The Effect of 2,3-Diphosphoglycerate on the Tetramer-Dimer Equilibrium of Liganded Hemoglobin*

ROBERT D. GRAY

From the Department of Biochemistry, University of Louisville School of Medicine, Health Sciences Center, Louisville, Kentucky 40201

SUMMARY

The effect of 2,3-diphospho-D-glycerate (P₂-glycerate) on the kinetic behavior of deoxyhemoglobin generated by the rapid dissociation of gaseous ligand (either by flash photolysis of carbon monoxide hemoglobin (HbCO) or by deoxygenation of oxyhemoglobin (HbO₂)) in the presence of sodium dithionite is consistent with a stabilization of the tetrameric state of liganded hemoglobin by the organic phosphate. The fraction (α) of rapidly reacting hemoglobin produced by pulsed laser photolysis of phosphate-free HbCO in 0.05 M 2,2'-bis(hydroxymethyl)-2,2',2''-nitroloethanol-0.1 M NaCl, pH 7.0, was independent of CO concentration below about 500 μM, but increased systematically with dilution of the hemoprotein. The apparent tetramer-dimer dissociation constant (K₂) calculated from the dependence of α on [HbCO] was 3.6 ± 1.0 μM in the absence of phosphates and decreased to 1.4 ± 0.3 μM when 1 mM P₂-glycerate was added. These values of K₂ are similar to estimates for liganded hemoglobin obtained in sedimentation experiments conducted at pH 7.0 in 0.1 M phosphate (Edelstein, S. J., Rehmer, M. J., Olson, J. S., and Gibson, Q. H. (1970) J. Biol. Chem. 245, 4372-4381) and in 0.1 M Tris-0.09 M NaCl (Kellett, G. L. (1971) J. Mol. Biol. 59, 401-424).

The magnitude of the Soret absorbance drift accompanying deoxygenation of dilute solutions of HbO₂ in the presence of sodium dithionite was decreased by added 1 mM P₂-glycerate, and the second order rate constant characterizing the drift phase was increased at 20°C from 0.54 ± 0.09 μM⁻¹ s⁻¹ to 1.57 ± 0.18 μM⁻¹ s⁻¹ by the organic phosphate. Since the drift has been shown to result from the formation of deoxy tetramers from deoxy αβ dimers (Kellett, G. L., and Cutfreund, H. (1970) Nature 227, 921-926), this result is also consistent with the proposed phosphate inhibition of dimer formation by liganded hemoglobin.

In 1939 Roughton (1) reported that deoxyHb⁺ produced by dissociation of oxygen from HbO₂ in the presence of sodium

* This work was supported by National Science Foundation Grant GB-32283.

1 The abbreviations used are: deoxyHb⁺, deoxyhemoglobin; HbCO, carbon monoxide hemoglobin; HbO₂, oxyhemoglobin.

(Received for publication, September 26, 1973)
stripped HbCO, both in order to interpret the kinetics and also to appreciate fully the role of organic phosphates in hemoglobin-ligand equilibria. The results of the kinetic experiments reported here (subject, of course, to the inherent inability of kinetic data alone to define unambiguously chemical structures) suggest that stripped, liganded hemoglobin dissociates to rapidly reacting dimers to a greater degree than when P₂-glycerate or inorganic phosphate is present.²

The following reaction scheme may facilitate presentation and discussion of the results.

\[
\begin{align*}
\text{CO} & \stackrel{k_{f}}{\rightarrow} \text{HbCO} \\
\text{HbCO} & \stackrel{k_{r}}{\rightarrow} \text{CO} \\
\text{HbCO} & \rightarrow 2 \text{CO}
\end{align*}
\]

In the scheme, \(k_{f}\) and \(k_{r}\) are the rate constants for formation of dimers from liganded and unliganded tetramers, respectively; \(k_{f}\) and \(k_{r}\) are the corresponding rate constants for the dimer-dimer association reaction. The pseudo-first-order rate constant for CO binding to \(\alpha\beta\) dimers is \(k_{f}\) and that for the slower binding of CO to tetramers is \(k_{r}\). A CO concentration is used so that insignificant adjustment of the tetramer-dimer equilibrium occurs during the cyclic displacement of bound CO by the flash and the reassociation dark reaction. The equilibrium constants for tetramer-dimer dissociation of liganded and unliganded hemoglobin are \(K_{f}\) and \(K_{r}\), respectively, where \(K_{f} \gg K_{r}\) (8, 16).

