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The Interaction of 2,3-Diphosphoglycerate with Human Hemoglobin

EFFECTS ON THE ALKALINE AND ACID BOHR EFFECT

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SUMMARY

A model is presented which is able to describe satisfactorily the increase of the alkaline and acid Bohr effect due to the binding by human hemoglobin of 2,3-diphosphoglycerate. From an analysis of this extra Bohr effect it could be concluded that the histidines H21(143) β are very probably responsible for the increase in alkaline Bohr effect. The second ionization of the phosphate groups of 2,3-diphosphoglycerate is responsible for the increase in acid Bohr effect. For K_a the pH-independent part of the apparent association constant of the deoxyhemoglobin-diphosphoglycerate complex, we estimated a value of $8 \times 10^5 \text{ M}^{-1}$ at 25° and an ionic strength of 0.1. It appeared further that the binding of diphosphoglycerate by oxyhemoglobin is under these experimental conditions negligible as compared with the binding to deoxyhemoglobin. From binding experiments on deoxyhemoglobin with diphosphoglycerate at 4° and at an ionic strength of 0.1 a value for K_a of $(6.5 \pm 1.5) \times 10^5 \text{ M}^{-1}$ could be estimated. Measurements of the rate of reaction of 1-fluoro-2,4-dinitrobenzene with the α -NH₂ groups of deoxyhemoglobin showed that in the presence of 2,3-diphosphoglycerate the reactivity of the α -NH₂ group of the β chain was strongly depressed.

groups are very probably the two terminal NH₂ groups, the two histidines H21 (11) and the lysines EF6 (9, 10). In view of this we explained our data in terms of pK shifts (due to salt-bridge formation) of the second ionization of the phosphate groups of DPG and of the two histidines H21. In a recent report Riggs (12) has proposed a model describing the mechanism of the enhancement of the alkaline Bohr effect due to the binding of DPG. In his proposal he takes only into account the change in proton charge of deoxyhemoglobin upon DPG binding. He stated explicitly that the change in ionization of DPG with pH was ignored. Consequently his model predicts that the acid Bohr effect will not be affected by DPG binding. Since we did find a change in acid Bohr effect in the presence of DPG, the purpose of this paper is to present an extended model, in which we will also take into account the change in ionization of DPG with pH.

The model will be checked using the experimental Bohr data reported earlier (8). In order to correlate the Bohr data with binding data for deoxyhemoglobin under similar experimental conditions we have carried out binding experiments in the pH region 7 to 8.

In addition we want to present extra evidence for the involvement of the α -NH₂ group of the β chain in the DPG binding. The chemical evidence reported in the literature is based on the observation that binding of DPG decreases strongly upon blocking of this group (4, 13, 14) and on the fact that various hemoglobins, which have the α -NH₂ group of the β chain acetylated or blocked in another way, show strongly diminished affinity for DPG (15, 16). We wish to report on the reversed situation that upon DPG binding the reactivity of the α -NH₂ group of the β chain toward reagents is diminished. The reaction studied is that of 1-fluoro-2,4-dinitrobenzene with the α -NH₂ of both chains.

MATERIALS AND METHODS

Hemoglobin Preparation—Human hemoglobin was prepared by the toluene method (17). The solutions were dialyzed against distilled water and subsequently freed from DPG by repeated passing, using a recycling system, through a mixed bed ion exchange column (Amberlite IRA 400 and IR 120). The number of moles of DPG present per tetramer, indicated by n , was 0.03 as judged by the spectrophotometrical method of Bartlett (18). By processing myoglobin through the Bartlett procedure it ap-

The effect of 2,3-diphosphoglycerate¹ in lowering the oxygen affinity of hemoglobin has been the subject of many recent studies (1-4). At first, it was believed (2, 5) that DPG was inhibiting the oxygen binding without affecting the well known pH dependence of the oxygen affinity of hemoglobin, the so-called Bohr effect. Benesch *et al.* (6) were the first to report on a change in Bohr effect when DPG was present. This result was confirmed by others (4, 7). Recently (8) we have reported that due to the binding of DPG also the acid Bohr effect was enhanced and to a similar extent as the increase in alkaline Bohr effect. The very likely binding site for DPG in deoxyhemoglobin is on the dyad axis at the entrance of the central cavity (9, 10), where the five negative charges of DPG can form saltbridges with positively charged groups of the β chains; these

¹ The abbreviations used are: DPG, 2,3-diphosphoglycerate; FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, 2,4-dinitrophenyl.

peared that the residual absorption which gives rise to this value of $n = 0.03$, is very probably entirely due to heme degradation. Correcting for this residual absorption n was zero in all cases.

