EFFECT OF 2,3-DIPHOSPHOGLYCERATE ON THE COOPERATIVITY IN OXYGEN BINDING OF HUMAN ADULT HEMOGLOBIN

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Summary: Oxygen equilibrium curves of human adult hemoglobin (Hb A) have been analysed by the Scatchard and Hill plots and four successive association constants for the binding of oxygen have been determined by fitting the polts with simulated curves calculated by a digital computer. 2,3-Diphosphoglycerate (DPG) markedly reduces the affinity of Hb A to the 1st, 2nd, and 3rd molecules of oxygen without affecting the affinity to the 4th molecule, which is similar to the oxygen affinity of the isolated β chains. Thus, the over-all free energy of interaction increases by about 1400 cal per site and the maximum slope of the Hill plot, n, increases significantly in the presence of 2×10^{-3} M DPG.

Since the discovery of the remarkable effect of organic phosphates, especially DPG, in lowering the oxygen affinity of Hb A (1), much attention has been directed to the mechanism and physiological role of the phenomenon (2, 3, 4). It is now believed that the effect of DPG originates from the preferential binding of the phosphate to deoxy Hb A, not to fully oxygenated Hb A, in a mole-for-mole ratio (2). This will undoubtedly reduce the affinity of deoxy Hb A to the 1st oxygen molecule. However, the effect of DPG on the oxygen affinity of Hb A in an intermediate stage of oxygenation is still uncertain. The present report is an approach to this problem from an equilibrium point of view based on the Adair's successive oxygenation theory (5).

Hb A prepared from the blood of normal human adults was freed from phosphates as described by Benesch *et al.* (6). The oxygen equilibrium curves were determined by the method of Imai *et al.* (7) on 1.5×10^{-5} M Hb A (as teteramer) in 0.01 M Tris-HCl buffer at 25° in the presence and absence of 2×10^{-3} M DPG. Special care was directed to the precise determination of the top and bottom of the curves. The isolation of α and β chains from Hb A and the determination of the oxygen equilibrium of the isolated chains were performed as described previously (8). The fractional saturation of hemoglobin with oxygen, Y, and the corresponding oxygen pressure, p, thus obtained were analysed by the Scatchard (9) and Hill plots (10), *i.e.* log Q *vs.* 4Y and log $[Y/(1 - Y)]$ *vs.* log p plots, respectively, where $Q = y/[(1 - Y)p]$.

According to Adair (5), the oxygen equilibrium of hemoglobin can be expressed in terms of the simplest set of four reversible reactions

$$Hb_4(O_2)_{i-1} + O_2 \rightleftharpoons Hb_4(O_2)_i, \qquad i = 1 \text{ to } 4. \tag{1}$$

Let k_i be the intrinsic microscopic association constant for the i-th equilibrium, Y is expressed as follows:

$$Y = \frac{k_1p + 3k_1k_2p^2 + 3k_1k_2k_3p^3 + k_1k_2k_3k_4p^4}{1 + 4k_1p + 6k_1k_2p^2 + 4k_1k_2k_3p^3 + k_1k_2k_3k_4p^4} \tag{2}$$

Further, it has been proved by Edsall *et al.* (9) that

$$\lim_{Y\to 0} Q = k_1, \qquad \lim_{Y\to 1} Q = k_4, \tag{3}$$

$$\lim_{Y\to 0} d(\ln Q)/d(4Y) = 3(k_2 - k_1)/4k_1, \quad \lim_{Y\to 1} d(\ln Q)/d(4Y) = 3(k_4 - k_3)/4k_3. \tag{4}$$

Thus, a preliminary set of k's was obtained from the extrapolated intercepts of the Scatchard plot on both the ordinates and the limiting slopes of the plot. These estimates were subjected to further adjustment in order to give the best fit to the experimental data over the whole range covered by the measurements in both the Scatchard and Hill plots. A Hitachi Hitac-10 digital computer was used for the calculation.

