Kinetic Analysis of the Reaction of Cytochrome cc' with Carbon Monoxide

(Received for publication, October 13, 1965)

QUENTIN H. GIBSON AND MARTIN D. KAMEN

From the Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and the Department of Chemistry, University of California, La Jolla, California

SUMMARY

The equilibria and kinetics of the reaction of the diheme proteins cytochrome cc' derived from Rhodospirillum rubrum and Chromatium with carbon monoxide have been examined. There are wide differences in many respects. The pigment from Chromatium is half saturated by 0.2 μM CO, while that of R. rubrum requires a concentration of 400 μM. The kinetics of the combination reaction also differs. In stopped flow experiments, the rate for R. rubrum cytochrome cc' is independent of CO concentration over the range examined, while that for the Chromatium pigment is proportional to CO concentration and yielded a second order rate constant of 260 m−1 sec−1 at room temperature. Flash photochemical experiments gave biphasic recombination reactions, with rates independent of carbon monoxide concentration in both cases. The reactions are much faster than those observed by the stopped flow method.

The velocity of dissociation of CO was measured for the pigment from R. rubrum by dilution and was 0.009 sec−1, at 22° and pH 7.4. The equilibrium between CO and both proteins could be displaced by steady illumination from medium intensity sources such as tungsten filaments. In the Chromatium pigment, the rates of photodissociation and recombination were independent of light intensity. They varied with CO concentration and were numerically equal at CO concentrations greater than 1 × 10−4M. In R. rubrum cytochrome cc' the photochemical behavior was analogous to that of myoglobin in that there was the usual dependence of rate of dissociation on light intensity.

Preliminary experiments on the reaction of the reduced hemoproteins with oxygen and on the reaction of the carbon monoxide complexes with oxygen have given complicated results which cannot be summarized briefly. The findings may be explained by the hypothesis that a bound carbon monoxide molecule, after dissociation from the heme as judged spectrophotometrically, retains an association with the protein molecule. The conclusions resulting from this scheme are discussed in detail.

The diheme protein, cytochrome cc', binds no ligands other than carbon monoxide and nitric oxide (1). Data obtained in studies of ultraviolet and visible absorption spectra (2), determinations of magnetic susceptibilities (3), and electron spin resonance characteristics (3) identify cytochrome cc' as an "unsaturated" type of heme protein, which should be capable of reaction with all the ligands usually found to combine with other heme proteins, such as myoglobins, catalases, and cytochrome oxidase. The restricted character of ligand binding exhibited by cytochrome cc' has been attributed to stereochemical factors peculiar to this type of heme protein (1).

In this paper, we present the results of some preliminary experiment with two representative forms of cytochrome cc', one derived from the facultative phototroph, Rhodospirillum rubrum, and the other from the obligate photoanaerobe, Chromatium, strain 1). The reactions of ferrocytochrome cc' with carbon monoxide have been studied, and some observations on the oxidation of the hemoproteins by molecular oxygen and potassium ferricyanide are included.

Our findings cannot be explained on the basis of a simple, one-step reversible reaction of the type, heme + CO ⇔ heme-CO, such as has been found adequate for an understanding of ligand reactions of most other heme proteins. It is necessary to postulate a two-stage equilibrium system in which the amount of carbon monoxide bound and the extent of absorbance change are not strictly proportional.

EXPERIMENTAL PROCEDURE AND RESULTS

Materials and Methods—The heme proteins were prepared by procedures described elsewhere (1). Test solutions were made by appropriate dilution with Tris1 or phosphate buffers, as described in the text. Details of the flash photolysis and stopped flow apparatus have already been given (4, 5).

Equilibrium Studies on Binding of Carbon Monoxide—The reaction between CO and cytochrome cc' is known to be reversible and photosensitive (6). If the difference in absorbance between the reduced protein and that of the complex is ΔA0, at a ligand concentration c, and the difference in absorbance between the reduced form and that of the complex (100% association) is ΔA9, then, provided the association curve is of the usual hyperbolic form, we can write the relation

\[
\frac{1}{\Delta A_e} = \frac{1}{\Delta A_0} + \frac{K}{c}
\]

1 The abbreviations used are: Tris, tris(hydroxymethyl)aminomethane chloride; R-cyto-cc', Rhodospirillum rubrum cytochrome cc'; C-cyto-cc', Chromatium cytochrome cc'.
where \( K \) is the dissociation constant. A plot of \( 1/\Delta A_\lambda \) with respect to \( 1/c \) should give a straight line with slope \( K \).