Reaction of Human Hemoglobin with FDNB and Characterization of Products—In the kinetic experiments the rate of reaction of FDNB with deoxyhemoglobin and with the deoxyhemoglobin-DPG complex was measured as described for the free α chains (19). Solutions of deoxyhemoglobin were prepared with a concentration of 2×10^{-4} M and with n varying from 0 to 4; 10 ml of these solutions were added under anaerobic conditions to a mixture of 5 ml of 0.2 M phosphate buffer and 5 ml of 10^{-2} M FDNB solution in a jacketed vessel thermostated at 25° . Before adding the hemoglobin solution, the FDNB solution was made oxygen-free by bubbling nitrogen through. At regular time intervals 2-ml samples were pipetted into test tubes containing 6 ml of 0.2 M phosphate buffer at pH 5.4 at 0° , kept in an ice bucket. In this way the reaction was stopped completely. Immediately after sample taking deoxyhemoglobin was converted to carboxyhemoglobin. The excess FDNB was removed by dialyzing the samples at 4° against three changes of 2 liters of 0.02 M phosphate buffer at pH 5.4. Control FDNB solutions dialyzed in the same way showed a residual absorbance near 0.002 at 353 nm, which is the wave length of maximum absorbance of DNP-substituted terminal NH_2 groups (20, 21), and were used as blanks in the spectrophotometrical measurements. The reaction rate was followed by measuring the ratio $R = A_{353}/A_{540}$, where A_{353} is the absorbance of the hemoglobin-DNP derivatives at 353 nm and A_{540} the absorbance at 540 nm. The ratio R is indicative for the amount of α - NH_2 groups substituted, since only A_{353} does change upon reaction. In addition to the measuring of the over-all kinetics of the hemoglobin tetramer with FDNB, the absolute amounts of the DNP-substituted α and β chains were measured by isolating the separate chains using the chromatographic procedure of Clegg *et al.* (22, 23). FDNB was obtained from Sigma and was used without further purification. For all experiments freshly prepared FDNB solutions (10 mM) in 0.1 M NaCl were used.

DPG-binding Experiments—Four milliliters of a buffered (0.05 M Tris-HCl buffer) solution containing oxyhemoglobin and DPG both in a concentration of about 1 mM were dialyzed against 10 ml of Tris buffer in a test tube. Prior to the experiment 1 mg of dithionite per ml was added to the inner and outer solution in order to deoxygenate both compartments and to convert oxyhemoglobin into deoxyhemoglobin. The total ionic strength was kept at 0.1 with KCl. The test tubes were sealed and shaken for 72 hours at 4° . The time needed to reach equilibrium was checked in control experiments. After reaching equilibrium, the DPG concentration in the outer solution was measured by the Bartlett procedure (18) and the number of moles bound per hemoglobin tetramer calculated.

DPG was obtained as salt from Calbiochem and was converted into the acid form by repeated passing through Amberlite IR 120.

Calculations—First we want to extend the Riggs model by taking into account the change in ionization of DPG with pH. The two second ionization steps of the phosphate groups must be those responsible for the extra acid Bohr effect (8). It is noteworthy to say here that the extra Bohr effect (ΔZ versus pH) as shown in Fig. 1 has been obtained by subtracting the Bohr effect measured without DPG from that observed in the presence of DPG. Since the extra acid Bohr effect is equal in magnitude to the extra alkaline Bohr effect (see Fig. 1) it will be obvious that the enhanced alkaline Bohr effect also can be described in terms

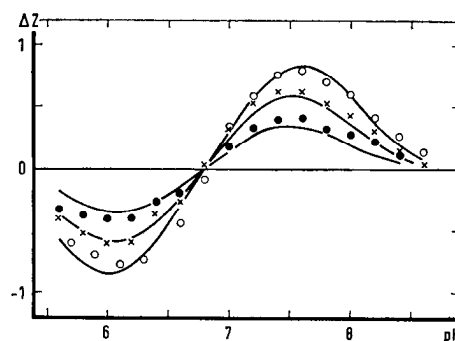


FIG. 1. The extra Bohr effect due to the binding of DPG by deoxyhemoglobin; hemoglobin concentration 2.5×10^{-4} M (tetramer basis). The ratio n between the concentration of DPG and hemoglobin was 0.5 (●), 1.0 (×), and 2.0 (○). Drawn lines were calculated with $\text{p}K_1 = 6.95$, $\text{p}K_2 = 6.65$, $K_d = 8 \times 10^5 \text{ M}^{-1}$, and $K_0 = 2 \times 10^4 \text{ M}^{-1}$.

of only two ionizable groups. A priori both the two histidines H21 and the two α - NH_2 groups of the β chains in the tetramer could be responsible, but we have reasons to believe (see below) that the contribution of the NH_2 termini is small as compared to that of the histidines H21.

