The Deoxygenation Kinetics of Hemoglobin Partially Saturated with Carbon Monoxide

EFFECT OF 2,3-DIPHOSPHOGLYCERATE*

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SUMMARY

The deoxygenation rate of hemoglobin solutions partially saturated with carbon monoxide, in the presence and absence of 2,3-diphosphoglycerate (2,3-DPG) at 23° and pH 7.0 and 8.0 have been measured. When the rate is calculated over the initial 30% of the reaction, solutions containing 98% HbO₂ show an increase in the rate as hemoglobin deoxygenates both at neutral and alkaline pH. Partial saturation with CO severely compromises this variation at both pH values. Indeed, for a solution containing about 15% HbO₂, no variation in the rate over the initial 30% of the reaction or thereafter, is observed. These results for the initial portion of the reaction are in agreement with the earlier studies of Gibson and Roughton (1955) Proc. Roy. Soc. London B Biol. Sci. 143, 310. The values of the deoxygenation rate constant calculated between 85 and 40% saturation (k in s⁻¹) at pH 8.0, in the absence of 2,3-DPG, are 19.5 ± 1.0 s⁻¹ for the 98% HbO₂ condition and 14.2 ± 0.7 s⁻¹ for the 85% HbCO-15% HbO₂ case. 2,3-DPG has no significant effect on k at pH 8.0. At pH 7.0, with no 2,3-DPG, the value of k for the 98% condition is 36.0 ± 1.8 s⁻¹, and for the 85% HbCO-15% HbO₂ case, it is 17.9 ± 0.9 s⁻¹. In the presence of a 10:1 molar ratio of 2,3-DPG to hemoglobin tetramer, the effect of partial saturation with CO is even more pronounced as evidenced by a value of 52.4 ± 2.0 s⁻¹ for 98% HbO₂ and 18.0 ± 0.8 s⁻¹ for 85% HbCO-15% HbO₂. Thus solutions containing Hb₄O₂(CO)₃ as a major initial species show no effect of 2,3-DPG at pH 7.0. The effect of this organic phosphate on k becomes significant as the concentration of the species Hb₄O₂(CO)₃ increases. These results support the view that there is no effect of 2,3-DPG on the release of the first oxygen from the fully liganded conformation. They also suggest that 2,3-DPG exerts its influence on the rate of removal of the second and subsequent oxygen molecules. This kinetic result is consistent with the findings of MacQuarrie and Gibson (1971) J. Biol. Chem. 246, 5832), that a 2,3-DPG analogue dissociates from its combination with hemoglobin after the binding of the third heme ligand. Since 2,3-DPG binding is thought to be dependent on the quaternary structure of hemoglobin, these results taken together would indicate that the transition from the oxy to the deoxy quaternary structure, at pH 7.0, occurs very early in the deoxygenation process, probably with the loss of the first heme ligand from fully liganded hemoglobin.

Studies on the properties of hemoglobin bound to CO are among the first studies reported on hemoglobin and can be traced back to the work of Haldane and Lorrain-Smith in 1895 (1). It has been shown that partial saturation of a hemoglobin solution with CO causes oxygen to bind to the remaining hemes with a higher affinity and results in a less sigmoid curve (2). Corroboration of these equilibrium observations was obtained from the work of Gibson and Roughton (4) who, in 1955, reported measurements of the deoxygenation rates of sheep hemoglobin solutions partially saturated with CO. The results of their investigation showed that the variation in the pseudo-first order deoxygenation rate constant, which is always seen in the early portion of the oxyhemoglobin-dithionite reaction, was considerably reduced when measured on solutions containing a mixture of 25% HbO₂ and 75% HbCO. Indeed, not only was the variation in the rate constant nearly eliminated, but the value of the over-all rate constant was also much reduced from normal. These results also offered good evidence that the function of one heme is closely dependent on the function of its neighbors in the molecule.