In Fig. 1, the data obtained for R-cyto-c' are seen to accord well with this expectation. The value of \( K \) can be deduced as \( 4 \times 10^{-4} \text{M} \), which indicates that R-cyto-c' has an unusually low affinity for CO, compared to that of a typical unsaturated heme protein such as myoglobin, for which \( K \) is approximately \( 3 \times 10^{-4} \text{M} \) (7). At 1 atm of CO, R cyto-c' is only about 70% saturated, so that an increase of CO pressure to 1.5 atm, on the basis of an extrapolation of data in Fig. 1, would increase the saturation by approximately 11 per cent. This prediction agrees well with a previous observation (1), wherein such an increase in CO pressure increased the degree of saturation about 8%.

On the other hand, C-cyto-c' exhibited an affinity for CO much like myoglobin, with \( K \) estimated at approximately \( 2 \times 10^{-4} \text{M} \). We could not determine the equilibrium curve accurately with the means available, but there was no doubt that the two cytochromes cc' exhibited a remarkable difference in equilibrium behavior with CO; the Chromatium protein had an affinity for CO some three orders of magnitude larger than that of the R. rubrum protein, a result mirrored in the kinetic characteristics of the two proteins, as documented in the following sections.

Combination with CO as Observed in Stopped Flow Experiments—Because of the low affinity of R-cyto-c' for CO, we were limited in the range of pressures of CO usable in the study of the kinetics of its combination with CO. It was found that, over the range 1 mm to 500 \( \mu \text{M} \) CO, the approach to equilibrium at 26.5° had a half-time (\( t_1/2 \)) of 20 sec and was independent of CO concentration. This unexpected lack of dependence on CO concentration allowed us to assign only a pseudo-first order constant of 0.033 sec\(^{-1}\) for the combination reaction, a slow rate as compared with those typical of other heme proteins (8) measured at comparable concentrations of CO.

The reaction of C-cyto-c' was also slow but was dependent on CO concentration over the much wider range of CO concentration used; the rate was accurately proportional to CO concentration from 38 \( \mu \text{M} \) to 500 \( \mu \text{M} \) CO, allowing calculation of a second order rate constant of 0.033 sec\(^{-1}\) for the pseudo-first order combination rate constant with 1 atm of CO may be quoted as approximately 0.03 sec\(^{-1}\). Thus, both proteins reacted at comparable rates with CO but responded differently to changes in CO concentration.

Flash Photochemical Experiments—Preliminary experiments were performed to determine whether, with the apparatus available, a reasonable proportion of the carbon monoxide compounds could be photodissociated. The energy input to the flash tubes was varied, and the change in absorbance produced by the discharge was measured. Writing the amount of compound breakdown as \( x \), the flash energy as \( J \), and the total quantity of the photosensitive compound initially present as \( a \),

\[
\frac{a - x}{a} = e^{-kj}
\]

where \( k \) is a constant which includes the quantum yield of the compound under study, as well as the geometrical efficiency of light transfer, luminous efficiency of the lamp, and overlap of the emission and absorption spectra.

Table I shows that the photosensitivity of both R. rubrum and Chromatium compounds was similar. The results were fitted best by assuming the removal of about 75% of the CO in each case by the maximum flash energy available. Exact comparison with static measurements was difficult because the measuring beam used in the flash apparatus produced appreciable photochemical dissociation, especially with R-cyto-c'. The results were in contrast to those with the carbon monoxide compound of cytochrome c oxidase (4), however, as the effective quantum yield for both cytochromes cc' was estimated to be only one-eighth as great as that for oxidase.
The experiments were conducted with a dichromate-shielded flash so that only the α-bands of the heme protein were used for excitation. The published spectra (10) do not suggest that differences in overlap between the exciting light and the absorption bands of the CO compounds of the oxidase, on the one hand, and of R. rubrum and Chromatium proteins, on the other, can account for the difference in quantum yield, which must be attributed rather to an intrinsic photochemical difference between cytochromes c' and the oxidase.