**EXPERIMENTAL PROCEDURE**

**Materials**

**Preparation of Hemoglobin**—Hemolysates were prepared from fresh human blood by the method described by Gibson (14), with the exception that stroma were removed by centrifugation after the addition of 0.25 volume of 5% NaCl. Polyacrylamide electrophoresis of the resulting hemolyzate in 5% gels at pH 9.5, followed by staining with Coomassie blue II250 in 10% trichloroacetic acid, showed a major band corresponding to HbA and two minor components.

Small molecules were removed by gel filtration of the hemolysate using Sephadex G-25 equilibrated with 2.5 mM bis-tris-0.1 M NaCl, pH 7.4 to 7.6.

Aliquots of the resulting stripped hemoglobin (at least 0.1 µmole of HbA) were analyzed for total phosphate by method of Ames (18) as modified by Gray and Gibson (19). In most cases no phosphate could be detected after gel filtration; sometimes traces of phosphate remained which were the equivalent of approximately 3 to 4% of the hemoglobin tetramers.

Working solutions were prepared by diluting the stock hemolysate (2 to 4 mM heme equivalents) directly into the desired buffer solution. Hemoglobin concentration was estimated spectrophotometrically using the extinction coefficients of Banerjee et al. (20).

**Reagents**—Bis-tris was obtained from Aldrich Chemical Co., Milwaukee, Wis., or from Sigma Chemical Co., St. Louis, Mo. P₂-glycerate was obtained from Sigma as the pentacyclohexylammonium salt; stock solutions of approximately 10 mM were prepared as described by Benesch et al. (11) and analyzed to determine the exact phosphate concentration (19). CO was a product of Matheson Co., Joliet, Ill., and prepurified N₂ was obtained from Air Products and Chemicals, Inc., Emusus, Pa. Solutions containing CO were prepared by mixing, in the desired proportions, buffers previously equilibrated with water vapor-saturated CO or N₂. The solubility of CO was taken to be 1.0 mM at 20° and atmospheric pressure (21). Sodium dithionite (Mannox Brand)

³ While this work was in progress, an abstract of light-scattering experiments was published which indicates that P₂-glycerate inhibits dimerization of liganded hemoglobin (15).

² The intensity of the measuring light beam did not significantly alter the results; see Table 1.
collection started 100 ms after flow stopping. A reference voltage was collected 30 or 60 s later. This procedure had the advantage that by omitting observation of the initial deoxygenation ($\Delta A \approx 1$) reaction, a greater sensitivity could be used to observe the slow reaction ($\Delta A \approx 0.1$). Estimation of the actual absorbance change in the slow phase was difficult because: (a) at 429 nm the slow changes amounted usually to less than 10% of the total $\Delta A$; (b) the slow second order reaction followed a first order reaction which made it impossible to establish accurately the "zero" time for the second order reaction; (c) occasional artifacts resulting from dithionite were experienced. The requirements adopted for using in the slow phase was difficult because: (a) at 429 nm the slow changes amounted usually to less than 10% of the total AA at 429 nm; (b) no detectable difference in the $\Delta A$ measured whether the final reference voltage was collected 30 or 60 s after the last data point. An estimate of $\Delta A$ due to the slow second order reaction was obtained by extrapolating plots of the reciprocal of the absorbance change versus time to zero time.

**RESULTS**

**Laser Photolysis of HbCO**—The kinetics of recombination of stripped human Hb subsequent to photolysis in the presence and absence of P$_2$-glycerate is illustrated in Fig. 1. The kinetics was measured at 437 nm, a wavelength isosbestic for both the rapidly and slowly reacting species. In the absence of phosphates the rapid species contributed 32.5 ± 0.4% to the observed absorbance change; when the sample was supplemented with 623 μM P$_2$-glycerate, the percentage of rapid material decreased to 25.3 ± 0.6%. This analysis can, at best, be only approximate since it is well known that the second order rate constant for CO binding to Hb depends upon the fractional saturation with ligand (26). However, it does serve to point out the effect of P$_2$-glycerate. In experiments shown in Table I, 0.1 M phosphate also depressed the proportion of rapidly reacting hemoglobin to about the same extent.

