THERMODYNAMICS OF 2,3-DIPHOSPHOGLYCERATE ASSOCIATION WITH HUMAN OXY- AND DEOXYHEMOGLOBIN

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SUMMARY: The association of 2,3-diphosphoglycerate with oxy- and deoxyhemoglobin was studied by means of ultrafiltration and microcalorimetry. It was found that in addition to parameters that are known to influence the binding of 2,3-diphosphoglycerate to both species of hemoglobin (such as pH, temperature and concentration of competing anion), the association is also strongly dependent on the hemoglobin concentration. The difference between the apparent association constants for the formation of the complex of the organic phosphate with oxy- and deoxyhemoglobin is relatively small. At pH 7.3, 25° C and 0.154 M chloride this difference is only 0.6 kcal/mole of free energy favoring the Hb·DPG complex. This free energy difference increases with decreasing pH but is not strongly affected by hemoglobin concentration. The enthalpy change for the formation of the 2,3-diphosphoglycerate complex with deoxyhemoglobin is 8-10 kcal/mole more exothermic than the complex with oxyhemoglobin.

Regulation of oxygenation of human hemoglobin by 2,3-diphosphoglycerate (DPG)* is understood in a qualitative way (1), and the X-ray diffraction study by Arnone (2) contributes much to the understanding of the structural properties of the Hb·DPG complex. However, several thermodynamic parameters for the interaction of DPG and other organic phosphates with oxy- and deoxyhemoglobin have not been established. Results obtained in different laboratories disagree not only in terms of absolute affinities for the interaction between DPG and Hb and HbO_2, but also in regard to the

*Abbreviations: DPG, 2,3-diphosphoglycerate; Hb, deoxyhemoglobin; HbO_2, oxyhemoglobin; BISTRIS, bis (2-hydroxyethyl) iminotris (hydroxy-methyl) methane

difference between these affinities (3). The latter difference is a measurement of the free energy that is available for regulation of oxygen binding under any given set of conditions. It is likely that some of the discrepancies in the literature can be explained by the variety of experimental conditions used. The results obtained in this investigation have been compared with those obtained in other laboratories and these comparisons are summarized in Tables 1 and 2. Such a comparison also emphasizes the importance of the hemoglobin concentration as an experimental variable (Fig. 2).

MATERIALS AND METHODS: The hemoglobin used in this investigation was prepared from three week old red cells. Hemoglobin was prepared as described elsewhere (5,6). Minor components of human adult hemoglobin were not removed.

The 2,3-diphosphoglycerate was purchased from Boehringer-Mannheim Corp. in the form of the pentacyclohexylammonium salt. The DPG was converted to the free acid by means of a Amberlite 120 H^+ column. The pH was adjusted to neutrality with NaOH.

The ultrafiltration experiments were carried out in equipment purchased from Amicon Corp. (Ultrafiltration Cells Model 12 and 52).

Table 1

Association constants (M^{-1}) for the binding of DPG to Hb and HbO_2 in low concentration of competing anion

		This work: 0.025 M BISTRIS, 0.004 M Cl^-, pH 7.2, 25° C $c_{prot.}$: 0.6 mM	Ref. 7: 0.050 M cacodylate, pH 6.5, 5° C $c_{prot.}$: 0.08 mM
DEOXY-	K_1	6.5×10^4 (n=1)	1.4×10^5 (n=1)
HEMOGLOBIN	K_2	1.5×10^3 (n=4)	3.6×10^3 (n=2)
OXY-	K_1	6.5×10^4 (n=1)	7.0×10^4 (n=1)
HEMOGLOBIN	K_2	1.5×10^3 (n=4)	1.1×10^3 (n=2)

Table 2

Summary of association constants (M^{-1}) for the binding of DPG to human oxy- and deoxyhemoglobin in the presence of 'physiological' concentration of chloride ion.

	THIS WORK			Ref. (10)	Ref. (9)	Ref. (11)
pH	7.0	7.3	7.6	7.3	7.2	ca. 7.2
Temp.	---------- 25 ----------			20	37	20 - 25
Buffer	------ 0.025 M BISTRIS ----			0.050 M BISTRIS	0.001 M* P_i	none
Chloride (M)	-------- 0.154 ----------			0.10	0.136	0.10
Protein conc. (mM)	--------- 0.60 ----------			0.10	0.40	0.25
Hb K_1***	1.5×10^4	7.5×10^3	2.9×10^3	6.7×10^4	3×10^{3}**	--
Hb K_2	2.0×10^2	2.5×10^2	2.8×10^2			
HbO_2 K_1	3.3×10^3	2.3×10^3	1.6×10^3	---	1×10^{3}**	2.3×10^{3}**
HbO_2 K_2	2×10^1	3×10^1	5×10^1			

*This buffer also contained 0.003 M Mg^{2+}.
**Association constants calculated using results in ref 9 and 11. One site was assumed.
***Best fit association constants using one strong and four weak sites.