The rate of the recombination reaction with CO after the flash was studied next. With R-cyto-c', all the experiments were done with solutions equilibrated with 1 atm of CO. The reaction was biphasic, with approximately equal proportions of slow and fast phases. The division between slow and fast phases was independent of the observing wave length, and of the proportion of the complex broken down by the photolysis flash. The rate of the rapid phase at 25° was 7 sec⁻¹, and of the slow phase, 0.015 sec⁻¹. In order to determine the dark equilibrium value after the flash, it was necessary to include an interference filter in the observing beam between the light source and the sample; otherwise the photochemical effect of the observing beam led to the calculation of a spuriously large rate constant for the slow phase of the recombination reaction.

With C-cyto-c', several sets of experiments were performed at different concentrations of carbon monoxide. The recombination reactions which followed flash photodissociation were again biphasic, as with R-cyto-c', but were much faster. With 1 atm of CO the rate of the rapid phase was $6 \times 10^4$ sec⁻¹, and of the slow phase, $6 \times 10^3$ sec⁻¹, or about 2 $\times 10^3$ and 2 $\times 10^2$ times faster, respectively, than in the flow experiments. When the concentration of CO was reduced, the proportion of the rapid phase diminished, but the rates of the reaction were not substantially altered until partial pressures below 0.1 atm had been reached.

These points are illustrated in Fig. 2. This experiment shows that the two phases of the reaction cannot be explained by heterogeneity of the preparation, because in that case the partition of the change in absorbance between slow and fast phases would not depend on the concentration of CO. It seems more probable that the rapid phase is due to the transient existence of a form of the reduced cytochrome c' which reacts more rapidly with CO than the normal reduced form does, as has already been described in the case of hemoglobin (11).

Thus, the course of the CO recombination reaction, after flash dissociation, was independent of CO concentration above about 100 μm but slowed at lower concentrations, whereas in the stopped flow experiments recombination was proportional to CO concentration and was much slower.

**Dissociation of Carbon Monoxide from Complexes**—A direct measurement of the rate of dissociation of the R-cyto-c' complex was made by equilibrating a small volume of a concentrated solution of the protein with CO, mixing with 30 volumes of deoxygenated buffer in an anaerobic cuvette, and then following the dissociation reaction at 416 mp. This wave length is the maximum of the difference spectrum (CO, reduced, minus reduced) (10). The affinity for CO was so low that, even starting with an initial pressure of 1 atm, the final saturation fell to below 10%. The rate constant for dissociation obtained at 22° and pH 7.4 in 0.1 M phosphate buffer was 0.090 sec⁻¹ (Fig. 3).

As will be described in a later section, the reaction of the reduced C-cyto-c' with oxygen was considerably more rapid than its reaction with CO. If, then, a solution of the carbon monoxide compound in equilibrium with a low partial pressure of CO were mixed with oxygen, most of the reduced heme formed by dissociation of CO should be oxidized and so become unable to recombine with CO. The theory of this method for determining the rate of dissociation of ligand has been fully dealt with by Gibson and Roughton (12). The reaction of the complex of C-cyto-c' was slow (0.001 sec⁻¹) and was approximately first order over the greater part of its course (Fig. 4). The oxidation of the CO complex of R-cyto-c' was also examined in the same
way and gave a markedly biphasic reaction with rate constants of 0.035 sec\(^{-1}\) and 0.0015 sec\(^{-1}\) for the fast and slow phases, respectively.

**Oxidation of the CO-Cytochrome cc' Complexes by Ferricyanide**—In treating the reactions of hemoglobin-ligand complexes, it has been assumed generally that the ligand protects the ferrous iron from oxidation by ferricyanide, and that only the free form reacts. Indeed, the rate of reaction of CO-myoglobin with ferricyanide has been used (7) as a means of measuring the velocity of dissociation of CO from the complex. With the cytochromes cc', the situation appears less simple. Although CO protects the protein to some extent, the rate of oxidation in the presence of ferricyanide is much greater than in the presence of oxygen and is proportional to the concentration of ferricyanide used. For example, with C-cyto-c' the rate of oxidation with 15 \(\mu M\) ferricyanide is about 0.1 sec\(^{-1}\), as compared with 0.001 sec\(^{-1}\) for oxidation by 150 \(\mu M\) oxygen. The behavior of R-cyto-c' is quite similar. The reactions are illustrated in Figs. 5 and 6.