In deriving the proper set of equations we denote the concentration of DPG molecules in which both phosphate groups have 1 proton bound as (DH_2) , that of the DPG molecules in which one of the phosphate groups has 1 proton bound as (DH) and the concentration of completely ionized DPG molecules as (D) . The dissociation of these protons is ruled by the microscopic dissociation constant K_1 of the second ionization of the phosphate groups. In the same way let $(\text{Hb}H_2)$ be the concentration of hemoglobin with the two relevant groups in the protonated form, $(\text{Hb}H)$ that of hemoglobin with one of the two groups protonated, (Hb) that with both groups ionized, and K_2 the microscopic dissociation constant of the two groups on the tetramer. With these notations it follows that h_d , the number of protons bound by deoxyhemoglobin, is given by

$$h_d = 2 \frac{K_d X [a_H K_1 / (K_2 (K_1 + a_H))]^2 + (a_H / K_2)^2 + a_H / K_2}{K_d X [a_H K_1 / (K_2 (K_1 + a_H))]^2 + (a_H / K_2 + 1)^2} \quad (1)$$

where X is the total concentration of free DPG, a_H is the proton activity, and K_d is the pH-independent association constant of the hemoglobin-DPG complex, defined by

$$K_d = \frac{(\text{Hb}H_2 D)}{(\text{Hb}H_2)(D)} \quad (2)$$

For r_d , the number of protons bound by DPG calculated per hemoglobin tetramer, we can write

$$r_d = \frac{2(DH_2) + (DH)}{c_{\text{Hb}}} \quad (3)$$

where c_{Hb} is the total concentration of hemoglobin. In case DPG binds also to oxyhemoglobin we arrive at a similar set of equations for h_0 and r_0 , the number of protons bound by oxyhemoglobin and DPG, respectively, except that K_0 replaces K_d .

ΔZ , the total extra Bohr effect due to DPG binding, is now given by

$$\Delta Z = (h_d - h_0) + (r_d - r_0) \quad (4)$$

This key relation differs from the Riggs model in so far that the

Riggs model does not include the term $r_d - r_o$. The equations given by Riggs (12) can easily be obtained from the above equation by assigning to K_1 an infinite value.

RESULTS

Extra Bohr Effect—Based on the model described we have carried out several computer calculations in order to see if the experimental data, reported earlier by us (8), would sufficiently be accounted for by this extended model. The shape of the additional Bohr effect curve due to the binding of DPG is shown in Fig. 1. The curves shown in Fig. 2 are calculated with a constant value for K_d and varying values for K_o . For pK_1 and pK_2 we used the values estimated as outlined below. The most important conclusion which can be drawn from Fig. 2 is that the extended model presented here is indeed able to match the overall shape of the experimental curves.

In order to evaluate the proper values of the several parameters we proceeded as follows. From the mathematical treatment given in the previous section it can be proven that the cross-over point, which is the pH where $\Delta Z = 0$, is equal to $(pK_1 + pK_2)/2$. Since pK_1 can directly be measured by titration of a solution of DPG, pK_2 can be estimated from the pH value of this cross-over point. For the mean value of pK_1 we found from accurate titration experiments at 25° and at an ionic strength of 0.1 (KCl) a value of 6.95 which is 0.15 lower than reported by Benesch *et al.* (6). From this pK_1 value and the crossover point at pH = 6.8 we calculated $pK_2 = 6.65$.

At this point we are left with the problem to get proper values for K_d and K_o ; in the binding experiments on deoxyhemoglobin the only parameter which has to be varied in order to fit the data is K_d , since K_1 and K_2 can be estimated from the Bohr curve. Here the analysis is not troubled by the extra parameter K_o . Therefore, in order to fit the Bohr data we chose as starting value for K_d the value obtained from the binding experiments at 4°. There we found (see below) $K_d = (6.5 \pm 1.5) \times 10^5 \text{ M}^{-1}$. Subsequently we calculated several curves with varying values for K_d and K_o . The best fit of the Bohr data shown in Fig. 1 was obtained with $K_d = 8 \times 10^5 \text{ M}^{-1}$ and with a much lower value for K_o , *viz.* $K_o = 2 \times 10^4 \text{ M}^{-1}$. That K_o should be much smaller than K_d is shown in Fig. 2, where curves are shown with $K_d = 8 \times 10^5$ and various values for K_o . The figure shows clearly that when K_o is half the value of K_d , as has been reported (24, 25), hardly any extra Bohr effect would be detected. The observation that K_d and K_o should differ a lot in order to get the additional Bohr effect supports the evidence presented (1, 26, 27), that at near-physiological ionic strength the binding of DPG by oxyhemoglobin can be neglected as compared with the binding by deoxyhemoglobin.

