Dissociation of CO from Carboxyhemoglobin*

Vijay S. Sharma, Mary R. Schmidt, and Helen M. Ranney

From the Department of Medicine, University of California at San Diego, La Jolla, California 92039

The reaction between carboxyhemoglobin and reduced microperoxidase (MP): Hb(CO)4 + 4MP = Hb4 + 4MPCO, recently reported by us, has been further studied. By generating species Hb4(CO), Hb4(CO)4, and Hb(CO)4 in the stopped flow cuvette by the reaction of dithionite with the species of the general formula Hb4(O2)4(CO)y (x + y = 4) in the presence of microperoxidase it has been possible to determine the stepwise CO dissociation rate constants k1, k2, k3, and k4. The overall CO dissociation rate constant k, which is the same in this system as k, is not affected by 2,3-diphosphoglyceric acid. The activation energy of the reaction is 21,400 cal in 15–25°C range. The ratio ΔΔ/ΔpH is ~3 in 6.5 to 7.5 pH range. The kinetic data indicate that, compared to HbO2, the contribution to the cooperativity of the dissociation rate constants of carboxyhemoglobin is greatly reduced. The ligand-dependent differences in the reactions of Hb with CO, O2, and NO suggest that in the combination reactions the ligand plays an active role in the rate-limiting step.

The most commonly used gaseous ligands for studying the functional behavior and ligand-reaction mechanism of hemoglobin are O2, CO, and NO. Of these three, only for oxygen the complete set of stepwise and overall ligand combination and dissociation rate constants have been obtained. Ligand combination rate constants for CO and NO have also been determined. The results show that the "on" rate constants are greatly dependent on the nature of the ligand. Thus, NO reacts fastest and shows no cooperativity in its combination reaction rate constants (1). O2, which is slower than NO by a factor of 10, shows only minimal cooperativity in its "on" rate constants (k'/k'' = 2) (2). CO, which is slowest in reacting with hemoglobin, shows appreciable cooperativity in its combination reaction (k'/k'' = 10) (3). It is interesting to note that the ligand combination rate constants follow the order of the σ electron accepting ability of the ligands (NO > O2 > CO) and they suggest the possible importance of electronic factors in the rate-limiting step.

A similar comparison of dissociation rate constants is not possible. This is because for CO and NO only the dissociation of the first ligand from fully saturated hemoglobin has been studied (4, 5). The remaining three stepwise dissociation constants and the overall dissociation rate constant of CO-hemoglobin and NO-hemoglobin have not been obtained mainly due to the lack of a suitable reagent that binds CO or NO faster and more strongly than hemoglobin. There is little doubt that the rate-limiting step in ligand dissociation reactions is the splitting of the metal (iron) donor (O2 or CO or NO) bond. In the case of oxygen it is known that as the ligand dissociation reaction proceeds, the rates become faster due to the changing electronic, or steric environment of successive heme pockets in the Hb tetramer, or both. Whether this is true for CO and NO is not certain as no direct measurements of overall and stepwise dissociation rate constants have been made, and k1 and k2, the only measured dissociation rate constants for CO and NO, for the release of the first ligand from fully saturated hemoglobin Hb4(O2)4 give no information regarding cooperativity or lack of it in the ligand dissociation reaction of Hb tetramer. In fact, the values of k1 and k2 are similar to the corresponding values of noncooperative species such as α and β chains (6).

In a recent communication (7) we reported a new method for determining the overall dissociation rate constant of CO from carboxyhemoglobin. For CO-hemoglobin we observed that the overall dissociation rate constant k is the same as the statistically corrected value of k as reported by Antonini and Gibson (4). In this paper we report the results of some further studies of the CO dissociation reaction: the effect of temperature, pH, and 2,3-DPG. By studying the CO dissociation from solutions partially saturated with CO and O2, it has been possible to extend the method for determining the stepwise dissociation rate constants k1, k2, k3, and k4. The results suggest that as compared to oxygen, the dissociation reactions of CO contribute less to cooperativity. The kinetic parameters for COHb are compared with those of oxyhemoglobin and the implications of ligand-dependent differences in the rate constants for the reaction mechanism are discussed.