Measurements were also made of the rate of ferricyanide-induced oxidation of the free reduced proteins by the flash photo-chemical method. The results are given also in Figs. 5 and 6. The photoflash generated a significant amount of free reduced protein which reacted exceedingly rapidly \((t_1 = 30\) to 50 \(\mu sec\) for 10 \(\mu M\) ferricyanide). When the supply of free protein was exhausted, the rate of reaction reverted to the slower value characteristic of the oxidation of the CO-bound cytochrome cc'. It is noteworthy that the photoflashes induced this rapid reaction in less than half of the total cytochrome-CO complex present at the time when the flash was fired, although, as already discussed, about 75% was believed to have been broken down under the conditions of the experiment. This difference probably was not due to direct competition with CO, as the rate of the reaction with ferricyanide was about 10 times faster than with CO even with C-cyto-c'.

**Reactions of Reduced Cytochromes cc' with Oxygen**—These reactions are complex in the case of both proteins, and depend on the nature of the reducing agent as well as on the nature of the proteins and the concentration of oxygen. For this reason only preliminary observations are presented here.

R-cyto-c' reacts rather slowly with ascorbic acid, about 2 to 3 hours being required for complete reduction. The reaction of the reduced material with oxygen depends on the time interval between the addition of ascorbate and the performance of the experiment. With short times of reduction the reaction is obviously biphasic. However, because of the slow reaction with ascorbate, it is impractical to examine the solutions less than 20 min or so after the addition of the reducer. If the ascorbate cytochrome cc' solution is kept at room temperature \((25^\circ)\) for several hours, the rapid phase increases at the expense of the slow one, and after 4 to 6 hours a rapid monophasic reaction is seen. Some of the characteristics of this rapid reaction have been examined. It was found that (a) the oxidation of the cytochrome cc' appears to be preceded by a short lag phase of the order of 5 msec; (b) the rate of the oxidation reaction is independent of oxygen concentration down to oxygen concentrations of the order of 5 \(\times 10^{-4}\) M; (c) the difference spectrum obtained from the kinetic experiments agrees well with the static difference spectrum (Fig. 7), and the course of the absorbance change with time is independent of the wave length of the measuring light.

These effects of ascorbate do not require that the protein be re-
duced by ascorbate; they develop in just the same way if the pigment is reduced with minimal dithionite and ascorbate then is added to the solution. They do not develop readily at low temperatures, and no change in behavior was observed when a dithionite-reduced solution was kept overnight at 2° in the presence of ascorbate.

When R-cyto-c' was reduced with minimal dithionite, the product showed a small initial rapid reaction with oxygen which did not change significantly in extent on storage of the solution at room temperature for up to 6 hours. The rate of the main reaction was approximately proportional to oxygen concentration and gave a second order rate constant of $2 \times 10^{-4} M^{-1} s^{-1}$ at 26°, for oxygen concentrations between 600 $\mu M$ and 120 $\mu M$. This result is illustrated in Fig. 8. The rate observed for the main reaction agrees, within an order of magnitude, with an earlier finding that the half time for autoxidation of partially reduced R-cyto-c' could be estimated as approximately 3 sec, when hydrogen and palladium were used as the reducing system (6).

In view of the complications encountered in studying the reaction of R-cyto-c' with oxygen, experiments were performed which established that oxidation was associated with the loss of the power to bind CO. The reduced protein was mixed simultaneously with oxygen and carbon monoxide, and the resulting reaction was followed at 408 $\mu M$ (isosbestic for the change (reduced minus oxidized)) and at 424 $\mu M$ (isosbestic for the change (reduced minus CO complex)). It was found that the CO combination reaction was small in extent and ceased when the oxidation reaction was complete.

**FIG. 7.** The difference spectrum (ferro- minus ferri-) for R-cyto-c'. The continuous line is taken from the data of Bartsch (10); the points represent the results of stopped flow kinetic experiments at different wave lengths. The results have been normalised to bring the point at 435 $\mu M$ onto the line. The stopped flow experiments were carried out with 7.2 $\mu M$ pigment reduced with 1 $mm$ ascorbate in 0.1 $M$ phosphate buffer, pH 7.4; the mixture was allowed to stand for 6 hours before the experiment was performed. The optical path was 2 cm, and the temperature, 20°.