However, the dissociation of oxygen from oxyhemoglobin, as measured by the dithionite reaction, has a behavior closely consistent with a first order process. This fact and the results of Gibson and Roughton (4) suggest that the dissociation of the
first oxygen molecule is the rate-limiting process and is followed by the rapid dissociation of the remaining oxygen molecules (9). Therefore, the observed first order rate of dissociation depends on two factors: the rate of dissociation of the first oxygen molecule \( k_2 \) and the difference between the off rate for the first oxygen and the subsequent ones (the difference between \( k_2 \) and \( k_2, k_3, k_4 \)). Clearly then, a change in the observed rate of dissociation of oxygen from oxyhemoglobin (say as a function of pH or as a function of the type of hemoglobin) also must depend on one of these two factors. The rate of deoxygenation of fetal hemoglobin is considerably greater than that for adult hemoglobin (in the absence of 2,3-DPG) (6). This difference is associated with a large value of the initial rate of deoxygenation for fetal as compared with adult hemoglobin (7). On the other hand, while protons (8) and 2,3-DPG (9) (but not carbamino formation (10)) increase the deoxygenation rate of hemoglobin, they have little or no effect on the initial off rates (9, 11, 12). One is led to the conclusion that protons and 2,3-DPG cause the over-all rate to increase by producing an increase in the remaining off rates (\( k_2, k_3, k_4 \)). An important question to be answered is at what point in the reactions are these nonheme ligands exerting this apparent influence? It is this question which is approached herein. We elected to extend the basic approach used by Gibson and Roughton (4) on sheep hemoglobin by studying human hemoglobin partially saturated with CO at neutral and alkaline pH values without and with saturating concentrations of 2,3-DPG. While this work was in progress, two papers were published which address themselves to related questions. One by Tyma et al. (13) investigated the values of the four equilibrium constants without and with organic phosphates. The second, by MacQuarrie and Gibson (14) approached the question by binding a fluorescent 2,3-DPG analogue (8-hydroxy-1,3,6-pyrene trisulfonate (HPT)) to deoxygenated hemoglobin and measuring the rate of appearance of fluorescence associated with the rate of binding of CO. Both of these papers suggest that 2,3-DPG dissociates from its complex with hemoglobin after the binding of the third ligand. We present, herein, our findings from the deoxygenation point of view.

METHODS

Preparation of Hemoglobin Solutions—Fresh blood was drawn from one of us (D.H.M.) who is a healthy nonsmoker. The blood was centrifuged at 1,500 \( \times g \) for 10 min and the plasma was discarded. The cells were then washed three times in isotonic NaCl. After the third washing and centrifugation the clear supernatant was discarded and 4 volumes of deionized water were added per volume of packed cells. The solution was agitated and allowed to stand for about 2 hours at 4°. The hemolyzed red blood cells were then centrifuged at 34,000 \( \times g \) for 20 min to remove stroma. Portions of the clear hemolysate were chromatographed on G-100 Sephadex in 0.1 m Tris-HCl and then dialyzed overnight in a large volume of 0.05 m Tris-HCl buffer at either pH 7.0 or 8.0. This technique for stripping hemolysates was found to remove any detectable amount of 2,3-DPG as determined using the sensitive fluorometric technique of Keitt (15). These hemoglobin solutions were then split into equal portions. One portion, at each pH, was then flushed with CO gas (Matheson Co.), which was bubbled through water three times before entering the flasks containing the hemoglobin to prevent evaporation. Also, 1 mM stock solutions of 2,3-DPG in 0.05 m Tris-HCl were prepared as described before (9). Using these solutions of fully saturated HbCO and HbO₂, as well as the 2,3-DPG solutions, hemoglobin solutions were mixed and allowed to equilibrate for 1 hour at room temperature in sealed containers. The final total concentration of hemoglobin at pH 7.0 and 8.0 was 0.02 mM (tetramer) and the concentration of 2,3-DPG, when present, was 0.2 mM. The hemoglobin solutions were scanned spectrophotometrically in a Cary 14 recording spectrophotometer from 650 to 490 nm. The fraction of HbCO in the solution was determined using Equation 1:

\[
[HbCO] = \frac{A_{560} - r}{s - r}
\]

where \( A_{560} \) is the absorbance of the solution at 560 nm; \( r \) is the molar extinction coefficient for HbO₂ and HbCO; and \( s \) is the molar extinction coefficient for HbCO at 560 nm (\( \lambda = 560 \text{ nm} \)). The average extinction coefficients are given in terms of tetrameric hemoglobin. The total concentration of hemoglobin, \( H_b \), was determined by using the absorbance of the sample at 498 nm (which is an isosbestic point for HbO₂ and HbCO) and the molar extinction coefficient at 498 nm (\( \Lambda = 498 \text{ m}^{-1} \text{ cm}^{-1} \)). The average fraction of HbCO for the samples used in this study was 0.013, 0.280, 0.640, 0.849.