MATERIALS AND METHODS

The hemolysates were prepared by the method of Drabkin (8). The samples were dialysed overnight at 4°C against 0.1 M phosphate buffer of appropriate pH. Hemoglobin solutions were stripped of phosphates by passing the hemolysate through a Sephadex (G-25) column equili-

*This work was supported by Grants AM17348-3 and AMHL 18761-1 from the National Institutes of Health.
brated with bis-Tris buffer of pH 7.4 containing 0.1 M sodium chloride and dialyzing the sample thus obtained overnight against water at 4°. The pentacyclobexxalaminium salt of 2,3-DPG (tetrhydrate, Grade A) was obtained from Calbiochem and was converted to the sodium salt form by shaking it at pH 7 to 8 with an excess of analytical grade cationexchange resin AG 50W-X2 (100 to 200 mesh, Bio-Rex) in the sodium form. The solutions were stored at 4°.

All kinetic runs were made at 20° unless otherwise stated, on a Durrum stopped flow apparatus using a 2-cm light path cuvette and a tungsten light source as described previously (9).

Solutions of CO-hemoglobin were prepared by mixing calculated amounts of deoxy Hb and CO solutions in a known volume of deoxygenated buffer. The last traces of oxygen were removed, when necessary, by adding a few grains of dithionite to the syringes containing the HbCO solution.

Microperoxidase (Sigma-MP-11) solutions were prepared by dissolving a weighed amount of microperoxidase in buffer, stirring it for 15 to 30 min and then deaerating it thoroughly by bubbling nitrogen. The optical density of a solution of 1 mg of ferric microperoxidase in 20 ml of buffer (pH 7.0, 0.1 M phosphate) was 0.190 at 525 nm and 9.587 at 405 nm. The reagent was reduced by adding a few crystals of dithionite to the deaerated solution.

Partially saturated HbCO solutions were prepared by two methods. In the first method a calculated amount (less than 1 eq of hemoglobin concentration on heme basis) of CO solution of known molarity was added to a measured volume of deoxy Hb solution in a syringe. The last traces of O2 were removed by adding a few crystals of dithionite to the solution. In the second method, a calculated amount of CO solution was added to a solution of oxyhemoglobin in deaerated buffer. The fast removal of oxygen was attained when this solution was mixed rapidly in the stopped flow apparatus with a solution containing microperoxidase and a much greater concentration of dithionite.

The optical density of a solution of 1 mg of ferric microperoxidase in buffer, stirring it for 15 to 30 min and then deaerating it thoroughly by bubbling nitrogen. The optical density of a solution of 1 mg of ferric microperoxidase in 20 ml of buffer (pH 7.0, 0.1 M phosphate) was 0.190 at 525 nm and 9.587 at 405 nm. The reagent was reduced by adding a few crystals of dithionite to the deaerated solution.

Partially saturated HbCO solutions were prepared by two methods. In the first method a calculated amount (less than 1 eq of hemoglobin concentration on heme basis) of CO solution of known molarity was added to a measured volume of deoxy Hb solution in a syringe. The last traces of O2 were removed by adding a few crystals of dithionite to the solution. In the second method, a calculated amount of CO solution was added to a solution of oxyhemoglobin in deaerated buffer. The fast removal of oxygen was attained when this solution was mixed rapidly in the stopped flow apparatus with a solution containing microperoxidase and a much greater concentration of dithionite.

The spectrum reported in Fig. 1 was recorded on a Cary 14 spectrophotometer. All concentrations are on a heme basis, and were determined by using the extinction coefficient of 14.3 x 10^3 at 540 nm for oxyhemoglobin.

Treatment of Data—First order rate constants were calculated from the slope of ln (A = A_0) versus time plots. The last 10% of the reaction was studied by delaying the scope-trigger and enlarging the absorbance change due to the last 10% of the reaction.

