% deoxygenation. The extent of spontaneous hydrolysis of 1,3-DPG was determined in each experiment. Initial and final samples were removed from the chamber anaerobically and added to a reaction mixture containing the components of the diphosphoglycerate mutase assay (22). Deoxygenated 1,3-DPG did not exceed 5% during the experimental period. The absence of diphosphoglycerate mutase in the hemoglobin solution was confirmed since at the end of an experiment there was no increase of radioactivity in acid-stable form.

Studies with [1,3-32P]DPG—The compound was prepared as reported (27). The specific activity was 1.2 x 10^9 cpm per pmol. The extent of spontaneous hydrolysis of 1,3-DPG was determined in each experiment. Initial and final samples were removed from the chamber anaerobically and added to a reaction mixture containing the components of the diphosphoglycerate mutase assay (22). Deoxygenated 1,3-DPG did not exceed 5% during the experimental period. The absence of diphosphoglycerate mutase in the hemoglobin solution was confirmed since at the end of an experiment there was no increase of radioactivity in acid-stable form.

Studies with [γ-32P]ATP—The compound was synthesized from ATP by the combined reactions of glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase. It was purified on a DEAE-cellulose column equilibrated with 0.01 M glycylglycine buffer, pH 7.5. The specific activity was 1.0 x 10^10 cpm per pmol. During the experiments there was no hydrolysis of ATP. This was true even in the presence of magnesium in an incubation which finally contained 9.5 x 10^-3 M ATP and 1.3 x 10^-3 M Mg^2+. Studies with [γ-32P]ATP and ATP2-—The specific activity was 2.5 x 10^8 cpm per pmol. To determine whether there had been adenylyl kinase activity, at the end of an experiment the contents of the chamber were deproteinized with trichloroacetic acid. After ether extraction and neutralization the sample was put on a Dowex 1 (Cl-) column. No counts were eluted in the ATP region.

Studies with Glucose-1,6-32P2—The compound was prepared from glucose-1,6-32P2 and 1000 units of phosphoglucomutase. The specific activity was 2.2 x 10^8 cpm per pmol. No hydrolysis was observed during the course of the incubation.

Studies with MgATP—The magnesium complex of 2,3-DPG is a competitive inhibitor of the substrate in the diphosphoglycerate phosphatase reaction. The dissociation constant of the complex, calculated from the effect of magnesium on the apparent Kd of 2,3-DPG, is 2.3 x 10^-3 M (pH 7.5, μ = 0.1, 25°C). This is close to the values obtained by other procedures (31, 32). The magnesium complex of 1,3-DPG is a competitive inhibitor of the noncomplexed substrate in the diphosphoglycerate mutase reaction. From the effect of magnesium on the apparent Kd of 1,3-DPG, dissociation constant found for the magnesium 1,3-DPG complex is 5.4 x 10^-4 M (pH 7.5, μ = 0.1, 25°C).

The dissociation constants for the ATP complexes are from Phillips et al. (33). The values applicable to the experimental conditions (pH 7.2, μ = 0.12, 25°C) are: 2.9 x 10^-4 M for MgATP; 1.90 x 10^-4 M for MgATP2-; and ATP/ATP2- = 1.46. At 37° these values are: 2.12 x 10^-4 M, 1.64 x 10^-4 M, and 1.66.

RESULTS

Effect of Hemoglobin Concentration on Binding of 2,3-DPG to Deoxyhemoglobin—Dissociation constants were determined with ionic strength and pH at physiological levels, by measuring the rates of dialysis of 2,3-[32P]DPG as the amount of phosphorylated compound was increased by the addition of non-radioactive com-
and indicated that there is one binding site for 2,3-DPG under the conditions of these studies.

Additional increments of nonradioactive 2,3-DPG gave, successively (m M), 0.05, 1.11, 1.05, and 47.2. Analyses of the hemoglobin indicated the following distribution at the beginning and end of the experiment: deoxymyoglobin, 4.75 X 10^-5 and 5.78 X 10^-4 M; methemoglobin, 0.11 X 10^-5 and 0.17 X 10^-5 M. The dissociation constant from the slope is 3.55 X 10^-4 M and the y intercept indicates n = 0.93. Temperature, 21°. B and F, bound and free 2,3-DPG.

