The Effect of 2,3-Diphosphoglyceric Acid on the Changes in β-β Interactions in Hemoglobin during Oxygenation*

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SUMMARY

During the binding of oxygen to the artificial intermediate of saturation of hemoglobin, α2β2, a significant change in the interactions between the two β subunits is observed. The same change in interaction is found both in the presence and absence of the hemoglobin effector 2,3-diphosphoglyceric acid (DPG). The observed change in β-β interactions even in the absence of DPG suggests that contacts between the two β chains contribute to cooperativity as well as a bridge formed by the DPG molecule.

The study of the mechanism of oxygen binding by hemoglobin has in recent years centered on the interactions between the four hemoglobin subunits. During this period a variety of experimental approaches have all indicated that during the course of oxygenation the conformation of each subunit changes when oxygen is bound to that subunit (1-4), so that the cooperative oxygen-binding mechanism is best described by a sequential change model (5). This model relates cooperativity to changes in the strengths of various interactions between subunits as each subunit changes shape. We have previously reported some experiments which measured some of the changes in subunit interactions (6). These results, similar to those obtained by two other groups (7, 8), showed that there was a significant change in the interaction between the two β chains but little or no change in the interactions of the two α chains during oxygenation.

The finding of a change in the β-β interaction during oxygenation emerged at the same time that the Benesches and their co-workers demonstrated that organic phosphates such as 2,3-diphosphoglyceric acid bound to hemoglobin and affected the midpoint of the saturation curve (9). Further investigations demonstrated that only 1 molecule of DPG is bound to each hemoglobin molecule and only to the deoxy form (10). Additional experiments led the Benesches to propose that DPG was bound between the two β chains (11), and Perutz has reported that DPG could be bound in the cavity between the two β subunits in the deoxy form (12). Bunn and Briehl (13) have studied DPG binding to normal and modified hemoglobins and concluded that the α-amino groups of the two β chains are involved in the binding.

Even though the position of the oxygen binding curve is shifted to higher oxygen pressures in the presence of DPG or even phosphate buffer, there is little or no change in the shape of the binding curve (9) and hence in the degree of cooperativity among the subunits. In view of the fact that a small but significant change in β-β interaction can be measured during oxygenation (6) it was of interest to examine whether the observed interactions between the two β subunits were the result of direct contacts of amino acid side chains or were mediated through a bridge of DPG or two phosphate ions. The nature of the β-β interaction could be ascertained by measuring the change in the β-β binding energy in the presence and absence of DPG and other phosphate compounds.

The basic approach to these questions was to use artificial intermediates of saturation formed by freezing some of the subunits (in this case, the α chains) into an oxygenated conformational state. This approach, first developed by Antonini et al. (14), was the basis of our previous measurement of α-α and β-β interactions (6). In Fig. 1 the method for measuring the change in β-β interaction in the intermediate α2β2 is illustrated. Oxygen is bound only by the unmodified β chains. Since the same changes in α1β1 and α1β2 interactions occur following the binding of each oxygen molecule, these changes cancel out in measuring the ratio of the two binding constants, K1 and K2. Thus, a value for Kββ/Kαα is obtained. If oxygen saturation is measured in the presence and absence of DPG under conditions when all other phosphate has been removed, the effect of DPG on the β-β linkage will be seen by the change in the ratio of the two binding constants.

EXPERIMENTAL RESULTS

Experimental methods and procedures for preparation of hemoglobin hybrids were as described in previous publication (6). Measurements of oxygen saturation were made in the presence of 0.2 M HEPES buffer, 0.06 M NaCl, and 5 × 10^-4 M EDTA, pH 7.2. In this solution the isolated subunits and the recombined α2β2 tetramers were found to be more stable than in a totally unbuffered solution. HEPES buffer was not totally analogous to unbuffered solution since the saturation...
(A) Sequential Model of $O_2$ Binding to Mixed State Hemoglobins

\[ K_1 = \frac{2K_{BB_1}K_{AB_2}K_{AB_1}K_{BB_2}}{K_{AB_2}K_{BB_1}K_{BB_2}} \left( \frac{K_S}{K_T} \right)^\beta \]

\[ K_2 = \frac{1}{2} \frac{K_{BB_1}^2K_{BB_2}}{K_{BB_1}K_{BB_2}K_{AB_1}K_{AB_2}} \left( \frac{K_S}{K_T} \right)^\beta \]