Fig. 2 shows that the fraction of rapid material depends on the wavelength of the observing light. The lines are calculated from the data in Fig. 2a of Cassoly and Gibson (27), assuming that the rapid species possesses the HbCO-hemoglobin to about the same extent.

spectrum of the isolated α and β chains and the slow species has the HbCO-deoxyHb difference spectrum of cooperative hemoglobin tetramers. The experimental points of Fig. 2 show that the spectral characteristics of the rapidly and slowly reacting species compare favorably with that expected if the unliganded rapidly reacting species has the chain like HbCO-deoxyHb difference spectrum characteristic of noncooperative hemoglobin derivatives.

The relationship between HbCO concentration and the fraction of rapid reaction, $\alpha$, is shown in Fig. 3 for stripped hemoglobin (open circles) and for hemoglobin in the presence of P$_2$-glycerate (filled circles). A value of $K_{1,2}$ was calculated for each data point in Fig. 3 using the expression $\alpha[HbCO]/(1 - \alpha)$; average values for $K_{1,2}$ of 3.6 ± 1.0 μM for stripped and 1.4 ± 0.3 μM for hemoglobin with P$_2$-glycerate were obtained. The agreement between the behavior observed and that predicted on the basis of the dimerization scheme suggests that the rapidly reacting hemoglobin observed subsequent to flash photolysis of stripped HbCO can be accounted for entirely by assuming it to be the αβ dimer.

**TABLE I**

*Photolysis of carbon monoxide hemoglobin*

| Experiment | Addition | Fraction of Hb reacting rapidly | $k_1$ | $k_2$ | $K_{1,2}$ |
|------------|----------|---------------------------------|-------|-------|-----------|
| 1          | None     | 0.447                           | 0.15  | 0.28  | 3.70      |
| 2          | None     | 0.448                           | 0.21  | 0.23  | 3.70      |
| 3          | 0.1 M phosphate | 0.318                      | 0.23  | 0.23  | 1.55      |
| 4          | 0.1 M phosphate | 0.323                      | 0.23  | 0.23  | 1.60      |
| 5          | 0.1 M P$_2$-glycerate | 0.313                      | 0.21  | 0.21  | 1.90      |

* Intensity of measuring light reduced by 47% compared to Experiment 1.

a Bis-tris and NaCl omitted.

![Fig. 1. Normalized kinetics of the recombination of CO with deoxyHb subsequent to the laser photolysis of stripped human HbCO. Conditions: [CO] = 100 μM; [Hb] = 14.5 μM; [P$_2$-glycerate] = 0 [●—●]; [Hb] = 13.5 μM, [P$_2$-glycerate] = 623 μM ([O—O]); 0.05 M bis-tris-0.1 M NaCl, pH 7.0, 21 ± 2°.](http://www.jbc.org/)

![Fig. 2. Dependence of the fraction of stripped deoxyHb reacting rapidly with CO after photolysis on the wavelength of the measuring light. Conditions: [Hb] = 13.6 μM; [CO] = 100 μM; 0.05 M bis-tris-0.1 M NaCl, pH 7.0, 21 ± 2°.](http://www.jbc.org/)
FIG. 4. Dependence of the fraction of deoxyHb reacting slowly with CO subsequent to photolysis of HbCO on P₃-glycerate concentration. Conditions: [CO] = 100 μM; [Hb] = 9.0 μM (●—●); [Hb] = 15.0 μM (O—O); 0.05 M bis-tris-0.1 M NaCl, pH 7.0, 21 ± 2°C. The temperature was 21 ± 2°C. The rate constants are the apparent first order constants estimated by least squares analysis of the average of at least four successive experiments as described in the text.