As shown in Figs. 1 and 2 the fittness of the simulated curves to the experimental points is satisfactory. The values of k's thus determined are summarized in Table 1. As expected, DPG reduces k_1 to about one-tenth. The phosphate also decreases k_2 in nearly the same ratio and k_3 much more. Interestingly, however, k_4 shows no or little, if any, change even in the presence of functionally saturating concentration of DPG. Moreover, k_4 was

Table 1. Intrinsic microscopic association constants (mmHg^{-1}) for the equilibrium of oxygen with Hb A at pH 7.4 and 25°.

	k_1	k_2	k_3	k_4
stripped	0.079	0.295	0.75	4.35
in 2×10^{-3} M DPG	0.008	0.037	0.02	4.35

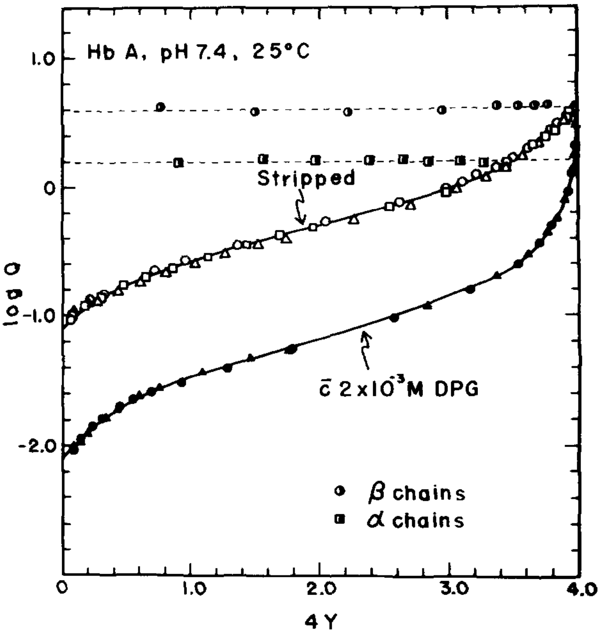

Fig. 1. Scatchard plots of the oxygenation of stripped Hb A in the absence and presence of 2×10^{-3} M DPG. pH 7.4 and 25°. Different symbols refer to separate experiments on different samples. Solid lines are the simulated curves constructed by using the values of k's shown in Table 1. Plots for the isolated chains (0.1 M phosphate, pH 7.5, 25°) are given for comparison.

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insensitive to pH change in the range of 7.0 to 7.8 irrespectively of the presence and absence of DPG. The insensitivity of k_4 to DPG is consistent with the results and idea described in two recent papers. In the one, Gibson (11) has shown that inorganic phosphate reduces the rate of dissociation of the 2nd, 3rd, and 4th oxygen molecules leaving oxy Hb A without affecting the rate of the 1st molecule (i.e. the 4th molecule in the direction of the combination). In the other, Perutz (12) has proposed a model for the sequence of cooperative oxygen binding of Hb A in which DPG combined with deoxy Hb A is expelled after the 2nd heme has been oxygenated.

Fig. 2 illustrates the estimation of the over-all free energy of interaction by the method of Wyman (10). The position of the asymptotes of unit slope required for the estimation can be determined precisely by extending the range of the simulation to both the extreme ends. This procedure is especially useful in the case such as with DPG where the Hill plot does not flatten out even at the extreme upper end and the position of the asymptote can not be obtained from the experimental points alone. In this connection, it is worthwhile to mention that the Hill plots of any oxygen equilibrium curve have a common upper or lower asymptote if their k_4 or k_1 is identical.

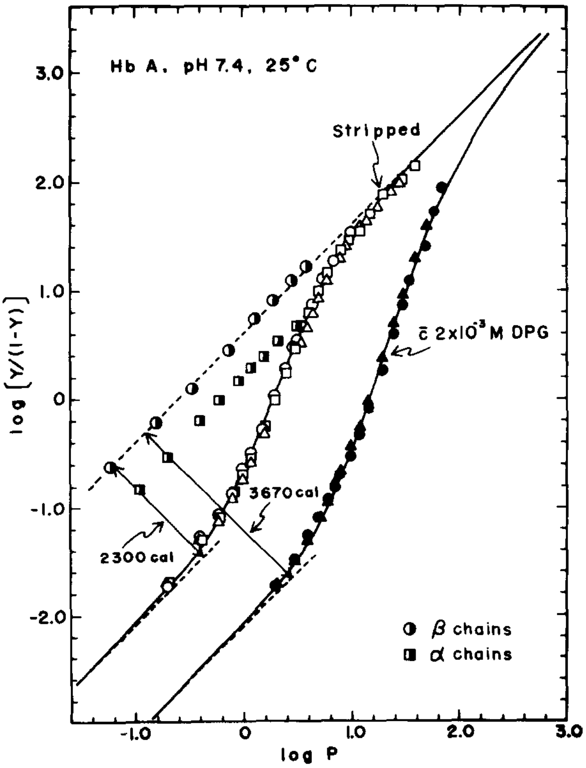

Fig. 2. Hill plots of the data shown in Fig. 1. All conditions as for Fig. 1.