Where:

- $K_{AB_1}, K_{BB_1}$ refer to $\alpha_1-\beta_1$ interactions and
- $K_{AB_2}, K_{BB_2}$ refer to $\alpha_2-\beta_2$ interactions

(B) Concerted Model of $O_2$ Binding to Mixed State Hemoglobins

\[ K_1 = \frac{2(LK_{ST} + KS_R)}{L + 1} \]

\[ K_2 = \frac{1}{2} \frac{(LK_{ST}^2 + KS_R^2)}{(LK_{ST} + KS_R)} \]

Fig. 1. Schematic illustration of the binding of oxygen to the artificial half-saturated hemoglobin, $\alpha_{2CN}\beta_2$. (A) Sequential Model. The two $\alpha$ chains have been converted to the cyanomet form, analogous in structure to the oxy form of hemoglobin, prior to the binding of oxygen. The binding of oxygen to the two $\beta$ subunits causes the same changes in $\alpha-\beta$ interactions in Step 1 and Step 2, no changes in $\alpha-\alpha$ interactions, but different $\beta-\beta$ interactions in each step. The ratio of the two binding steps, $K_1$ and $K_2$, will therefore yield a value for the changes in $\beta-\beta$ interactions during oxygenation. (B) Concerted Model. The $R$ and $T$ state equilibria is shifted towards the $R$ form by conversion of the two $\alpha$ subunits to the oxy form. Binding of $O_2$ shifts the equilibrium further.
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In pH results from the release of protons during oxygenation (9). was added where indicated to a final concentration of $8 \times 10^{-5}$ M NaCl and $5 \times 10^{-4}$ M EDTA. Hemoglobin concentration in terms of heme equivalents was approximately $8 \times 10^{-5}$ M. The observation that β-β interactions are maintained in the presence or absence of phosphate ions or 2,3 diphosphoglyceric acid suggests that the changes in interaction between the two β subunits during oxygenation are not mediated through a phosphate or organic phosphate bridge. There must be some direct changes in the interactions between the amino acid side chains of the two β subunits which accompany the sequential changes during oxygen binding. It should be emphasized, of course, that, since the cooperativity depends on the change in subunit contacts, the contacts may be present only in the deoxy form and may be broken in the oxy form. Furthermore, these results lead to the conclusion that the binding site for DPG does not involve all of the amino acid side chains of the two β subunits which are implicated in changes in stabilization of the two subunits.

Recently Perutz (12) has suggested that the binding of DPG involves three β chain amino acid groups: the α-amino group of the NH$_2$-terminal valine (1), the ε-amino group of lysine (82), and histidine (143). Perutz also implicates a salt linkage between histidine (146) and the ε-amino group of lysine (140). Since the β-β interactions are observed to change whether organic phosphate or phosphate ion is present or not, there appear to be additional amino acid contacts which are either broken or formed during the oxygenation process.

One additional salt linkage which has been previously suggested from crystallographic observations (15) is between the carboxyl group of the COOH-terminal histidine (146) and one ε chain and the ε-amino group of lysine (132) in the other β chain. Such a bond may be important in the change of interaction energy between the two β subunits.

The demonstration of a change in β-β interactions during the oxygenation of α$^i_{4CNβ_2}$ was taken as evidence that ligand binding to hemoglobin follows a mechanism of sequential conformational changes rather than a concerted pathway (6). The effect of DPG further substantiates this conclusion.