Dependence of fraction of rapidly reacting stripped hemoglobin on [CO]

| [CO] (μM) | Fraction rapid hemoglobin (a) | kₕ | kₛ |
|-----------|-------------------------------|-----|-----|
| 105       | 0.143                         | 616 | 25.3|
| 205       | 0.373                         | 1340| 50.8|
| 316       | 0.423                         | 2005| 72.5|
| 516       | 0.433                         | 3650| 112.7|
| 802       | 0.470                         | 4045| 166.4|

An approximate equilibrium constant for binding of P₃-glycerate to stripped HbCO in 0.05 M bis-tris-0.1 M NaCl, pH 7.0, can be obtained by observing the fraction of photolytically produced rapid hemoglobin as a function of P₃-glycerate concentration (Fig. 4). The concentration of P₃-glycerate required to give half the maximal decrease in rapid hemoglobin is in the range 100 to 300 μM. This relatively high concentration reveals that the interaction is weak compared to phosphate binding by deoxy Hb (28). The difference in the two curves of Fig. 4 was not investigated further but may result from the use of different hemoglobin preparations.

The effect of CO concentration on the fraction of rapidly reacting stripped hemoglobin was also tested. Increasing CO from an excess of 5-fold to nearly 30-fold had only a small effect on α (Table II). This experiment indicates that a first order conformational change which converts rapidly to slowly reacting hemoglobin after the photolytic flash does not become rate-limiting in the absence of phosphates in the range of CO concentrations tested.

Deoxygenation of HbO₂—Gibson and Roughton (discussed in Ref. 3) noted that slow absorbance changes accompany the fast deoxygenation of HbO₂ by sodium dithionite under certain carefully controlled conditions. The magnitude of this so-called "drift phase" depends on the wavelength of observation and also on the concentration of HbO₂ (28, 29). Kellett and Gutfreund (29) correlated the size of the drift phenomenon with the fraction of oxygenated αβ dimers (determined by sedimentation equilibrium (30) in 0.01 M Tris-0.09 M NaCl, pH 7.0, 1 mM EDTA, pH 7.0). Their results suggested that the absorbance changes following the deoxygenation process were the result of the relatively slow (kₙ₁ = 0.43 μM⁻¹ s⁻¹) association of the unliganded dimers produced by deoxygenation of liganded hemoglobin in dilute solution. The effect of phosphates on the magnitude or the kinetics of the drift phase was not reported. Consequently, this author repeated the deoxygenation experiments using stripped HbO₂ in the presence and absence of P₃-glycerate.

Figs. 5 and 6 show the wavelength dependence of the deoxygenation reaction of stripped HbO₂ by dithionite. Fig. 5 illustrates that the apparent rate of deoxygenation of stripped HbO₂ progressively decreases toward the end of the reaction when observed at 429 nm. In contrast, no such deceleration is found when the reaction is followed at 437 nm. This result shows the absence of dithionite-dependent artifacts within the time range of these experiments (~60 s).

FIG. 6. Shows that the kinetic difference spectrum of the slow phase after HbO₂ deoxygenation exhibits the characteristics expected of conversion of a noncooperative derivative to a cooperative form of unliganded hemoglobin (2).

When the magnitude of the absorbance change due to the slow phase is plotted as a function of the initial concentration of HbO₂, the results of Fig. 7 are obtained. In Fig. 7 the lines corresponding to stripped and P₃-glycerate-supplemented solutions of hemoglobin are the theoretical ones based on ΔG°m = 12 (determined by the method of Kellett and Gutfreund (29) in 1 M NaCl) and Kₖₙ1,k₂ = 3.6 μM for stripped and 1.4 μM for phosphate-liganded hemoglobin. The results of the deoxygenation experiments are thus consistent with the interpretation of the
The kinetic data of Fig. 8 are presented as additional evidence supporting this conclusion. When the reciprocal of the absorbance change at 429 nm is plotted as a function of time after mixing with dithionite, a linear relationship is obtained over at least 85% of the drift reaction. Such kinetic behavior, indicative of a second order reaction of the type $2A \rightarrow B$, is most reasonably interpreted as the association of unliganded dimers to give cooperative unliganded tetramers. The experiments illustrated in Fig. 8 also show that P$_2$-glycerate increases the rate of dimer association. The bimolecular rate constants calculated from the slopes of a series of such experiments, in conjunction with the value of $\Delta \varepsilon$ given above, were $0.54 \pm 0.09 \mu M^{-1} s^{-1}$.
and P2-glycerate-supplemented hemoglobin at 20°, respectively. Note also that values of the intercepts are inversely related to the initial HbO₂ concentration and are decreased in magnitude by the dissociating agent NaCl and increased by P₂-glycerate.