Deoxygenation Kinetic Measurements—The deoxygenation kinetics was measured in a Durrum-Gibson stopped flow apparatus at 560 nm with a 20-mm path length, with monochromator slits set at 0.4 nm. The reactions were performed at 23° using sodium dithionite (1 g per liter) as a deoxygenating agent, in 0.05 m Tris-HCl, taking great care in its preparation. The solutions of hemoglobin with the various concentrations of HbCO, at pH 7.0 and 8.0, with and without 2,3-DPG, were transferred anaerobically to the stopped flow apparatus. At least six independent experiments per experimental point reported, were performed on these hemoglobin preparations.

Analysis of Kinetic Data—The polaroid pictures, which were taken of each reaction trace on the recording oscilloscope, were analyzed by first converting transmittance to absorbance. At the reaction wave length, deoxyhemoglobin (Hb) and HbCO have an isosbestic point. Thus, any change in absorbance will be due to the loss of HbO₂ alone. Therefore the fractional saturation with oxygen may be calculated using the following equation:

\[
Y = \frac{A_b - A_a}{A_a - A_a}
\]

where \( A_b, A_a \) are the absorbances at the beginning, at any time, \( t \), and at the end of the reaction, respectively. The values for \( Y \) were determined and were fitted to the first order exponential equation:

\[
Y = e^{-kt}
\]

from which \( k \), in \( s^{-1} \), is determined. Except where indicated, \( k \) was determined for all data between 85 and 40% saturation with O₂.

Calculation of Initial Species—It is important for the interpretation of these data to know the distribution of species present...
Table I

Percentage of species initially present in the various mixtures

These values were calculated from the spectrophotometric measurements of the fraction of HbCO and HbO2 in the mixtures with the expressions from the binomial expansion. The data and details are given in the text.

<table>
<thead>
<tr>
<th>Species present</th>
<th>HbO2</th>
<th>HbO2(CO)</th>
<th>HbO2(CO)2</th>
<th>HbO2(CO)3</th>
<th>HbO2(CO)4</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbO2</td>
<td>94.91</td>
<td>26.87</td>
<td>1.68</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>HbO2(CO)</td>
<td>5.00</td>
<td>41.80</td>
<td>11.96</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td>HbO2(CO)2</td>
<td>0.12</td>
<td>24.39</td>
<td>31.85</td>
<td>9.86</td>
<td></td>
</tr>
<tr>
<td>HbO2(CO)3</td>
<td>6.34</td>
<td>37.74</td>
<td>36.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbO2(CO)4</td>
<td>0.62</td>
<td>16.77</td>
<td>51.96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Log Periods—As can be seen in Figs. 1 to 4, significant lag periods are present in certain instances. The factors which determine the length of these lag times are (a) PO2, (b) pH, and (c) concentration of dithionite, as first shown by Hartridge and Roughton (17). Although these variables influence the length of the lag period, the experiments of Hartridge and Roughton (17) show that the reaction of dithionite with free oxygen in solution is not rate-determining with respect to the deoxygenation reaction. For the reaction of 98% HbO2 at pH 7.0 (Fig. 1), the lag time is small (about 5 ms). As the concentration of HbCO increases, which is also accompanied by a slight fall in the PO2 of the solution, the lag period disappears. The disappearance of the lag is probably the result of the fall in PO2, which would be noticeable on a lag time which was short to begin with. Such an effect, however, was minimal at pH 8.0, where the lag is seen to shorten slightly, but not disappear as the concentration of HbCO increases. This latter result is due to the slight pH
FIG. 4. Deoxygenation kinetics of human hemoglobin containing initially 15.1% HbO₂. The conditions and meaning of the symbols are the same as in Fig. 1.