**FIG. 8.** The reaction of the R-cyto-c' with oxygen. The pigment was reduced with minimal dithionite and mixed with buffer equilibrated with 240 $\mu M$ O$_2$ (A) and with 1.2 mM O$_2$ (B). The ordinate is the difference (absorbance at time $t$ minus absorbance 5 min after mixing) measured at 426 $\mu M$. The concentration of pigment was 1 $\mu M$ before mixing (b.m.), in 0.1 $M$ phosphate buffer, pH 7.4; optical path, 2 cm; 26°.

**FIG. 9.** The reaction of C-cyto-c' with oxygen. A, reaction of pigment reduced with minimal dithionite when mixed with buffer containing 1.2 mM, 240 $\mu M$, 60 $\mu M$, and 30 $\mu M$ O$_2$. The course of the reaction was the same, within experimental error, with all these concentrations. The ordinate is the difference (absorbance at time $t$ minus absorbance after 1 min) measured at 429 $\mu M$ pigment concentration, 1 $\mu M$ before mixing, in 0.1 $M$ phosphate buffer, pH 7.4; optical path, 2 cm; 26°. B, reaction with oxygen measured at 424 $\mu M$ by the flash photochemical method with the CO pigment at 1.5 $\mu M$ reduced with minimal dithionite in equilibrium with pCO of 25 mm mixed with an equal volume of 0.1 $M$ phosphate buffer, pH 7.4, in equilibrium with 1 atm O$_2$ before firing the flash. The reaction was followed in an 8-cm light path at 424 $\mu M$ (isosbestic for the reduced and CO complexes of the pigment at 26°).

The reactions of C-cyto-c' with oxygen could not be studied in material reduced with ascorbate, because the rate of reduction was quite similar to the rate of oxidation; the effects observed corresponded to the approach to, and departure from, a steady state. When the protein was reduced with minimal dithionite, the rate of reaction was independent of oxygen concentration over the range from 600 $\mu M$ to 150 $\mu M$. The reaction was markedly biphasic, as shown in Fig. 9.

The reaction of the reduced forms of the cytochromes c' with oxygen was further studied by the flash method. A solution of the carbon monoxide complex of the protein was mixed with oxygen, and the mixture was placed in the observation tube of the flash apparatus. On firing the flash, the oxidation reaction was observed at 424 $\mu M$ (isosbestic for the change (reduced minus CO complex)). With C-cyto-c' a rapid reaction was observed. The
set-l with 120 PM oxygen. The amplitude of the change was small, however, and corresponded to the oxidation of rather small points indicated, a flash discharge of 710 joules was fired at the solution. The observations were made with an 8-cm optical path at 26°.

The observations were made with an 8-cm optical path at 26° in 0.1 M phosphate buffer, pH 7.4. The reactions were followed at 432 rnp in a Bausch and Lomb small grating monochromator together with an interference filter to protect the observing photomultiplier from the photolysis light.

Photodissociation of cytochrome c' - CO complexes by steady illumination

The effect of illumination by unfiltered light from a tungsten filament on the equilibrium between 3 PM C-cyto-c' and 4 PM R-cyto-c' with CO is shown. The experiments were performed at 26° in 0.1 M phosphate buffer, pH 7.4. The reactions were followed at 432 mnp in a Bausch and Lomb small grating monochromator together with an interference filter to protect the observing photomultiplier from the photolysis light.

The methods used are given in the text. The combination order constants. The value for the R. rubrum compound (by flow), and so are presented as first order constants. The value for the R. rubrum compound (by flow) is the rate for the approach to equilibrium after mixing.

The methods used are given in the text. The combination rates for CO were independent of CO concentration except for the Chromatium pigment (by flow), and so are presented as first order constants. The value for the R. rubrum compound (by flow) is the rate for the approach to equilibrium after mixing.

Table II

<table>
<thead>
<tr>
<th>Material</th>
<th>CO dissociation</th>
<th>CO association</th>
<th>Disassociation in light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatium</td>
<td>sec⁻¹</td>
<td>sec⁻¹</td>
<td>%</td>
</tr>
<tr>
<td>150</td>
<td>0.17</td>
<td>0.14</td>
<td>52</td>
</tr>
<tr>
<td>340</td>
<td>0.25</td>
<td>0.25</td>
<td>35</td>
</tr>
<tr>
<td>730</td>
<td>0.45</td>
<td>0.45</td>
<td>14</td>
</tr>
<tr>
<td>730</td>
<td>0.5</td>
<td>0.052</td>
<td>80</td>
</tr>
</tbody>
</table>

Table III

Summary of rate constants for reactions with CO

The methods used are given in the text. The combination rates for CO were independent of CO concentration except for the Chromatium pigment (by flow), and so are presented as first order constants. The value for the R. rubrum compound (by flow) is the rate for the approach to equilibrium after mixing.