Both Visking dialysis membranes and Amicon PM 30 membranes were used with similar results. DPG was analysed as total phosphate using the method of Bartlett (4). The procedure is discussed in detail elsewhere (5). All experiments were carried out at 25° C.

Microcalorimetric experiments were carried out in a batch calorimeter of the heat conduction type. The amount of heat that was measured was typically in the range of 2-20 millicalories (5, 6).

Deoxyhemoglobin was prepared either by equilibration under nitrogen or by means of addition of solid sodium dithionite followed by ion exchange chromatography under nitrogen.

RESULTS AND DISCUSSION: Ultrafiltration experiments carried out with dilute hemoglobin (less than 1.0 mM) and with very low concen-

Table 3

Calorimetry of the binding of DPG to oxy- and deoxyhemoglobin. In the oxyhemoglobin experiments a 0.025 M BISTRIS buffer pH 7.2 was used, chloride concentration was 0.004 M. The hemoglobin concentration was 0.61 mM. In the deoxyhemoglobin experiments a 0.05 M BISTRIS buffer was used, pH 7.2, chloride concentration was 0.008 M. The hemoglobin concentration was 0.56 mM.

Conc. DPG (mM)	No. of exp.	Fraction DPG bound	ΔH kcal/mole DPG bound	Stand. dev. kcal/mole
	O X Y H E M O G L O B I N			
0.242	8	96.7	-4.4	0.27
0.413	4	95.5	-3.7	0.12
0.610	9	93.4	-2.9	0.21
	D E O X Y H E M O G L O B I N			
0.257	5	94.7	-13.5	0.9
0.373	3	93.4	-12.5	0.6
0.513	9	92.2	-11.5	0.6

tration of competing anions are summarized in Table 1. The results obtained in this laboratory can be compared with those obtained by Chanutin and Hermann (7). A direct comparison of the two sets of data is difficult since the experimental condition were different, but several important observations can be made. Thus, the difference between the association constants for the strong site of organic phosphate binding is small or nonexisting in low concentration of competing anion. Weaker secondary sites exist for the binding of DPG on both the liganded and unliganded protein. A larger difference between the two association constants for the strong sites is observed at low temperature. This is to be expected since the enthalpy for the binding of DPG to deoxyhemoglobin is more exothermic than binding to oxyhemoglobin (see Table 3).

Results obtained for the binding of DPG to Hb and HbO_2 in the

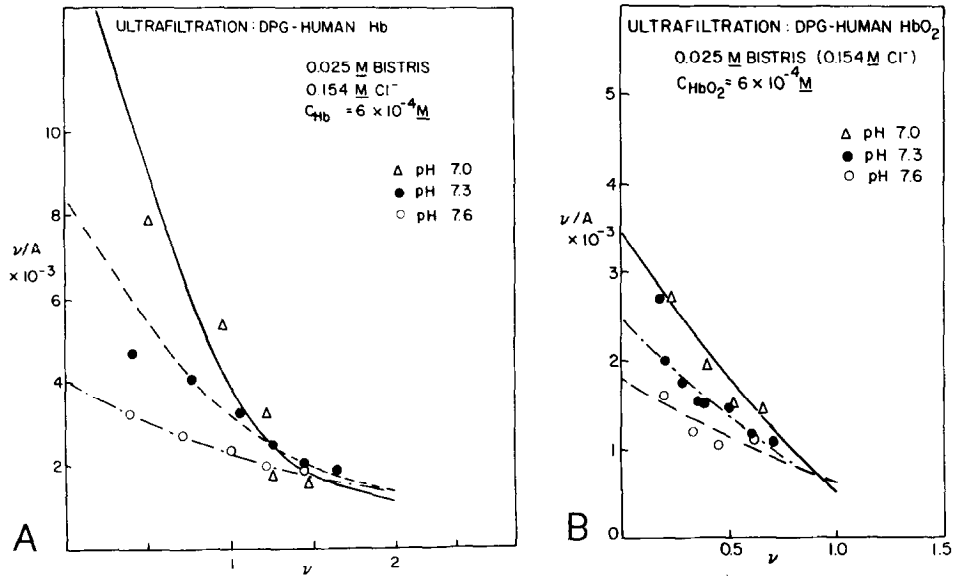

Figure 1. Binding of DPG to human deoxy- (Fig. 1a) and oxyhemoglobin (Fig. 1b).