The Hill coefficient for the binding of oxygen to α$^i_{4CNβ_2}$ is related to the binding constants $K_1$ and $K_2$ by Equation 1. If oxygen binding follows the sequential model (cf. Fig. 1A) and DPG binds to the various mixed state hemoglobins as in Equation 2, then the Hill coefficient equation becomes Equation 3.

$$n_H = \frac{4}{\sqrt{\frac{K_1}{K_2} + 2}}$$

$$K_4 = \frac{(Hb^+SD)}{(Hb^-SD)} K_1 = \frac{(Hb^+SD)}{(Hb^-SD)} K_II = \frac{(Hb^+S_2D)}{(Hb^-S_2D)}$$

In Table 1, the midpoints of the saturation curves are listed. The ratios of the midpoint in the presence of DPG to that in the absence of a phosphate compound is 1.7 in the case of α$^i_{3CNβ_2}$ and 1.85 for α$^i_{3CNβ_3}$. The fact that a similar shift in the midpoint of saturation occurs for both α$^i_{3CNβ_2}$ and α$^i_{3CNβ_3}$ when DPG is added to the solution is a direct demonstration that DPG exerts its effect on the two β chains regardless of the state of oxygenation of the α subunits. This observation is consistent with the fact that DPG is bound between the two β chains (10–12).

### Table 1

|        | (PO$_2$)$_i$ | Ratio of (PO$_2$)$_i$ (−DPG) to (PO$_2$)$_i$ (−DPG) |
|--------|-------------|--------------------------------------------------|
| α$^i_{3CNβ_2}$ | 11.0        | 6.5                                              |
| α$^i_{4CNβ_2}$ | 4.4         | 2.4                                              |

In each case, the hemoglobin concentration was approximately $8 \times 10^{-5}$ M. Although there is no change in the slope, the presence of DPG clearly shifts the midpoint of the saturation curve.

In the case of α$^i_{4CNβ_2}$ a similar shift of the midpoint is observed when DPG is added. In both the presence and absence of DPG, however, the slope of the Hill plot is 1.3. As we observed before (6), a slope of 1.3 represents a significant and consistently observed deviation from 1.0, the expected slope if there were no changes in β-β interactions during oxygenation. It would thus appear that the β-β interaction changes remain constant whether DPG or other phosphates are present or not.

The effect of DPG on α$^i_{4CNβ_2}$ is related to the binding constants $K_1$ and $K_2$ by Equation 1. If oxygen binding follows the sequential model (cf. Fig. 1A) and DPG binds to the various mixed state hemoglobins as in Equation 2, then the Hill coefficient equation becomes Equation 3.

$$n_H = \frac{4}{\sqrt{\frac{K_1}{K_2} + 2}}$$

$$K_4 = \frac{(Hb^+SD)}{(Hb^-SD)} K_1 = \frac{(Hb^+S_2D)}{(Hb^-S_2D)} K_II = \frac{(Hb^+S_2D)}{(Hb^-S_2D)}$$
To satisfy the observation that \( n_H \) did not change in the presence or absence of DPG, the condition that

\[
\frac{(1 + K_{1[D]})}{(1 + K_{1[D]})(1 + K_{1[D]})} = 1
\]

must be satisfied. At a high concentration of DPG, Equation 4 becomes

\[
\frac{K_1^p}{K_1} = 1
\]

which states that the binding of DPG must change progressively (or not at all) as the ligand state of hemoglobin changes.

The effect of DPG on the midpoint of \( O_2 \) binding is given in Equation 6.

\[
S_{0.5} = \frac{1}{\sqrt{K_{1[D]} + K_{1[D]}}}
\]

Since the midpoint of \( O_2 \) binding is shifted to higher concentrations of \( O_2 \) in the presence of DPG, fulfillment of the conditions described in Equations 5 and 6 lead to the conclusion that \( K_1 > K_1 \), i.e. binding of DPG is strongest to the least liganded form. The observations that there was no change in \( n_H \) but a significant shift in the binding curve midpoint when DPG is introduced thus are consistent with a sequential model.