The results, although preliminary, indicate clearly that a working hypothesis to comprehend the behavior of both proteins cannot be based on a simple, one-step equilibrium of the type

\[
\text{Heme-CO} \xrightarrow{k_1} \text{Heme} + \text{CO} \quad (1)
\]

which is so often found adequate to rationalize the ligand-binding reactions of other "unsaturated" heme proteins. It is necessary to cite only a few of our findings in support of this conclusion. (a) Whereas the rate of combination of R-cyto-c' with CO is independent of CO concentration, C-cyto-c' combination with CO depends on the CO concentration. (b) In photodissociation under steady illumination of CO - C-cyto-c', the rate constants both for association and dissociation increase with CO concentration and run parallel to one another. (c) Flash dissociation of either CO-protein complex in the presence of oxygen is not followed by the expected oxidation and consequent loss of photo-
The kinetic characteristics of CO binding by both cytochromes $cc'$ can be rationalized by a scheme based on a two-step process, which may be written

$$
\text{Heme-CO} \xrightarrow{k_1 - k_3} \text{heme} \cdots \text{CO} \xrightarrow{k_2 - k_4} \text{heme + CO} \quad (2)
$$

I II III

The crucial feature of this formulation is that there is an association of ligand with protein, governed by the equilibrium $II \leftrightarrow III$, which does not involve a spectrophotometric change, such a change being assigned wholly to the equilibrium $I \leftrightarrow III$. Only the constant $k_1$ is assumed responsive to illumination, whereas the constants $k_2$, $k_3$, and $k_4$ are unaffected. Because these experiments are concerned only with kinetic analysis, the chemical nature of the intermediate, $II$, is irrelevant.

Assume that $x$, $y$, and $z$ are the mole fractions of States I, II, and III, respectively, so that $x + y + z = 1$. Then $x$ is the proportional saturation measured spectrophotometrically, and $(x + y)$ is the proportional saturation which could, in principle, be determined by gasometry, if it were assumed that compound $z$ contains only 1 molecule of CO per heme.

It follows that

$$
\frac{1}{x} = 1 + \frac{k_1}{k_3} \left[ 1 + \frac{k_2}{k_4[k(CO)]} \right] \quad (3)
$$

and

$$
\frac{1}{x + y} = 1 + \frac{k_1k_3}{([(k_1 + k_2)k_4[C(CO)]} \quad (4)
$$

where $[C(CO)]$ is the molar concentration of carbon monoxide. Note that, in a two-step process of this kind, spectrophotometric saturation can never be attained, the value approaching the limit $[1 + (k_1/k_3)]$ as the concentration of CO becomes infinite.

On the other hand, the gasometric saturation as defined in Equation 4 does approach 100% at infinite CO concentration, provided that only 2 molecules of CO are bound per mole of protein, or 1 molecule per mol of heme. It is possible, however, that Compound II may contain 2 or more molecules of CO per heme. In this case gasometric saturation approaches a limiting value determined by the stoichiometry and by the mechanism assumed for the reaction.

Rough estimates of the values of the four constants can be made in the following manner for the C-cyto-$c'$ reaction. $k_1$ is the spontaneous dissociation rate constant which increases on illumination. It is given by the stopped flow experiments (Fig. 4) as $0.001$ sec$^{-1}$, which increases on illumination (Table II) to 0.5 sec$^{-1}$. $k_2$, as discussed later, may be identified with the lesser of the two rate constants for recombination found in the flash photolysis experiments (Fig. 2); its value is 600 sec$^{-1}$. $k_3$ is related to $k_1$ by Equation 3, the latter being the second order recombination constant, $2.6 \times 10^6$ sec$^{-1}$, determined in the stopped flow experiments (see "Combination with CO as Observed in Stopped Flow Experiments"). An accurate value for the proportional saturation at any concentration of CO is not known from our present data, but a reasonable estimate places the half-saturation value at a CO concentration of approximately $2 \times 10^{-3}$ M. Hence, from Equation 3, we can assign the value of about 60 sec$^{-1}$ to $k_3$.