Fig. 2 (center). Scatchard plot of binding of 2,3-DPG to a near physiological level of deoxymyoglobin. The total hemoglobin concentration was 5.15 mM. 2,3-[U-32P]DPG (specific activity 2.30 X 10^4 cpm per pmol) was added to 12.12 mM, followed by increments to give, successively (mM 2,3-DPG), 1.87 X 10^-4 M (specific activity 4.36 X 10^4 cpm per pmol). Additional increments of 2,3-DPG gave, successively (m M), 1.34, 5.96, 10.6, and 56.0. Temperature, 23°. The y intercept indicates 1.2 mM 2,3-DPG bound. The dissociation constant is 2.96 X 10^-6 M.

**binding of phosphorylated compounds to oxyhemoglobin**—In the presence of air, weak binding of 2,3-DPG to hemoglobin was observed. The oxyhemoglobin tetramer has one binding site for 2,3-DPG under the conditions of these studies (Table I). The average value is (4.22 ± 0.44) X 10^-4 M with no trend toward weaker binding at higher protein concentrations as proposed by Garby and deVerder (10). The Scatchard plot for the experiment in which the hemoglobin was 4 mM (Fig. 2) reinforces the conclusion that under the conditions of these experiments there is only one binding site on the deoxymyoglobin tetramer. Measurements made in the range of 2,3-DPG concentration that would readily have shown additional binding failed as tight as that of the tight site indicated no additional binding.

The K_diss measured at 37° was (10.3 ± 0.20) X 10^-5 M (Table I). The value of ΔH calculated from these values is −11.2 Cal.

**Dissociation Constants of Deoxymyoglobin with Other Phosphorylated Metabolites**—The dissociation constant of ATP with deoxymyoglobin measured at 25° was 8.54 X 10^-3 M, which is somewhat weaker than the binding to 2,3-DPG under the same conditions (Table II). The affinities of ADP, 1,3-DPG, and glucose 1,6-P_2 at 25° were still weaker, with dissociation constants of 5.60 X 10^-4 M, 6.45 X 10^-4 M, and 9.93 X 10^-4 M, respectively. The binding affinity of P_i was too weak to be determined under the experimental conditions, i.e. the K_diss is larger than 3 X 10^-4 M. The same binding site appeared to be functioning in all cases since any compound could be used to release any other.
one can calculate the expected absorbance changes at these wave-lengths as 2.03 and 1.41. However the addition of 2,3-DPG caused no change in absorbance from 610 to 700 nm. It is concluded that the binding observed is to hemoglobin in the oxy form.

Effects of Magnesium on Binding of Phosphorylated Compounds to Hemoglobin—The effects of magnesium on the binding of 2,3-DPG and ATP to hemoglobin were considered since in the cell magnesium will compete with hemoglobin for these and other phosphorylated compounds. Since the magnesium complexes of 1,3-DPG, 2,3-DPG, and ATP are charged, they might also bind to hemoglobin. These studies were done at a constant pH of 7.2, and the binding constants to hemoglobin included all ionic forms present. No binding of the magnesium complex of 1,3-DPG to deoxyhemoglobin was detected at 25°; also there was no binding of 2,3-DPG complexes to deoxyhemoglobin at 37°. Magnesium complexes of the ATP forms present at pH 7.2 are bound to deoxyhemoglobin with a binding affinity of 1.15 x 10^-4 M at 25°. Other workers have also found binding to oxyhemoglobin with a variety of techniques including gel filtration (19), ultrafiltration (20), calorimetry (34), pH-stat (35), and nuclear magnetic resonance (36). The equilibria measured in our experiments are rapid and not associated with changes in the visible spectrum from 610 to 700 nm. We were unable to observe the slow changes described by Lo and Schimmel (37) which were done in the presence of 3 mM MgCl2.