**DISCUSSION**

The liganded, or R allosteric conformation of hemoglobin (31, 32) binds CO at a rate about 80 times that of the T or deoxy conformation. Of the several mechanisms discussed in the introductory section of this paper in which phosphate compounds might influence the transition between the high and low affinity forms subsequent to ligand removal, the data suggest that the primary mechanism is a stabilization of the liganded tetrameric structure relative to the dimer in the presence of phosphates.

The dependence of α on [HbCO] (Fig. 3) follows the relationship predicted on the assumption that dilution of the protein solution results in dissociation of slowly reacting tetramers to rapidly reacting dimers. The value of $K_{1,2}^{T}$ calculated from α and [HbCO] is 3.6 ± 1.0 μM for stripped HbCO in 0.05 M bis-tris·0.1 M NaCl, pH 7.0. This value of $K_{1,2}^{T}$ is significantly different from that of 1.5 μM estimated by Edelstein et al. (8) from photolysis and sedimentation data obtained with unstripped HbCO in 0.1 M phosphate, pH 7.0, and closer to the value of 2.9 μM determined by Kellett (30) for stripped HbO₂ using the sedimentation equilibrium method and slightly different solution conditions (0.1 M Tris·0.09 M NaCl, 1 mM EDTA, pH 7.0). The data presented in Figs. 1 and 3 and Table I show that 1 mM P₂-glycerate added to stripped HbCO decreased the $K_{1,2}^{T}$ to values close to those observed earlier (8) in phosphate buffers.

The relatively high concentration of P₂-glycerate required to achieve half the maximum phosphate effect (about 250 μM, Fig. 4), taken with the fact that P₂-glycerate equilibrates rapidly with deoxyHb (18, 33, 34) also is consistent with the importance of an interaction between the phosphate compound and liganded, rather than ligand-free, hemoglobin. The association constant of P₂-glycerate and HbO₂ must be about 4 × 10⁴ M⁻¹ (assuming one binding site per tetramer) as judged from the data in Fig. 4. This value is consistent with equilibrium dialysis data of Caldwell and Nagel (35).

The relative independence of α with respect to [CO] eliminates the possibility that phosphates increase the rate of a first order $R \rightarrow T$ transformation. Although the data of Fig. 8 show that the rate of deoxydimer association is increased by P₂-glycerate, the increase is not so large that a significant fraction of dimeric deoxyHb could associate to form slowly reacting tetramers prior to CO binding. For example in 1 mM P₂-glycerate, the first half-life for dimer association would be approximately 60 ms when [HbCO] = 35 μM, whereas the half-time for CO binding to the rapidly reacting dimer was less than 10 ms.

The effect of P₂-glycerate on the drift phase following photolytic ant1 deoxygenation of stripped HbO₂ also supports the conclusion that liganded hemoglobin is stabilized by phosphate compounds. The reduction in the extent of the drift phase by P₂-glycerate (Figs. 7 and 8), the dependence on [HbO₂], and the apparent second order kinetics all are best interpreted by assum-
I am further indebted to Professor R. J. DeSa for generously supplying the wiring diagrams, computer programs, and advice for constructing the computer interface used in data collection. The University of Louisville Toward Greater Quality Committee supplied part of the financial support necessary to purchase the components for the interface.