This can easily be proved, since the asymptotes are expressed by the following equations:

$$\log [Y/(1 - Y)] = \log p + \log k_4, \qquad (5)$$

$$\log [Y/(1 - Y)] = \log p + \log k_1, \qquad (6)$$

respectively, which are derived by taking the limits of log $[Y/(1 - Y)]$ at $p \to \infty$ or $p \to 0$ by using eq. (2).

As seen from Fig. 2, 2×10^{-3} M DPG increases the over-all free energy of interaction by about 1400 cal per site, *i.e.* 5600 cal per mole of Hb A tetramer, Since DPG bears four negative charges per mole at pH 7.4 (2) and the bond energy per salt bridge has been assumed as 1000 to 2000 cal per mole (12), the increase of 5600 cal in free energy of interaction may reasonably be assigned to the formation of four additional salt bridges between Hb A and DPG. This value, however, is slightly lower than the free energy of DPG binding obtained from direct binding measurements (6400 cal per mole at 20° (6)). DPG increases so-called Hill's exponent, n, *i.e.* the maximum slope of the Hill plot, as well. Calculation of the slope on the simulated Hill plot at different log p revealed that the maximum value of the slope is 2.52 and 3.02 in the absence and presence of 2×10^{-3} M DPG, respectively.

According to the Perutz model (12), Hb A will be in a completely unconstrained conformation after the 3rd heme has been oxygenated. A comparison was made, therefore, between the value of k_4 for Hb A and the corresponding value for the isolated chains which are probably free from constraint and show no cooperativity at all. As shown in Fig. 1, the extrapolated intercept of the Scatchard plot of β chains, not of α chains, on the right hand ordinate coincides quite well with that for Hb A. Furthermore, the Hill plot for β chains lies exactly on the upper asymptote for Hb A. These results and the insensitivity of k_4 to pH change clearly indicate that the oxygen equilibrium function of Hb A at very near the full saturation is similar to that of the isolated β chains. The similarity in the upper part of the oxygen equilibrium curves between stripped Hb A and myoglobin has already been shown by Benesch et al. (13). Thus, it is now tempting to speculate that the hemoglobin subunit which combines the 4th and last oxygen molecule is β chains.

Similar studies on other organic phosphates and on various hemoglobins are now in progress.

References

1. Benesch, R., and Benesch, R.E., Biochem. Biophys. Res. Commun., 26, 162 (1967)
2. Benesch, R.E., Benesch, R., and Yu, C.I., Biochemistry, 8,2567 (1969)
3. Tyuma, I., and Shimizu, K., Fed. Proc., 29, 1112 (1970)
4. Brewer, J. ed., Red Cell Metabolism and Function, Plenum Press, new York (1970)
5. Adair, G.S., J. Biol. Chem., 63, 529 (1925)
6. Benesch, R., Benesch, R.E., and Yu, C.I., Proc Natl. Acad. Sci., 59, 526 (1968)
7. Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., and Kuroda, M., Biochim. Biophys. Acta, 200, 189 (1970)
8. Tyuma, I., Benesch, R.E., Benesch, R., Biochemistry, 5, 2957 (1966)
9. Edsall, J.T., Felsenfeld, G., Goodman, D.S., and Gurd, F.R.N., J. Am. Chem. Soc., 76, 3054 (1954)
10. Wyman, J., Advan. Protein Chem., 19, 223 (1964)
11. Gibson, Q.H., J. Biol. Chem., 245, 3285 (1970)
12. Perutz, M.F., Nature, 228, 726 (1970)
13. Benesch, R., Benesch, R.E., and Enoki, Y., Proc. Natl. Acad. Sci., 61, 1102 (1968)