FIG. 5. Plot of the deoxygenation kinetic constant, $k$ (in s⁻¹) from Equation 3 of text, calculated between 85 and 40% saturation versus the per cent HbO₂ initially present. These data come from the values given in Table II. The symbols have the same meaning as given in the legend to Fig. 1.

dependence of the reaction of dithionite with dissolved oxygen, a result noted in the original experiments of Hartridge and Roughton (17), and confirmed in this and other laboratories.

Effect of Carbon Monoxide on $k$—An inspection of Figs. 1 to 4 shows that as the concentration of HbCO increases, the over-all rate decreases. This is very apparent in Fig. 5 and Table II. It is of interest to know how $k$ varies during any particular reaction. This point is illustrated in Figs. 6 and 7, where the value of $k$ is calculated at various saturation levels during the initial portion of the reaction and is plotted versus the extent of the reaction. For a hemoglobin solution containing mostly HbO₂, there is a significant variation in $k$ early in the reaction both at pH 7.0 and at 8.0. These findings are in agreement with the earlier results of Gibson and Roughton (4) who studied the reaction only at alkaline pH. The greatest variation in $k$ is observed over about the initial 30% of the reaction. Further-

![Fig. 4](http://www.jbc.org/)

![Fig. 5](http://www.jbc.org/)

![Fig. 6](http://www.jbc.org/)

![Fig. 7](http://www.jbc.org/)

**TABLE II**

Values of the deoxygenation rate constants for the various conditions of partial saturation are given as mean and standard deviation. The conditions of hemoglobin and 2,3-DPG concentration are given in the text. Temperature was 23°.

<table>
<thead>
<tr>
<th>HbO₂ %</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k$ with DPG</td>
<td>$k$ no DPG</td>
</tr>
<tr>
<td>85.7</td>
<td>52.4 ± 2.0</td>
<td>36.0 ± 1.8</td>
</tr>
<tr>
<td>72.0</td>
<td>36.2 ± 1.7</td>
<td>26.2 ± 1.4</td>
</tr>
<tr>
<td>36.0</td>
<td>25.4 ± 1.1</td>
<td>18.8 ± 0.7</td>
</tr>
<tr>
<td>15.1</td>
<td>18.0 ± 0.8</td>
<td>17.9 ± 0.9</td>
</tr>
</tbody>
</table>

FIG. 6. Plot of $k$, from Equation 3 of text, versus the extent of the reaction for the case where 98.7% of the hemoglobin initially had oxygen bound. The constant $k$ was calculated starting with the first measurable change in absorbance. The symbols are: closed circle, pH 7.0, and open circle, pH 8.0. No 2,3-DPG was present in either case.

Fig. 7. Plot of $k$, from Equation 3 of text, versus the extent of the reaction for the case where 15.1% of the hemoglobin initially present had oxygen bound. The treatment of the data and meaning of the symbols are otherwise the same as those for Fig. 6.
occurs. It is also interesting to note that the rate at pH 7.0 is greater than at pH 8.0.

Effect of 2,3-DPG on k—At alkaline pH, 2,3-DPG has been shown to have little effect on the rate of deoxygenation of human hemoglobin (9). This result is again observed in the studies presented herein (Figs. 1 to 5).

The situation is different at neutral pH. In a solution containing 15% HbO2 and 85% HbCO, 48% of the hemoglobin molecules have some amount of oxygen bound (see Table I); about 77% of these molecules are in the form HbO2(CO)2. The large predominance of this form, along with the results shown in Fig. 4 (see also Fig. 5 and Table II), clearly indicates that 2,3-DPG does not influence the dissociation of the first oxygen molecule from fully liganded hemoglobin. This result is in good very agreement with other evidence which indicates that 2,3-DPG does not influence the dissociation of the first oxygen from oxyhemoglobin. This evidence includes the absence of an effect of 2,3-DPG (a) on the lag period in the dithionite reaction with oxyhemoglobin at pH 7.0 (9) (which reflects the constant k4 in the reaction HbO2 + k4 HbO2 + O2), (b) on k5 determined by CO replacement reactions (11), (c) on the deoxygenation rate of horse hemoglobin reacted with bis(N-maleimidomethyl)ether (18) (a hemoglobin "locked" in the liganded quaternary conformation independent of the state of ligation), and (d) on the equilibrium constant for binding the fourth oxygen molecule (13).