REFERENCES

1. Roughton, F. J. W. (1954) Proc. Roy. Soc. London B Biol. Sci. 145, 495-503
2. Brunori, M., Antonini, E., Wyman, J., and Anderson, S. K. (1968) J. Mol. Biol. 34, 357-359
3. Gibson, Q. H. (1950) Progr. Biophys. 9, 1-53
4. Muirhead, H., Cox, J. M., Mazarella, L., and Perutz, M. F. (1967) J. Mol. Biol. 28, 117-156
5. Gibson, Q. H. (1959) Biochem. J. 71, 293-303
6. Gibson, Q. H., and Antonini, E. (1967) J. Biol. Chem. 242, 4678-4681
7. Antonini, E., Chiancone, E., and Brunori, M. (1967) J. Biol. Chem. 242, 4360-4366
8. Edelestein, S. J., Rehmar, M. J., Olson, J. S., and Gibson, Q. H. (1970) J. Biol. Chem. 245, 4372-4381
9. Benesch, R., and Benesch, R. E. (1967) Biochem. Biophys. Res. Commun. 26, 162-167
10. Chanutin, A., and Curnish, R. R. (1967) Arch. Biochem. Biophys. 121, 96-102
11. Benesch, R., Benesch, R. E., and Yu, C. I. (1968) Proc Nat. Acad. Sci. U. S. A. 59, 526-532
12. Gibson, Q. H., and Parkhurst, L. J. (1968) J. Biol. Chem. 243, 5621-5624
13. Gray, R. D. (1970) J. Biol. Chem. 245, 2014-2021
14. MacQuarrie, R., and Gibson, Q. H. (1972) J. Biol. Chem. 247, 5686-5694
15. White, S. L., and Glauser, S. C. (1973) Fed. Proc. 32, 551
16. Thomas, J. O., and Edelestein, S. J. (1972) J. Biol. Chem. 247, 7570-7574
17. Gibson, Q. H. (1970) J. Biol. Chem. 245, 3285-3288
18. Ames, B. N. (1966) Methods Enzymol. 8, 115-117
19. Gray, R. D., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 7169-7174
20. Bannerjee, R., Alpert, Y., Leverrier, F., and Williams, R. P. (1969) Biochemistry 8, 2862-2867
21. Hodgman, C. D., ed. (1966) Handbook of Chemistry and Physics, p. 1666, 38th Ed, Chemical Rubber Publishing Co., Cleveland, Ohio
22. Gibson, Q. H., and Milnes, L. (1964) Biochem. J. 91, 161-171
23. DeSa, R. J. (1970) Anal. Biochem. 36, 293-303
24. DeSa, R. J. (1972) in Computers in Chemical and Biochemical Research (Kloppenstein, C. D., and Wilkins, C. L., eds) Vol. I, p. 185, Academic Press, New York
25. Bevington, P. R. (1969) Data Reduction and Error Analysis for the Physical Sciences, pp. 134-148, McGraw-Hill, New York
26. Dateman, J. B., and Roughton, F. J. W. (1955) Biochem. J. 64, 2622-2633
27. Cassoly, R., and Gibson, Q. H. (1972) J. Biol. Chem. 247, 7332-7341
28. Antonini, E., Brunori, M., and Anderson, S. (1968) J. Biol. Chem. 243, 1816-1822
29. Kellett, G. L., and Gutierrez, H. (1970) Nature 227, 921-926
30. Kellett, G. L. (1971) J. Mol. Biol. 59, 401-424
31. Monod, J., Wyman, J., and Changeaux, J.-P. (1965) J. Mol. Biol. 12, 88-118
32. Perutz, M. F. (1970) Nature 220, 729-739
33. Gray, R. D., and Gibson, Q. H. (1971) J. Biol. Chem. 246, 5176-5178
34. Gibson, Q. H. (1970) Biochem. Biophys. Res. Commun. 40, 1319-1324
35. Caldwell, P. R. B., and Nagel, R. L. (1973) J. Mol. Biol. 76, 605-711
36. Guidotti, G. (1967) J. Biol. Chem. 242, 3685-3693
37. Wyman, J. (1964) Advan. Protein Chem. 19, 223-286
38. Benesch, R. E., Benesch, R., and Yu, C.-I. (1969) Biochemistry 8, 2567-2571
39. Lindstrom, T. R., and Ho, C. (1973) Biochemistry 12, 134-139
40. Arnone, A. (1972) Nature 237, 146-149
41. Benesch, R. E., Benesch, R., Renthal, R. D., and Maeda, N. (1972) Biochemistry 11, 3576-3592
The Effect of 2,3-Diphosphoglycerate on the Tetramer-Dimer Equilibrium of Liganded Hemoglobin

Robert D. Gray

J. Biol. Chem. 1974, 249:2879-2885.

Access the most updated version of this article at http://www.jbc.org/content/249/9/2879

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/249/9/2879.full.html#ref-list-1