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

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SUMMARY: Binding of 2,3-diphosphoglycerate (DPG) to both deoxyhemoglobin (Hb) and oxyhemoglobin (HbO_2) is accompanied by an uptake of protons. A study of this proton uptake as a function of n, the mole to mole ratio of DPG and hemoglobin, yielded adsorption isotherms which could be described with one single association constant. It appeared that at pH 6.8 the proton uptake per molecule of DPG bound is larger for HbO_2 than for Hb. The data showed that the binding of DPG to HbO_2 is functionally significant.

DPG has a remarkable effect on the oxygen affinity of human hemoglobin; P_{50} the oxygen pressure at half saturation increases strongly on addition of DPG (1-3). It is now known that in addition to this effect DPG also increases both the alkaline (4-7) and acid Bohr effect (7). In a recent report (8) we have shown that the increase in alkaline Bohr effect is due to an uptake of protons which occurs upon binding of DPG to Hb and that the increase in acid Bohr effect is surprisingly due to an proton uptake occurring upon binding of DPG to HbO_2. These two results were confirmed by the observations of Kilmartin (9). The data showed however that at n=1.3 the influence of the binding of DPG to HbO_2 on the Bohr effect could almost be neglected at pH values above pH 7.3. In this paper we extend our study of the DPG effect to higher n values, up to a DPG concentration of 5×10^{-3} M. We measured the number of protons taken up upon a) mixing solutions of Hb and DPG, b) oxygenation of Hb in the presence of DPG, c) oxygenation of Hb in the absence of DPG and d) mixing solutions of HbO_2 and DPG. Indicating the number

of protons bound per tetramer along the several pathways by ΔZ_a, ΔZ_b, ΔZ_c and ΔZ_d the following equation will hold:

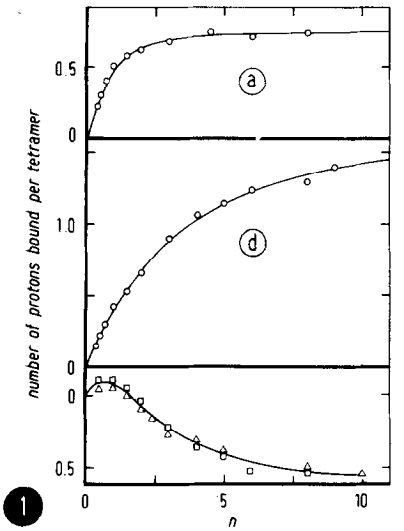
$$\Delta Z_a + \Delta Z_b = \Delta Z_c + \Delta Z_d \qquad (1)$$

Since ΔZ_a and ΔZ_d will be proportional to the number of DPG molecules bound, a determination of these quantities as a function of n will yield the association constants of the binding of DPG to Hb and HbO_2.

The pH stat procedure has been outlined in our previous report (8). In all experiments the concentration of hemoglobin was 2.5×10^{-4} M per tetramer. The highest DPG concentration used was 5×10^{-3} M (i.e. n=20), which is equal to the DPG concentration in vivo. When protons were bound ΔZ values were given a positive sign.

In Fig. 1 ΔZ_a and ΔZ_d values measured at pH 6.8 have been plotted vs. n. The shape of the two curves corresponds to normal binding isotherms and can be described with a single association constant for both Hb and HbO_2 (see below). Without doing a quantitative analysis the data indicate that at pH 6.8 Hb binds DPG stronger than HbO_2 and that at this pH the maximum proton uptake upon binding of DPG is at least twice as large for HbO_2 as for Hb.
From eqn. 1 it follows that the DPG induced Bohr effect $(\Delta Z_b - \Delta Z_c)$ should be equal to $(\Delta Z_d - \Delta Z_a)$. These two difference quantities have been plotted in Fig. 1 too (lower part); it can be seen that the agreement between the two independent sets of data is very good. The difference curve shows that at low values of n the DPG induced Bohr effect is negative; this is due to the large affinity of DPG to Hb; at high values of n, where the binding of DPG to HbO_2 becomes increasingly important the induced Bohr effect is positive.

The full lines of curves a and d were calculated using a non linear least squares fitting procedure. The curves were fitted using two parameters, viz. the association constants for the hemoglobin DPG complex and the maximum values for ΔZ_a and ΔZ_d for n going to infinity. Only one binding site was assumed to be present in both Hb and HbO_2. At pH 6.8 we found



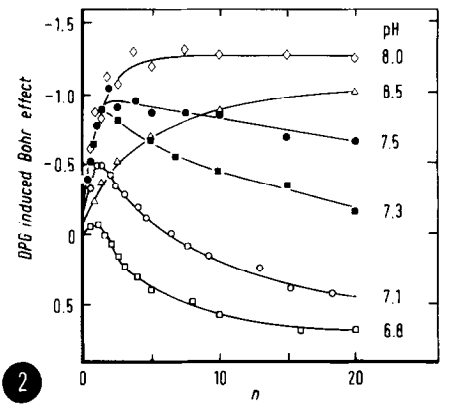


Fig. 1 Number of protons bound upon binding of DPG to Hb (curve a) and HbO_2 (curve d); n is the mole to mole ratio of DPG and hemoglobin; full lines were calculated (see text). In the lower part the DPG induced Bohr effect has been plotted: (Δ), directly observed values; (□), obtained by subtracting curve a from curve d; pH = 6.8, hemoglobin concentration 2.5 x 10^{-4} M (tetramer basis), KCl concentration 0.1 M, temp. 25°C.