As is observed in Figs. 3 and 5 and Table II, 2,3-DPG does have a significant effect on the value of k observed in a solution containing 36% HbO2 and 64% HbCO. In Table I we see that, in this case, about 83% of the hemoglobin molecules initially contain oxygen bound to some degree. Roughly 45% of these oxygen-containing species are in the form HbO2(CO)2, which cannot release oxygen at a faster rate when 2,3-DPG is added. However, approximately 32% of the hemoglobin is in the form HbO2(CO)2 in contrast to only 10% in the mixture containing 15% HbO2 and 85% HbCO. Thus, the significant increase in the deoxygenation rate observed with the 36% HbO2-64% HbCO mixture, upon addition of 2,3-DPG, may be accounted for by the increase in the initial species HbO2(CO)2 (Figs. 3 and 5 and Table II).

**DISCUSSION**

The results described here contribute more evidence to the list of observations from this and other laboratories that the sequential scheme for viewing the deoxygenation reaction, discussed by Gibson (5), can explain flow kinetic observations. Ideally, one would wish to be able to quantitatively interpret these and other dithionite reactions. However, in a recent paper by Hopfield et al. (19), the problem of quantitative interpretation of dithionite data is mentioned and it was pointed out that such treatment of the deoxygenation reaction using dithionite may be complicated by the lag period. Certainly, this is a concern. However, the elimination of the increase in k observed in Fig. 7 for solutions containing initially 13.1% HbO2 and 84.9% HbCO can be offered as evidence that the variation in k observed in the first 30 to 40% of the reaction (Fig. 6) is characteristic of hemoglobin and not of the reaction of dithionite with oxygen. Thus we shall attempt to make certain semiquantitative comparisons of the value of k, for hemoglobins which are noncooperative (i.e. n = 1), to see if a consistent view of the dithionite reaction emerges.

As pointed out by Antonini and Brunori (20), the time course of the dithionite reaction can be explained by assuming (a) that

<table>
<thead>
<tr>
<th>Hemoglobin type</th>
<th>Measured over-all rate k</th>
<th>Expected value of k as determined by (k4 + k5)/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human HbO2(CO)2, pH 7.0</td>
<td>17.14</td>
<td>17.14</td>
</tr>
<tr>
<td>Human HbO2(CO)2, pH 5.0</td>
<td>14.24</td>
<td>11.95</td>
</tr>
<tr>
<td>Human Bethesda, pH 7.0</td>
<td>17.18</td>
<td>17.18</td>
</tr>
<tr>
<td>Human Rainier, pH 7.0</td>
<td>6.14</td>
<td>17.18</td>
</tr>
<tr>
<td>Horse BME-reacted, pH 7.0</td>
<td>14.24</td>
<td>11.15</td>
</tr>
</tbody>
</table>

* From the studies presented herein.
* From Olson et al. (12); these are the values of (k4 + k5)/2 determined from the measurements of k4 and k5 by these authors for the release of the first oxygen molecule from the given native hemoglobin, i.e. either human or horse for this table.
* Same as Footnote b except for human hemoglobin at pH 9.0.
* Olson and Gibson (21).
* Salhany (22).
* Salhany (18).

The over-all deoxygenation rate, k (s⁻¹) (calculated between 85 and 40% saturation), for various noncooperative hemoglobins is compared with the expected value of k for a noncooperative hemoglobin (assuming that the affinity of the individual subunits is unaltered). The expected value of k comes from the measurements of (k4 + k5)/2 for the respective native oxyhemoglobin at 21–23°C. BME, bis(N-maleimidomethyl)ether.