The reaction time course of hemoglobin solutions partially saturated with CO and O2 were biphasic. The rate constants for the two components, which theoretically justified, were calculated by making a least square fit to the equation:

\[ A_t = A_0 + \sum_{i=1}^{n} C_i \exp(-\lambda_i t) \]  

where \( A_t \) is the absorbance at time \( t \), \( A_0 \) is the absorbance at infinite time, \( n \) is the number of components, and \( C_i \) and \( \lambda_i \) represent zero time amplitude and first order rate constants, respectively. The initial estimates of \( C_i \) and \( \lambda_i \) were obtained from the initial and final slopes of ln (A = A_0) versus time plots. The initial value of \( \sum(C_i) \) was obtained from the intercept \( \delta A \) versus \( t \) plot. The intercept drawn from the distal part of the same plot was used as the initial value of \( C_\infty \).

The least square refinements were made on Burrough's 6700 computer.

RESULTS

CO dissociation rates were studied by mixing reduced microperoxidase with HbCO and following the optical density changes occurring in the reaction:

\[ \text{HbCO} \rightarrow \text{Hb} + \text{CO} \]  
\[ \text{CO} + \text{MP} \rightarrow \text{MPCO} \]  
\[ \text{MP} \rightarrow \text{Hb} + \text{MPCO} \]  

Since microperoxidase (M) binds CO much faster and more strongly than hemoglobin (k_m = 2 x 10^11 M^-1 s^-1; k_m/k_d = 2 x 10^8 back-reactions in Steps I and I b become negligible particularly when microperoxidase is in slight excess over Hb.

Fig. 1 shows the spectrum of microperoxidase, CO-microperoxidase, Hb, and HbCO at 20°, 0.1 M phosphate buffer, pH 7.0. Concentration of all species = 5 μM.

It has been pointed out to us by Dr. Q. H. Gibson that the last 20% of the dissociation reaction would be expected to exhibit some acceleration because of the predominance of HbCO in this region. We have rechecked this observation and find no acceleration of the reaction. It may be that the suggested acceleration is within the accuracy of the graphical method of data treatment, or less likely, the acceleration is obscured by an unknown secondary reaction of microperoxidase in that part of the dissociation reaction. A more likely explanation lies in the kinetic heterogeneity of α and β chains. The dissociation from the slower dissociating chains dominates the last part of the reaction, and if the difference between \( I_1 \) and \( I_2 \) is not large enough, the net acceleration in the dissociation reaction could be too small to observe. The reason that such acceleration is observed in the dissociation of oxyhemoglobin is that \( k_1/k_2 \approx 40 \) as compared to \( I_1/I_2 \) (with statistical factors) of only 2.5 (see "Discussion").
Kinetics of Carboxyhemoglobin

**Table I**

<table>
<thead>
<tr>
<th>CO dissociation rate constants for Hb A and myoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>All concentrations are in micromolar of heme before mixing.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[Hb]</th>
<th>[CO]</th>
<th>Rate constant (s⁻¹) ± 0.001</th>
<th>nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3–30</td>
<td>0.009</td>
<td>419, 419, 437.8</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>0.009</td>
<td>437.8</td>
</tr>
<tr>
<td>80</td>
<td>110</td>
<td>0.009</td>
<td>556, 600</td>
</tr>
<tr>
<td>[Mb]</td>
<td>[CO]</td>
<td>Rate constant (s⁻¹) ± 0.001</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3–25</td>
<td>0.017</td>
<td>416, 419</td>
</tr>
</tbody>
</table>

**Effect of pH**—As the pH is lowered from 7.5 to 6.5 the CO dissociation rate constant is increased 3-fold (Table Ib). The corresponding effect for \( k \), \( k_a \), and \( j_a \) is much smaller (11).

**Effect of Temperature**—Table Ic shows the results of three experiments over the temperature range of 15–25°C at pH 7.0, 0.1 M phosphate buffer. The average value of \( Q_{10} \), the temperature coefficient of \( l \) per 10°C in the range of 15–20°C is 3.3, which is significantly lower than the value reported by Gibson and Roughton (\( Q_{10} \) for \( l_a \) - 4 (5)). \( Q_{10} \), if calculated from the values at 20°C and 25°C, is 4.2, in good agreement with \( Q_{10} \) for \( l \) reported by Gibson and Roughton (5). The energy of activation corresponding to \( Q_{10} \) of 3.3 is 21,400 cal.