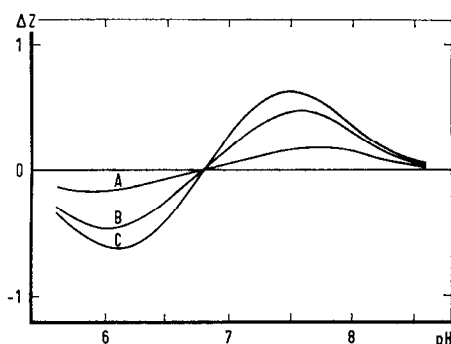


FIG. 2. The influence of increasing K_o values on the extra Bohr effect. The curves were calculated with $K_d = 8 \times 10^5 \text{ M}^{-1}$, $pK_1 = 6.95$, $pK_2 = 6.65$, and various K_o values. Curve A, $K_o = 4 \times 10^5 \text{ M}^{-1}$; Curve B, $K_o = 8 \times 10^4 \text{ M}^{-1}$; Curve C, $K_o = 5 \times 10^3 \text{ M}^{-1}$.

Binding Experiments—In Fig. 3 the pH dependence of the binding of DPG by deoxyhemoglobin is shown. The hemoglobin concentration was $2.5 \times 10^{-4} \text{ M}$ (tetramer basis) and the total DPG concentration $1.0 \times 10^{-4} \text{ M}$. The concentrations are given per total volume, which is the sum of the volumes of inner and outer compartment. The figure shows that above pH 7 the binding decreases with increasing pH, which is consistent with earlier reports (1, 2, 24). The drawn line is calculated with the same values for pK_1 , pK_2 , and K_d as those used to calculate the curves in Fig. 1. In other sets of experiments K_d varied between $5 \times 10^5 \text{ M}^{-1}$ and $8 \times 10^5 \text{ M}^{-1}$.

Kinetic Experiments—In Fig. 4 first order plots are shown for the reaction of the $\alpha\text{-NH}_2$ groups of the α and β chain of deoxyhemoglobin with FDNB in the presence and absence of DPG. We did not make an attempt to analyze the data quantitatively in terms of the intrinsic reaction constant and dissociation constants of both termini (19, 20). The figure shows, however, clearly that the reaction of FDNB with deoxyhemoglobin is decreased when DPG is present. The plot for deoxyhemoglobin without DPG is linear up to a degree of at least 50% substitution indicating that the rates of reaction of both termini are about equal resulting in a pseudohomogeneous reaction.

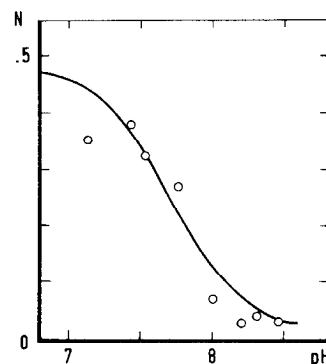


FIG. 3. A plot of N , the number of DPG molecules bound per tetramer by deoxyhemoglobin, versus pH at 4° and an ionic strength of 0.1. Hemoglobin concentration $2 \times 10^{-4} \text{ M}$; DPG concentration $1.0 \times 10^{-4} \text{ M}$. Drawn line was calculated with $pK_1 = 6.95$, $pK_2 = 6.65$, and $K_d = 8 \times 10^5 \text{ M}^{-1}$.

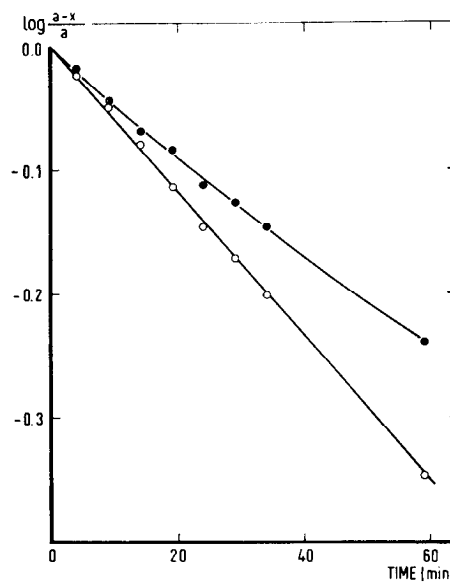


FIG. 4. First order plot of the reaction of FDNB with the $\alpha\text{-NH}_2$ groups of deoxyhemoglobin at pH 7, 25°, and an ionic strength of 0.1; in the presence of DPG (●); in the absence of DPG (○).