At pH 7.0, the observed rate constant, k4, was 17.9 s⁻¹ for a solution containing HbO2(CO)2 as the predominant (~77.0%) species initially present. This value of k is in excellent agreement with the value of (k4 + k5)/2 (i.e. the expected value shown in Table III) measured by Olson et al. (12) for human oxyhemoglobin at the same temperature and pH. At alkaline pH the value of k for HbO2(CO)2 was 14.2 s⁻¹ in these studies. Al-
though this value is only slightly less than that at pH 7.0, it is nevertheless significant (see Table II). The slower rate at pH 8.0 for HbO₂(CO₂) is also in agreement with what would be expected as determined by the results of Olson et al. (12). The reason their value listed in Table III is lower than that for the HbO₂(CO₂) solution is probably because their alkaline pH value was 9.0 rather than 8.0. The kinetic properties of two mutant hemoglobins have recently been determined. They are hemoglobin Bethesda α₂β₂ (145 Tyr → His) (21) and Rainier α₂β₂ (145 Tyr → Cys) (22). Both of these hemoglobins have n = 1. The deoxygenation rate for Bethesda was measured by Olson and Gibson to be about 18 s⁻¹, quite close to the expected value for oxygen release from a fully liganded (noncooperative) human hemoglobin. The value measured by Salhany (22) for Rainier was 6.1 s⁻¹, considerably below what would be expected for the value of k from a noncooperative human hemoglobin, suggesting that the liganded conformation of Rainier is abnormal (23, 24). Lastly, the kinetics of horse oxyhemoglobin reacted with bis(N-maleimidomethyl)ether is also presented in Table III, from the studies of Salhany (18). The value of k for this hemoglobin compares quite favorably with the value of (kₐ + kₕ)/2 for native horse hemoglobin measured by Olson et al. (12). bis(N-maleimidomethyl)ether-horse hemoglobin has been shown to be "locked" in the quaternary conformation of the fully liganded protein, independent of the state of ligation (25–27). Thus, from the comparison made in Table III, it appears that whenever a noncooperative hemoglobin (with the affinity of the individual subunits unaltered) releases oxygen, the value of the over-all rate will equal the value of (kₐ + kₕ)/2 for the respective native hemoglobin. Therefore, the value of the kinetic constant, k, calculated between 55 and 40% saturation, appears to give consistent results, which can at least, semiquantitatively, represent the deoxygenation kinetic behavior of hemoglobin.

As was pointed out above, the deoxygenation kinetics of solutions containing HbO₂(CO₂) as a major initial species can be viewed as if they were the rate of release of the first oxygen molecule from fully oxygenated hemoglobin. Thus the lack of an effect of 2,3-DPG on these solutions at pH 7.0 (Figs. 4 and 5) indicates that this organic phosphate has no influence on the release of the first oxygen molecule from the fully oxygenated conformation. It was also indicated above that the deoxygenation kinetics of bis(N-maleimidomethyl)ether-horse oxyhemoglobin may also be viewed as if they were the rate of release of the first oxygen molecule from fully liganded horse hemoglobin. It is therefore significant that the earlier studies of Salhany (18) showed that 2,3-DPG does not affect either the deoxygenation kinetics or the oxygen equilibrium of this chemically modified hemoglobin. The observation that a significant effect of 2,3-DPG was noticed associated with an increase in the species HbO₂(CO₂) (see Table I) may imply that 2,3-DPG does affect the deoxygenation of oxyhemoglobin at the point of the second oxygen molecule going off. Such a deoxygenation kinetic result is consistent with the recent ligand binding kinetics of MacQuarrie and Gibson (14), where they suggested that their 2,3-DPG analogue dissociates from its complex with hemoglobin after the binding of the third heme ligand. Since the results of this work and the work of others show that a necessary condition for 2,3-DPG to influence the oxygen interaction is the loss of the fully liganded conformation, it would then be suggested from this study that the conformational change occurs with the dissociation of the first heme ligand from the fully liganded hemoglobin.

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The Deoxygenation Kinetics of Hemoglobin Partially Saturated with Carbon Monoxide: EFFECT OF 2,3-DIPHOSPHOGLYCERATE
J. Mitchell Salhany, Daniel H. Mathers and Robert S. Eliot


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