**Effect of 2,3-DPG**—At pH 7.0, 0.1 M bis-Tris buffer, a 20-fold excess of 2,3-DPG (60 µM) has no effect on the CO dissociation rate constant \( l \) (Table Id) of carboxyhemoglobin (3 µM). A slight decrease in \( l \) in bis-Tris as compared to in phosphate buffer, is most probably due to the difference in the ionic strength of the two buffers.

**Effect of Partial Saturation**—Partially saturated CO-hemoglobin (50 and 25%) solutions, if prepared by the method one (see "Materials and Methods"), show a monophasic CO dissociation reaction and yield a rate of 0.009 ± 0.001 s⁻¹ in agreement.
ment with the values obtained from fully saturated hemoglobin solutions. On the other hand, if the partially saturated COHb solutions are prepared in the stopped flow cuvette by rapid deoxygenation of Hb(O)4 (CO)4 solution with dithionite, the reaction time course is no longer monophasic. At 75 to 90% saturation with O2 and 10 to 25% saturation with CO, the reaction time course is independent of Hb concentration. Equation II therefore, can be written as:

$$-0.75 \cdot k_4 [Hb_4(CO)_4] + k_4 ([Hb_4] - [Hb_4(CO)_4])$$

(III)

In the concentration range of 5 to 80 μM of hemoglobin (heme basis before mixing), the average of these experiments value of \(k_4\) was 0.03 ± 0.01 s⁻¹. Changing the tetrameric dissociation constant to 3 μM (13) gave \(k_4\) = 0.037 s⁻¹.

**Discussion**

The data listed in Table II show that in spite of important differences the relative values of CO dissociation rate constants qualitatively follow the same trend as the corresponding dissociation rate constants of oxyhemoglobin (2). Lack of any effect of 2,3-DPG on \(l\) suggests that \(l\) mainly represents the dissociation of the first ligand from fully liganded carboxyhemoglobin. A similar observation has been made regarding \(k_4\) of oxyhemoglobin (2). The value of \(k_4\) independently determined by the ligand exchange method is 0.031 s⁻¹ (20.5°, pH 7.1, in 0.1 M phosphate buffer (4)), which is 10 to 20% lower than the value obtained in the present study. This may be due to slight differences in the experimental conditions or it may indicate some contribution to \(l\) from \(L\). Since the difference is within the confidence limit of the rate constant \(l\), listed in Table II, it is difficult to attach much significance to it. No information is available about the values of \(k_1\), \(k_2\), \(k_3\) or the corresponding equilibrium constants (\(L_1\), \(L_2\), \(L_3\)) to provide a basis of comparison with the values of these constants obtained in the present study. However, the approximate range of these constants can be estimated from the following considerations.

In Table III, column \(L_a\) are listed the values of stepwise equilibrium constants calculated for species with Hb(CO) ratios of 1:1, 1:2, 1:3, and 1:4. These estimates were obtained using the kinetic constants for oxyhemoglobin (2) and by guest on October 30, 2017 http://www.jbc.org/ Downloaded from
L·-t determined by Gibson (2) and a partition coefficient of 300 (4). Column $L_i$ of the same table lists similar constants ($L_1 \cdots L_n$) estimated from the $K_i$ values reported by Roughton and Lyster (14). There are significant differences in the $K_i$ values from the two sources, which are obviously reflected in the estimated values of the corresponding $L_i$ constants. The constants $K_1$ and $K_2$ from Gibson's data differ by as much as a factor of 10 from those from the data of Roughton and Lyster. Column $L_i$ of Table III lists the values of $L_i$ constants estimated from the combination rate constants available in the literature or estimated from it and the dissociation rate constants determined in the present study.

It is evident that $L_i$ shows good agreement with the experimentally determined and estimated values of $L_1$, $L_2$, $L_3$, and $L_4$, which lie within a factor of 2 to 3 from those estimated from Gibson's (2) kinetic data. The values of $L_1$ and $L_2$ are intermediate between those estimated from the data of Gibson (2) and of Roughton and Lyster (14). The differences between the estimated values and those obtained on the basis of dissociation rate constants of the present study may arise because of the approximations made in the estimation of the constants or because of differences in the experimental conditions. For example, there is some doubt (15) regarding the validity of using the same partition function to estimate $L_i$ from the corresponding $K_i$ over the entire range of the dissociation curve. Also, Gibson's (2) kinetic data were obtained from hemoglobin solutions sufficiently concentrated to exclude dissociation of Hb tetramers into dimers, while the present study was made on relatively dilute (2 to 40 μM) hemoglobin solutions.