Several of the phosphorylated compounds studied bind weakly to oxyhemoglobin (Fig. 3 and Table II). Other workers have also found binding to oxyhemoglobin with a variety of techniques including gel filtration (19), ultrafiltration (20), calorimetry (34), pH-stat (35), and nuclear magnetic resonance (36). The equilibria measured in our experiments are rapid and not associated with changes in the visible spectrum from 610 to 700 nm. We were unable to observe the slow changes described by Lo and Schimmel (37). Others have observed (18, 38) that as the 2,3-DPG level increased, the PsO increased and then leveled off. This result is expected if there is binding to oxyhemoglobin; in the absence of a competing reaction for 2,3-DPG in addition to the two equilibria shown,

\[
Hb + 2,3\text{-DPG} \rightarrow Hb\cdot 2,3\text{-DPG}
\]

\[
Hb + n\text{O}_2 \rightleftharpoons Hb\cdot (O_2)_n
\]

higher levels of 2,3-DPG should continue to increase the PsO. Using our binding constants for 2,3-DPG to hemoglobin at 37° and a value of 2.7 for n, as suggested by Benesch et al. (39), we calculate that the limiting ratio for PsO at high 2,3-DPG is Pso when 2,3-DPG = 0, is 4. At the pH and salt concentration used in these studies, Scatchard plots (Figs. 1 to 3) show one binding site per tetramer for either deoxygenated or oxyhemoglobin. This site is very likely the same in both cases, although additional salt bridges stabilize the interaction with the deoxy configuration (40).

A summary of dissociation constants determined for 2,3-DPG and ATP with deoxyhemoglobin and oxyhemoglobin under near physiological conditions in this and other studies is given in Table III. No data are presented from Garby and deverdier (19) since binding constants are not readily derived from their studies which were done in the presence of 3 mM MgCl2. Values obtained for the binding of 2,3-DPG to oxyhemoglobin at 37° by three groups of workers are in good agreement, with a range of 4.00 x 10^-8 M to 4.75 x 10^-8 M. Dissociation constants for ATP under the same conditions ranged from 1.61 x 10^-3 M to 3.45 x 10^-3 M. All workers found tighter binding to deoxy-hemoglobin, although the range of values was larger than for oxyhemoglobin. The binding constants themselves, the relative affinities for oxy- and deoxyhemoglobin and the amounts of the given components will determine the extent of binding in the cell. A large effect of deoxygenation will only be observed if there is a large difference between the binding affinities of oxy and deoxyhemoglobin. Berger et al. (20) found dissociation constants for 2,3-DPG which differ by a factor of 20; for ATP there was a 7-fold difference. The ratios of our values are larger: 46 for 2,3-DPG and 27 for ATP, and give greater significance to binding as an effecter of red cell metabolism.

Using the dissociation constants obtained in this study, we
have calculated the amounts of 2,3-DPG and ATP that would be free or complexed to hemoglobin or magnesium in the cell under totally aerobic or totally anaerobic conditions (Fig. 4). Because of their much lower levels in the cell, other phosphorylated compounds will have little effect on the distribution of compounds will have little effect on the distribution of forms of 2,3-DPG and ATP. The dependence on the level of free magnesium is shown. In air, 40% of the 2,3-DPG is complexed to oxyhemoglobin. When the hemoglobin is assumed to be totally in the deoxy form, almost 90% of the 2,3-DPG is complexed to hemoglobin (Fig. 4A). Although the level of free magnesium has little effect on the total amount of free or bound 2,3-DPG (Fig. 4, A and B), the amount of 2,3-DPG-Mg complex present is significant relative to the level of free magnesium (Fig. 4C). With anaerobiosis, this magnesium is largely liberated and will affect the distribution of other ligands. The distribution of ATP is more dependent on the magnesium level. Less than 10% is bound to hemoglobin in air; anaerobically about 25% is bound. Levels of free ATP and MgATP (Fig. 4, E and F) decrease with deoxygenation. The actual free magnesium level of the cell may be derived from the apparent adenylate kinase equilibrium (42) which should be calculated from the portions of each nucleotide not bound to hemoglobin under such conditions (44).

**Fig. 4.** Computer simulated distribution of 2,3-DPG and ATP in totally oxygenated (O) or deoxygenated (D) red cells. The parameters assumed were (mM): hemoglobin (7), 2,3-DPG (6), ATP (2), and dissociation constants given in the text.

The glycolytic rate of the red cell increases under anaerobic conditions (44). Since 2,3-DPG is an inhibitor of phosphofructokinase (45), the decrease in free 2,3-DPG anaerobically will be a factor, in addition to the higher pH, that will allow an increase in the rate of that reaction. The net rate of the phosphoglycerate mutase reaction increases when the pH rises (13), which could contribute to the increase in 2,3-DPG levels observed under such conditions (44).

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