A consideration of the concerted mechanism (10) (cf. Fig. 1D) yields a less satisfactory conclusion. The Hill coefficient equation for the concerted model treated in its general (nonspecific) binding form is:

\[
n_H = \frac{4}{\sqrt{K_1(1 + K_{1[D]}) + 2}}
\]

To determine whether the binding of DPG must change progressively (or not at all) as the ligand state of hemoglobin changes.

The Hill coefficient equation for the concerted model treated in its general (nonspecific) binding form is:

\[
S_{0.5} = \frac{1}{\sqrt{K_{1[D]} + K_{1[D]}}}
\]

where \( L' = L(1 + K_{1[D]})(1 + K_{1[D]})+2 \) and \( K_{TP} \) and \( K_{RP} \) represent the association constants for DPG of the \( T \) and \( R \) forms, respectively.

It is argued that in a concerted mechanism the binding of one or more ligand to hemoglobin should stabilize the molecule in the \( R \) state. In this case, the absence of DPG should exert little or no effect, since the binding of DPG to the fully liganded molecule (that is, the \( R \) state) has been found to be very weak (11). One could postulate that the binding of DPG to \( \alpha^{	ext{CN}} \beta_2 \) would indeed be the same as to \( \alpha^{	ext{CN}} \beta_0 \beta_2 \) or to \( \alpha^{	ext{CN}} \beta_2 \). In this case, however, there should be no significant effect on the midpoint of saturation when DPG is added. Clearly, this is not the case.

The results reported here are in conflict with a recent report (17) based on the nuclear magnetic resonance heme spectrum of \( \alpha^{	ext{CN}} \beta_2 \), which postulates that the \( \beta \) chain heme are in an oxy form in the absence of DPG (in the presence of 2,2-bis(hydroxymethyl)-2,2',2'-nitroethanol buffer) but in the deoxy form when DPG is introduced. From this no further conformational changes would be expected when DPG is absent, thus leading to a loss of \( \beta \) interactions.

REFERENCES

1. \textsc{Ogawa, S., and McConnell, H.}, Proc. Nat. Acad. Sci. U. S. A., 55, 10 (1967).
2. \textsc{Shulman, R. G., Ogawa, S., Wuthrich, K., Yamane, T., Pribsch, J., and Blumberg, W. E.}, Science, 165, 251 (1969).
3. \textsc{Antonini, E., and Brunori, M.}, J. Biol. Chem., 244, 3000 (1969).
4. \textsc{Simon, S. R., and Cantor, C. R.}, Proc. Nat. Acad. Sci. U. S. A., 63, 209 (1969).
5. \textsc{Koshland, D. E., Jr., Nemethy, G., and Filmer, D.}, Biochemistry, 5, 355 (1966).
6. \textsc{Haber, J. E., and Koshland, D. E., Jr.}, Biochim. Biophys. Acta, 194, 329 (1969).
7. \textsc{Banerjee, R., and Cassoly, R.}, J. Mol. Biol., 42, 351 (1969).
8. \textsc{Brunori, M., Antonini, E., Wyman, J., and Winterhalter, K. H.}, J. Mol. Biol., 49, 461 (1969).
9. \textsc{Benesch, R., Benesch, R. E., and Yu, C. T.}, Proc. Nat. Acad. Sci. U. S. A., 59, 626 (1968).
10. \textsc{Benesch, R., and Benesch, R. E., Nature, 221, 618 (1969).
11. \textsc{Banerjee, R., and Cassoly, R.}, J. Mol. Biol., 42, 351 (1969).
12. \textsc{Dugas, H. F., and Druehl, R. W.}, J. Clin. Invest., 49, 1066 (1970).
13. \textsc{Antonini, E., Brunori, M., Wyman, J., and Noble, R. W.}, J. Biol. Chem., 241, 3230 (1966).
14. \textsc{Perutz, M. F., Proc. Royal Soc. London B Biol. Sci., 173, 113 (1969).
15. \textsc{Ogawa, S.}, J. Mol. Biol., 12, 88 (1965).
16. \textsc{Shulman, R. G.}, Biochem. Biophys. Res. Commun., 42, 9 (1971).
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