presence of 0.154 M chloride are illustrated in Fig. 1A and 1B. The experimental points could not be fitted assuming one site. A summary of the results obtained here, as well as selected results from other laboratories are presented in Table 2. The results obtained here indicate that the difference in association constant for the binding of DPG to Hb as compared to HbO_2 is a factor of 5 at pH 7.0 and decreases to a factor of two at pH 7.6. Thus the pH dependence for the binding of DPG to Hb is more pronounced than binding to HbO_2. This suggest some difference between the binding sites on the two forms of the protein. It is likely that the binding site on deoxyhemoglobin contains at least one more amino acid residue with pK near 7 than does oxyhemoglobin. The increase in affinity for DPG as pH decreases as well as the larger difference in affinity at lower pH has been observed by other workers (8, 9).

A comparison between the results obtained here and those obtained in other laboratories suggest some differences. Thus, for deoxy-

hemoglobin, at pH 7.3 Benesch and coworkers (10) obtained an association constant of 6.7×10^4 M^{-1} whereas in this investigation 7.5×10^3 was obtained. However, three variables were somewhat different. Benesch et. al. had a lower concentration of hemoglobin and chloride and the temperature was 20° C. All these changes tend to increase the observed association constant, and may when summed, raise it by as much as an order of a magnitude. However, the lack of binding of DPG by oxyhemoglobin as noted by Benesch et. al. can not be easily explained. The binding levels for oxyhemoglobin that are observed by Garby and deVerdier (9) as well as by Luque (11) and coworkers agree well with those obtained here. In addition, the results obtained by Garby and deVerdier suggest about a three fold increase in association constant upon deoxygenation at physiological pH, which is the same as obtained in this investigation. This corresponds to 0.6 kcal/mole of free energy that may be available for regulation of oxygenation. This is of the same order of magnitude as random thermal energy.

The influence of hemoglobin concentration on the binding levels of DPG for both species of hemoglobin is illustrated in Figure 2. This figure was constructed by calculating association constants using the results obtained by Garby and deVerdier (9) and by Luque et. al. (11), the reported association constant in ref. 10, as well as the results obtained in this investigation. The two lines through the points are the least square regression lines for the two species of hemoglobin. Figure 2 suggests, that despite the strong dependence of the two association constants on the hemoglobin concentration, the difference between them do not change on a log (i.e. free energy) scale. The amount of free energy favoring DPG binding to deoxyhemoglobin probably does not exceed one kcal/mole under physiological conditions, suggesting that DPG exerts a rather weak control of hemoglobin oxygenation. The binding levels in physiological concentration of hemoglobin are

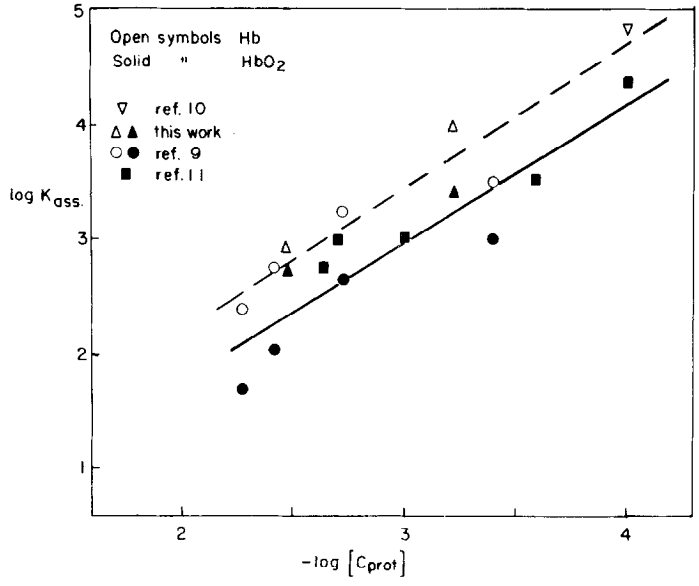

Figure 2. Dependence of the observed association constant for the binding of DPG to hemoglobin on protein concentration.

rather low. Using Fig. 2 one can estimate that about 50% of the DPG is bound in the deoxygenated cell and about 30% in the oxygenated cell, assuming that both hemoglobin and DPG concentration is 5 mM.