It is interesting to note that Roughton (15) estimated a value of 55 mm Hg for $L_1$ for sheep hemoglobin at pH 9.0. At pH 7, it was suggested that $L_1$ would equal $55/3 = 18.3$ mm Hg. Converted to moles per liter, $L_1$ is estimated to be 15 to 10$^8$ as compared to the value of 4.9 x 10$^9$ observed in the present study. In the same study (15), the authors estimated that $L_2$ should be approximately 3 times $L_1$. On this basis a value of 0.093 to 0.11 is predicted for $L_2$. This is in agreement with the experimentally determined value of $L_2$ (0.08 to 0.01 in). In a recent study on Hb Kansas (16), it was shown that Hb (CO)/Hb in the absence of IHP was in liganded conformation and in the presence of HPH the conformation changes to the unliganded state. The CO dissociation rate constants of the two states differ by a factor of 10. This is to be compared with the values of $L_1$ and $L_2$ without statistical factor listed in Table II, Column 2.

The values of $L_1$, $L_2$, $L_3$, and $L_4$ with statistical factors, Table II, Column 3) lie within a factor of 2.5 of each other. This is in marked contrast to oxyhemoglobin for which $k_i$ is approximately 40 times $k_2$ (2). The data indicate that as compared to oxyhemoglobin in CO-hemoglobin the level of cooperativity in dissociation rate constants is greatly reduced. Whitehead (17) reached a similar conclusion in analyzing the photodissociation of CO-hemoglobin. The data of Brunori et al. (18) on CO binding to Hb under photodissociating conditions suggested a similar trend but, in the absence of direct experimental evidence, the authors discounted it as at that time it ran against the current ideas on cooperativity which attributed cooperativity mainly to the dissociation rate constants.

Ligand-dependent differences in the reactions of hemoglobin suggest some interesting possibilities regarding the reaction mechanism. In 0.1 M phosphate at pH 7.0 for O$_2$ the cooperativity lies mainly in the dissociation rate constant $k_r/k_a = 40$, $k_r/k_a = 2$ (2). Oxygen in this respect resembles NO which lacks cooperativity in ligand combination rates (1). For CO the behavior is the opposite as the cooperativity lies mainly in the combination rate constants: $k'_r/k'_a = 17$ (3), $k'_r/k'_a = 2.5$ (Table II). This trend may have important implications for the mechanism of ligand combination reactions. In the first place, by analogy with model compounds (19), it indicates that in the combination reactions the rate-limiting step is an "associative" one, i.e. it involves bond formation rather than a bond-breaking process in which the ligand plays an active role. Secondly, it suggests that for ligands with π electron accepting ability such as O$_2$ and NO, the successive hemes in Hb$_4$ react with more or less equal ease. Poor π acceptors (relative to NO and O$_2$) such as CO react more slowly than O$_2$ and NO, and their reaction rates with successive hemes become faster as the ligation reaction proceeds. If it is assumed that the rate-limiting step in combination reactions is electronic in nature, it would mean that in the R form unliganded hemes have a significantly different electronic environment than in the T form. This is certainly consistent with the observation that high affinity Hb variants (R form) in reduced form, have different spectra in the Soret region than reduced HbA (T form) (20). Alternatively, it may be argued that ligand-dependent differences in combination rates arise from the different extent of steric hindrance at the binding site due to different orientations of O$_2$, NO, and CO in the heme pocket, and this steric hindrance may decrease as the ligation reaction proceeds. According to Perutz (21) the steric hindrance in ligand binding is provided by γ-methyl of valine E11 (67) in β subunits and therefore, if the ligand combination rates are sterically controlled, β chains should react slower than α chains. The kinetic data of Gibson et al. (22) on the other hand indicate that β chains react faster than α chains, suggesting that the γ-methyl of Val E11 (67) moves out of the way of the ligand faster than the slower rate-limiting step.

