THE INTERACTION OF 2,3-DIPHOSPHOGLYCERATE ON HUMAN DEOXY AND OXYHEMOGLOBIN

Simon H. de Bruin, Lambert H.M. Janssen and Gerard A.J. van Os

Department of Biophysical Chemistry
University of Nijmegen, Toernooiveld, Nijmegen, The Netherlands.

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SUMMARY: A very accurate pH stat method was used to measure the number of protons which are taken up when 2,3-diphosphoglycerate (DPG) reacts with deoxyhemoglobin (Hb) and oxyhemoglobin (HbO₂). As judged by this method it appeared that the effect of DPG on both Hb and HbO₂ is pH dependent with this difference that similar results are obtained at different pH ranges. The data showed that the additional Bohr effect and therefore the oxygen affinity of hemoglobin in the presence of DPG is not only due to the binding of DPG to Hb, but also due to the interaction of DPG with HbO₂.

It has been known for some time that DPG strongly decreases the ligand affinity of human hemoglobin (1,2) and enhances the alkaline Bohr effect above pH=7 (3,7). Recently we have presented evidence that below pH=7 DPG decreases the alkaline Bohr effect and increases the acid Bohr effect (6). From a phenomenological point of view the effect of DPG on the Bohr effect can be described by saying that due to the presence of DPG some additional alkaline and acid Bohr groups are introduced in the hemoglobin molecule approximately equal in number and in magnitude of their pK shifts. On a molecular level we explained (7) the change in Bohr effect by assuming that at high pH upon dissociation of DPG from Hb protons are released due to a shift in pK to lower values of the positively charged groups on the protein involved in the binding of DPG; these groups are located at the entrance of the central cavity (8,9). The other assumption we made was that at low pH protons are consumed when completely ionized DPG molecules bound to Hb get adjusted to their proper degree of ionization as soon as they dissociate from Hb.

In the mathematical description (7) proposed for this extra Bohr effect we made the assumption that the binding site for DPG was the same in HbO₂ and Hb. Based on this assumption we reached the conclusion that the binding of DPG to Hb was much stronger than to HbO₂. This conclusion was consistent with the observations of Benesch et al. (1) and Caldwell et al. (10) at least as far as the binding above pH 7 was concerned, even though not in keeping with other
Since the extra Bohr effect is a net result of the reaction of DPG with both Hb and HbO₂, we wish to report in this paper on a study about the number of protons bound at constant pH upon a) mixing a solution of Hb and a solution of DPG, b) oxygenation of Hb in the presence of DPG, c) oxygenation of Hb in the absence of DPG, d) mixing a solution of HbO₂ and a solution of DPG. The interrelation between these four processes is schematically produced in fig. 1. HbDPG and HbO₂DPG stand for the complexes of DPG with Hb and HbO₂ respectively. We will indicate the number of protons bound (on tetramer basis) along the several pathways by ΔZₐ, ΔZₐ, ΔZₐ, and ΔZₐ respectively. These quantities are correlated by the equation:

$$\Delta Z_a + \Delta Z_b = \Delta Z_c + \Delta Z_d$$  (1)

The extra Bohr effect we reported on (6) is equal to ΔZₐ - ΔZₐ and therefore to ΔZₐ - ΔZₐ. In our analysis (7) we assumed that for the entire range of pH levels ΔZₐ could be neglected as compared with ΔZₐ. The results obtained during our investigation proved this last assumption to be invalid at least for the data below pH 7.5.

In order to be able to measure the several proton exchange processes we developed a very accurate pH stat equipment as an extension to the titration equipment we described earlier (13). It would go beyond the scope of this paper to describe this pH stat equipment in detail; suffice it to say that the rate of revolution of the burette motor is linearly proportional to deviations from any particular fixed pH value. The sensitivity is such that acid or base is added as soon as this deviation amounts to only a few thousandths of a pH unit.

Solutions of human HbO₂ were prepared according to the toluene procedure
Fig. 2. Number of protons bound upon mixing DPG with deoxyhemoglobin (curve a, pathway a) and oxyhemoglobin (curve b, pathway b). Ionic strength 0.1 (KCl), hemoglobin concentration 1.2 x 10^{-3} M in heme; DPG concentration 4 x 10^{-4} M.

(14); after dialysis the hemoglobin solutions were deionized and freed from DPG as described (6). Solutions of Hb (2 x 10^{-3} M in heme; 0.1 M in KCl) were prepared by flushing argon through a rotating cylindrical tonometer. The time needed for complete deoxygenation was about 5 minutes. 4 ml of the solution were anaerobically transferred to a thermostated (25°C) reaction vessel and adjusted to a particular pH. When pathway d was studied 4 ml of a solution of HbO_2 were added. DPG (Calbiochem) was converted into the acid form by repeated passing through Amberlite IR 120 and subsequently neutralized with NaOH; KCl was added to adjust the solution to a 0.1 M concentration. For each experiment 2.7 ml of the DPG solution (10^{-3} M) was added to a vessel thermostated in line with the reaction vessel and adjusted to the pH of the hemoglobin solution. When pathway a was studied the DPG solution was carefully freed from oxygen by bubbling argon through. The DPG solutions were anaerobically transferred to the reaction vessel through a thin PVC tubing under argon pressure, and the amount of acid or base needed to keep the pH constant was recorded.

In order to ensure that the pH readings recorded did in fact correspond to the actual pH values, blanks were carried out in which 2.7 ml of a DPG solution (10^{-3} M) were mixed with solutions of phosphate buffer having the same buffer capacity as the hemoglobin solutions actually used. In all cases the amounts of acid or base needed to correct for possible misadjustments could be neglected.