Microcalorimetric results from experiments in which DPG was mixed with human oxy- and deoxyhemoglobin are summarized in Table 3. By using molar excess of hemoglobin tetramer, the binding to the strong site was maximized. Binding levels were calculated using ultrafiltration data obtained under identical or very similar conditions. Thus DPG binds to deoxyhemoglobin with an enthalpy change of about 9 kcal/mole more exothermic than to oxyhemoglobin. Recent results by Nelson et. al. (12) suggest a similar enthalpy change for the binding of DPG to deoxyhemoglobin. Nelson and coworkers did not observe any heat upon mixing DPG and oxyhemoglobin at pH 7.4. This is very likely due to a nearly athermal reaction under the conditions chosen, rather than to no binding of DPG to oxyhemoglobin. A small number of experiments were carried out in this laboratory at pH 7.2 and in the

Table 4

Thermodynamic parameters for the binding of DPG to the strong site on human oxy- and deoxyhemoglobin. The experimental conditions are the same as in Table 3.

	ΔG kcal/mole	ΔH kcal/mole	ΔS e. u.
Hb	-6.5 ± 0.2	-15.5 ± 0.8	-30 ± 3
HbO_2	-6.5 ± 0.2	-5.4 ± 0.3	4 ± 2

presence of 0.154 M chloride and yielded +0.7 ± 0.2 kcal/mole for the binding of DPG to oxyhemoglobin and -11 ± 2 kcal/mole for deoxyhemoglobin. Thus, no serious discrepancy exists between the two reports. The observed decrease in the enthalpy change as the concentration of ligand is increased is probably due to an increasing influence of weaker (presumably less exothermic) binding sites. In Table 4 the estimated thermodynamic parameters for the strong binding site on both species of hemoglobin are summarized. The values for the enthalpies were obtained by extrapolation to zero ligand concentration. The measured enthalpy may include heats of protonation of any of the ionizable species in solution. The binding of DPG to both species of hemoglobin is known to be accompanied by release of protons (13) and such proton shifts are likely to contribute to the total observed heat. The highly exothermic enthalpy change for the binding of DPG to deoxyhemoglobin implies that the free energy for this association is more temperature dependent than binding to oxyhemoglobin. Thus in the presence of low concentration of chloride ion the binding of DPG to HbO_2 will be favored at temperatures above 25° C. In high chloride concentration the situation is essentially the same. At pH 7.3, 37° C and in 0.15 M chloride the binding to deoxyhemoglobin will still be slightly favored but with an even smaller amount of free energy than

the ca. 0.6 kcal/mole that was obtained at 25° C. Although the free energy favoring DPG binding to deoxyhemoglobin, which is the free energy available for regulation of hemoglobin oxygenation, is dependent on a variety of conditions, it is apparent that the amount is smaller than has been thought hitherto.

REFERENCES

1. Perutz, M. (1970) Nature 228, 726-739.
2. Arnone, A. (1972) Nature 237, 146-149.
3. Brewer, G.J., and Eaton, J.W. (1971) Science 171, 1205-1211.
4. Bartlett, G.R. (1959) J. Biol. Chem. 234, 466-471.
5. Hedlund, B.E. (1973) Ph.D. Thesis, Univ. of Minnesota.
6. Hedlund, B.E., Danielson, C., and Lovrien, R. (1972) Biochemistry 11, 4660-4668.
7. Chanutin, A., and Hermann, E. (1969) Arch. Biochem. Biophys. 131, 180-184.
8. Benesch, R.E., Benesch, R., and Yu, C.I. (1969) Biochemistry 8, 2567-2571.
9. Garby, L., and deVerdier, C.H. (1971) Scand. J. Clin. Lab. Invest. 27, 345-350.
10. Benesch, R.E., Benesch, R., Renthal, R., and Gratzer, W.B. (1971) Nature New Biol. 234, 174-176.
11. Luque, J., Diederich, D., and Grisolia, S. (1969) Biochem. Biophys. Res. Commun. 36, 1019-1023.
12. Nelson, D.P., Miller, W.D., and Kiesow, L.A. (1974) J. Biol. Chem. 249, 4770-4775.
13. de Bruin, S.H., Rollema, H.S., Janssen, L.M.H., and van Os, G.A.J. (1974) Biochem. Biophys. Res. Commun. 58, 204-209.