In the presence of DPG the reactivity of deoxyhemoglobin is lowered and the plot becomes biphasic indicating a diminished reactivity of the NH_2 terminus of the β chain due to the shielding effect of this group by DPG. A priori a decreased reactivity due to the presence of DPG could also be caused by a change in reactivity of the $\alpha\text{-NH}_2$ group of the α chain. Therefore, substitution reactions were carried out at pH 7 under experimental conditions identical with those of the kinetic experiments reported in Fig. 4. The reaction was allowed to proceed for 1 hour. The reaction mixtures, with and without DPG, were processed through the chromatographic procedure of Clegg *et al.* (22, 23). The results are such, that each of the two peaks usually obtained (22, 23), corresponding with the separated α and β chains from which the heme groups have been removed, split up into two peaks corresponding with the substituted and unsubstituted α and β chain, respectively. The peaks containing the substituted chains were yellow colored while the peaks containing the unreacted material could only be detected by spectrophotometrical measurements at 280 nm. After collecting the samples extensive dialysis was carried out to remove all chemicals which might interfere with the spectrophotometrical measurements and the relative amounts of the materials under the four peaks were measured at 280 nm. It appeared that the ratio P of the amounts of substituted and unsubstituted α chains did not depend upon the presence of DPG and amounted to 1.58. On the contrary the ratio of the amounts of substituted and unsubstituted β chains changed from $P = 0.80$ in the absence of DPG to $P = 0.47$ in the presence of DPG. This seems to indicate that the depressed reactivity of deoxyhemoglobin toward FDNB is entirely due to a decrease in reactivity of the NH_2 terminus of the β chain.

DISCUSSION

The most important conclusion which can be drawn from the foregoing is that both the general features of the extra Bohr effect due to the binding of DPG and the results obtained in binding experiments are quite well consistent with the model presented. The K_d value as measured by us is about 5 times larger than the one calculated from the data of Benesch *et al.* (6), while it is about equal to the value which can be calculated from the data of Calwell *et al.* (27). We do not have an explanation for this discrepancy. In excellent agreement with the results reported in both papers we found that at near-physiological ionic strength K_0 can be neglected as compared with K_d . A consequence of the fact that at low ionic strength K_d is only twice the value of K_0 (24, 25) would be that the extra Bohr effect should decrease with decreasing ionic strength (see Fig. 3). From the value for K_d of $8 \times 10^5 \text{ M}^{-1}$ we calculated a mean binding free energy of 1.6 Cal per saltbridge present, which is a very reasonable value.

A very striking result is the value for pK_1 of 6.65 of the two groups of hemoglobin responsible for the extra alkaline Bohr effect. This pK value suggests strongly that these groups are the two histidines H21. If so, then the question arises why the $\alpha\text{-NH}_2$ group of the β chains do not contribute to the extra Bohr effect; the possible explanation that they are not involved in the binding of DPG is ruled out by the observation of Bunn and Briehl (15) who found that various human hemoglobins in which the NH_2 termini of the β chains are modified, showed depressed affinity for DPG. Also the results of Bauer (13) and Pace *et al.* (14) who found that DPG interferes with the binding of CO_2 by the NH_2 termini and the x-ray data of Perutz (9) and Arnone

(10) point in the same direction. Our kinetic data present further evidence for the part played by this group in binding DPG, since the results show clearly a diminished reactivity of this group toward FDNB in the presence of DPG. A possible explanation might be that the pK of the $\alpha\text{-NH}_2$ group of the β chain is higher than 6.65 so that this group will contribute to the alkaline Bohr effect at higher pH values than the group with a pK of 6.65. Since with increasing pH the binding of DPG by hemoglobin decreases (see Fig. 3) this contribution would consequently be diminished. In agreement with this would be that the degree of substitution by FDNB of the α chain is about twice as large as the degree of substitution of the β chain. The lower reactivity of the $\alpha\text{-NH}_2$ group of the β chain toward FDNB as compared to that of the α chain might, however, also be due to the binding of phosphate ions to the $\alpha\text{-NH}_2$ group of the β chain in deoxyhemoglobin. Kinetic experiments in non-phosphate buffers could give the answer to this difficulty.

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