Steric hindrance at the binding site is generally reflected in slower combination rates and higher dissociation rates. The combination rate constants for O$_2$ suggest very little change in steric hindrance in going from $k_2$ to $k'_2$. Yet the corresponding dissociation rate constants would suggest greatly increased steric hindrance at the binding site in Hb$_2$(O$_2$) than in Hb$_2$(O$_2$)$_2$. Similarly, on steric considerations it would appear, from dissociation rate constants, that in going from Hb$_2$ to Hb$_2$L the steric hindrance is more pronounced for oxygen ($k_r/k_a \approx 40$) than for CO ($k_r/k_a \approx 3$). This is quite contrary to what we should expect on the basis of bonding modes of O$_2$ and CO to hemoglobin as suggested by x-ray crystallographic studies (23). These studies indicate that the angle between the heme plane and the ligand is likely to be much smaller for O$_2$ (azide type of orientation of oxygen) than for CO, and therefore the possibility of steric hindrance on the distal side of heme by Val E11 or His E7 is greater for CO than for O$_2$. Is it possible that the metal—ligand bond in CO-Hb is too strong to be affected to the same extent as metal—oxygen bond in oxyhemoglobin, as the steric hindrance at the binding site increases in going from Hb$_2$L to Hb$_2$L? The recent kinetic studies (1) on the ligand combination reaction of NO with Hb$_2$ do not support this view. It is well known...
that NO forms a stronger bond with hemoglobin than CO and yet NO behaves like oxygen in its reactions with hemoglobin. It is estimated that \( J_{\text{NO}}/J_{\text{O}} \approx 30 \) (1). The inadequacy of the steric argument in explaining the ligand-dependent differences in the combination rate constants has also been mentioned by Cassoly and Gibson (1).

The kinetic and equilibrium data obtained from kinetic constants on oxyhemoglobin and carboxyhemoglobin suggest a model in which there is strong interaction between the two hemes of a pair of hemes and weaker interaction between the hemes belonging to different pairs (24). The binding constants of HbL and HbL, are similar to those of HbL, and HbL, respectively, although \( K_{\text{HbL}} \) is slightly higher than \( K_{\text{HbL*}} \) for both ligands (L = O2 or CO). Although the equilibrium dissociation curve fits satisfactorily the two state model (MWC) proposed by Monod et al. (25), it is least satisfactory in predicting the values of individual equilibrium constants. The two-state model requires that the values of stepwise kinetic and equilibrium parameters increase monotonically, a requirement that is not supported by the data (stepwise equilibrium constants obtained from rate constants, Table III, \( L^* \) and \( L^1 \)) available on HbO2 and HbCO. The ability of the MWC model to represent the ligand dissociation curve is most likely due to the insignificant contributions of HbL* and HbL, species to the shape of the ligand dissociation curve.

The \(^{19}F\)-NMR data (26) on CO binding to hemoglobin covalently labeled with fluorine at cysteine 893 yields the values of equilibrium constants (\( K_1 = 6.8, K_4 \) in T state) = 8, and \( K_4 = 100 \); all in mm \(^{-1} \)) which, considering the difference in the experimental conditions in the two studies, are in reasonable agreement with the constants obtained in this study: (\( K_1 = 4.6, K_4 = 4.9, K_4 = 190 \); all in mm \(^{-1} \)). These constants were obtained on the basis of "modified concerted transition model." The NMR (26) data indicate that the binding of the first ligand does not bring about any significant configurational change in other chains. It was therefore assumed that \( K_4 \) is not very different from \( K_1 \). The equilibrium constants obtained from the kinetic experiments suggest just the opposite. It may be that in partially liganded species the kinetic experiments measure the rate of formation of intermediates different from those observed in equilibrium studies.

**Acknowledgments**—We wish to thank Dr. Q. H. Gibson and Dr. R. W. Noble for their constructive criticism of the manuscript.

**REFERENCES**

Dissociation of CO from carboxyhemoglobin.
V S Sharma, M R Schmidt and H M Ranney


Access the most updated version of this article at http://www.jbc.org/content/251/14/4267

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/251/14/4267.full.html#ref-list-1