For pathway b a solution containing HbO_2 (2 x 10^{-3} M in heme), KCl (0.1 M) and DPG (6.5 x 10^{-4} M) was deoxygenated using the above mentioned tonometer. For each experiment 4 ml of this solution was transferred to the reaction vessel and subsequently oxygenated. The pH was kept constant by adding acid or base. Pathway c was carried out like b except for the absence
Fig. 3. The number of protons bound on oxygenation of deoxyhemoglobin (Bohr effect) in the presence (curve b) and absence (curve c) of DPG. Experimental conditions as mentioned in the legend to figure 2.

In all our experiments the mole to mole ratio of DPG to hemoglobin was 1.3. The hemoglobin concentrations were determined by drying in air at 105°C to constant weight, the DPG concentrations were measured by hydrogen ion titration.

In Figure 2 $\Delta Z_a$ and $\Delta Z_b$ are plotted vs. pH. The results are very surprising, for it can unambiguously be said that, judging by the results we obtained, the interaction between DPG and Hb is similar to the one between DPG and HbO$_2$. The main difference consists of a shift of the two curves with respect to each other amounting to about one pH unit. When pathway d was studied the data show more scattering than was seen in the study of pathway a; the curve, however, is well defined.

Since proton uptake as a result of the reaction with DPG means that some groups in hemoglobin change their pK to higher pK values, it is reasonable to assume that these groups are those responsible for the binding of DPG in both Hb and HbO$_2$. The binding site for DPG in Hb is well established (8,9). Let us
suppose that DPG binds to the same site in HbO\textsubscript{2}. In that case the pK shift upon binding should start from the same pK values in both Hb and HbO\textsubscript{2}, since there is no proof that the groups making up the binding site in Hb have different pK values in HbO\textsubscript{2}. Consequently curves \(a\) and \(d\) should have fallen in the same pH range. This is evidently not the case, so that we are led to the conclusion that the binding site for DPG in HbO\textsubscript{2} is different from the one in Hb. We should point out, however, that this conclusion is entirely based on the assumption that the binding groups in Hb do not show a pK shift upon ligation. In case evidence would be produced which shows that, quite on the contrary, DPG binds to the same site in Hb and HbO\textsubscript{2} our data would be an indication for a possible role of these groups as the still missing normal Bohr groups.

The data as presented in Figure 2 are accessible for an analysis in terms of the equations for linked functions as described by Wyman (15). Applying the general equation we can write:

\[
\left( \frac{\delta \Delta Z}{\delta \nu} \right)_{a_H} = - \left( \frac{\delta \ln (\text{DPG})}{\delta \ln a_H} \right)_{\nu}
\]

where \(\nu\) represents the number of DPG molecules bound by Hb or HbO\textsubscript{2}, \(a_H\) the proton activity and (DPG) the free DPG concentration. For \(\nu = 0.5\) we can write (DPG) = \(K^{-1}\) where \(K\) is the association constant of the hemoglobin-DPG complex. At constant pH \(\Delta Z\) will be proportional to \(\nu\) or \(\Delta Z = \Delta Z_{\nu=1} \cdot \nu\); \(\Delta Z_{\nu=1}\) is the number of protons bound when \(\nu=1\). For equation 2 we can write therefore:

\[
\frac{d \log K}{d \text{pH}} = \Delta Z_{\nu=1}
\]

\(\Delta Z\) is always positive at least above pH=6; so that \(\log K\) must increase with decreasing pH. The result is consistent with the observation in direct binding studies of several authors (3,7,10,11). Integrating curve \(a\) between pH 7.8 and 7 an increase in \(\log K\) of about 0.8 could be calculated while in direct binding studies this increase in \(\log K\) was in fact found to be about 1.0 (3). The agreement is satisfactory, especially when one bears in mind that \(\nu\), calculated using the binding constants as given by Benesch et al. (3), is only 0.7 to 0.8 on the average. The crossover point at pH=6 in the curve for Hb is probably due to the fact that at low pH the change in degree of ionization of the DPG molecule upon binding, accompanied by a release of protons, overcomes the proton uptake by the groups on hemoglobin involved in the binding of DPG.
**Fig. 4.** The additional DPG induced Bohr effect. The drawn line is obtained by subtracting curve a from curve d as shown in Fig. 2. The points are obtained by subtracting curve c from curve b as shown in Fig. 3.

In Fig. 3 $\Delta Z_a$ and $\Delta Z_b$ are plotted vs. pH. They represent the Bohr effect in the presence and absence of DPG respectively. The curves are very similar to those obtained from hydrogen ion titration curves (6).

In Fig. 4 $\Delta Z_d - \Delta Z_a$ and $\Delta Z_b - \Delta Z_c$ are plotted vs pH. As is seen the agreement is satisfactory and lies within the expected experimental accuracy. It must be emphasized here that the magnitude of $\Delta Z_a$ or $\Delta Z_b$ at a particular pH does not give any information as to the affinity of DPG to either Hb or HbO$_2$. To get information about the relative affinities of DPG to Hb and HbO$_2$ we have to study $\Delta Z$ of the several pathways at constant pH as a function of $n$; these experiments are in progress.

In summary we can say that the additional Bohr effect and therefore the oxygen affinity of hemoglobin in the presence of DPG is due to the interaction of DPG with both Hb and HbO$_2$. In this respect the data presented seem to reconcile some conflicting reports on the binding of DPG to HbO$_2$ (3,10,11,12).

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**REFERENCES**