Fig.2. The DPG induced Bohr effect ($\Delta Z_b - \Delta Z_c$) observed at various pH values; these pH values are indicated in the figure. KCl concentration 0.1 M; temp. 25°C.

for Hb K_{ass} = 1.7 x 10^4 M^{-1}, Δz_a^{max} = 0.77; for HbO_2 we calculated K_{ass} = 1.2 x 10^3 M^{-1}, Δz_d^{max} = 1.64. The fact that our data proved to be consistent with the assumption of one binding site in both Hb and HbO_2 is in agreement with the results obtained in direct binding studies (10-12), although some additional weaker binding sites have been observed (13, 14). The relatively large difference of more than a factor 10 between the two association constants is in better agreement with the results of Benesch and Benesch (10) and Benesch et al. (11) than with the results reported by Chanutin and Herman (13) and Garby and Verdier (14).

In Fig. 2 the DPG induced Bohr effect is shown at various pH values. The data show that in going from low to high pH the contribution to the Bohr effect of the binding to HbO_2 decreases

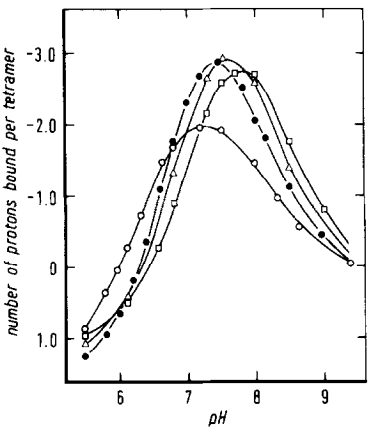


Fig. 3. The Bohr effect as observed at various values of n: (o) n=o; (•) n=1.3; (Δ) n=5; (□) n=20; KCl concentration 0.1 M; temp. 25°C.

in proportion to the increasing contribution of the binding of DPG to Hb. Above pH 8 only the latter is observed. It must be noticed here that our data prove that the mechanism of the enhancement of the alkaline Bohr effect by DPG as proposed by Riggs (15) was partly correct; in this model it is assumed that both HbO_2 and Hb bind DPG under uptake of protons. However it was also assumed that the pH dependence of this proton uptake was equal for both Hb and HbO_2; according to this mechanism only the alkaline Bohr effect would be affected by the DPG binding to Hb and HbO_2. This is evidently not the case.

The curves obtained near the physiological pH show that at high n values the induced Bohr effect tends to go to zero. This is consistent with the observation of Benesch et al. (14) that the values for $\Delta \log p_{50}/\Delta pH$ were identical at high and zero DPG concentration, while at intermediate concentrations larger values were observed than at n=0; this phenomenon was explained by assuming that it was caused by the pH dependence of the binding of DPG to Hb; our data show that the actual reason is that at pH 7.3 the two contributions to the Bohr effect of the binding of DPG to Hb and HbO_2 cancel out.

Our results invalidate the assumption made in reports on the influence of DPG on the oxygen saturation curves of hemoglobin (16, 17) viz. that DPG only binds to Hb and to hemoglobin partially saturated with one or two ligands. The hemoglobin and DPG con-

centrations used in these oxygenation experiments were such that comparison is possible to conditions existing at n=10 in the experiments described in this paper. Fig. 3 clearly shows that at pH 7.3 the binding of DPG to HbO_2 cannot be considered as functionally insignificant; from this it follows that the Adair constants are affected by this binding, which is in contrast to the assumption mentioned above.

In Fig. 3 Bohr curves are shown at various values of n. The data show that the curves get displaced to the right when n increases. At high n values the curve is considerably different from previously reported curves (6-9) obtained at n values near one. It might be noted again, that, if the interaction of DPG with HbO_2 would have been negligible, the increase in alkaline Bohr effect would have been much larger at neutral pH than actually is observed. In view of this the conclusion is inevitable that log p_{50} is strongly influenced by the interaction of DPG with HbO_2 - note: the difference in log p_{50} between pH 9 and any other pH can be calculated by integrating the curves shown in Fig. 3 from pH 9 to that pH -.

At pH values below pH 6 we see that at high n values the curves tend to coincide with the curve measured at n=0; we think that this is due to the fact that in this pH region protons are released when DPG binds to Hb, whereas above this pH protons are taken up (8).

The nature of the DPG binding site in Hb is well established. It is at the entrance of the central cavity, where a cluster of positively charged groups form saltbridges with the negatively charged groups of DPG (18). In preliminary experiments we studied the influence of the presence of DPG on the reactivity of the α-NH_2 group of the α chain. In the presence of DPG we found a diminished reactivity. As a result we are inclined to think that the α-NH_2 group of the α chain is involved in the binding of DPG in HbO_2. If this is true, HbO_2 has two identical binding sites for DPG. It will be obvious that the simulated curve for the binding of DPG to HbO_2 as shown in Fig. 1 can be fitted equally well assuming two identical sites with a maximum value for ΔZ_d^{max} of 0.82 per site instead of 1.64 in the case of one binding site.

In a previous paper we have shown that the DPG binding site in HbO_2 must be absent in Hb (8). The α-NH_2 group of the α chain

fulfills this requirement; for in HbO_2 this group is free to move, whereas in Hb it forms a saltbridge with the carboxyl group of Arg HC3 (141)α(18). More experiments will be needed to establish the nature of the DPG binding site in HbO_2.

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