In the case of R-cyto-$c'$, we find $k_1 = 9 \times 10^2$ sec$^{-1}$ (Fig. 3) and $k_2 = 1 - 1.5 \times 10^2$ sec$^{-1}$ (see section referred to above). We cannot state separate values for $k_3$ and $k_4$, but the ratio $k_3/k_4$ can be specified from the knowledge that the CO concentration for half-saturation is 400 μM (Fig. 1). Hence, $k_3/k_4 = 7 \times 10^{-4}$ M.

The first advantage of the two-step scheme is a simple rationalization of the variant behavior in recombination reactions of the two proteins. When the rate is limited by $k_2$, rather than $k_4$, the recombination can be expected to be independent of the ambient CO concentration, as appears to be the case for C-cyto-$c'$ in its recombination reaction after photolysis. On the other hand, when $k_4$ governs the over-all rate, dependence on CO concentration can be expected, and is observed in the stopped flow experiments when CO in the medium reacts with protein.

R-cyto-$c'$ recombination reactions appear to require that the CO concentration-invariant rate observed in the stopped flow experiments be identified with $k_2$. The biphasic character of the reactions of both proteins after photolysis will be discussed below.

The photoinduced dissociations on continuous illumination can be understood by noting that the effect of illumination in the case of R-cyto-$c'$ is to produce a relatively large concentration of Species II compared to Species III ($k_2/k_4 = 7 \times 10^{-4}$) and a small concentration of Species II in the case of C-cyto-$c'$ ($k_3/k_4 = 2.3 \times 10^{-3}$). Thus, when the R-cyto-$c'$-CO complex is illuminated, the result is an accumulation of Species II with the CO still associated with the protein. We expect, then, that the rate and extent of dissociation should depend on light intensity.

The rate of recombination, however, should be independent of extent of dissociation and also of ambient CO concentration. This final point could not be experimentally verified because of the low affinity of the home groups for CO.

By contrast, Species II is always a minority species in C-cyto-$c'$. Here the effect of illumination is to populate mostly Species III. Thus, we expect that the rate and extent of dissociation should be influenced by ambient CO concentration but not by light intensity. The demonstration that the association and dissociation rates should parallel each other and in fact nearly equal each other can be appreciated on a qualitative basis but will require computer analysis for quantitative determination. It is evident that the pseudo-first order rate constants for the approach to both light and dark equilibrium positions increase with CO concentration in the expected manner.

In spontaneous dissociation experiments, the rate-limiting step for both proteins may be identified with $k_1$, so that dissociation in the light does not occur at the same rate as in the dark.

The biphasic character of the recombination reactions after photolysis (Fig. 2; see also "Combination with CO as Observed in Stopped Flow Experiments") poses questions which can be resolved completely only by more detailed knowledge of structural factors. However, the existence of a rapid initial reaction appears to require only that the immediate product of photolysis be an excited form of the reduced protein which can react with CO more rapidly than can the normal form. Such a phenomenon has been noted previously in the case of hemoglobin (11). Hence, it appears reasonable to assign $k_4$ to the lesser of the two recombination constants in the reaction System I $\equiv$ II, as we have done above.

To extend further the scheme presented, we may direct attention to the remarkable results obtained in experiments such as
those depicted in Fig. 10, in which flash photolysis of CO-R-
cyto-c' fails to have an appreciable effect on oxidation by the
ambient oxygen, and in which almost immediate re-forma-
tion of the complex occurs although one would not expect the freshly
dissociated CO to be available for reaction. It seems that the
CO, although its bond to the heme has been disrupted, nonethe-
less retains a close association with the heme group and is not
lost by diffusion into the medium, as is the case for every other
heme protein known to bind CO. One may suppose that the
equilibrium II ↔ III represents a hindered diffusion away from,
or to, the protein, governed by the constants k8 and k9. More
specifically, one may suppose that the protein structure envelops
both heme groups so that they are in a relatively empty region
bound only at one extraplanar coordinate position, the other
position being open or perhaps bound to water. Ligands would
have limited accessibility in such a structure as has been sug-
gested in previous researches (1) to explain the fact that cyto-
chromes cc' bind only CO and NO among the great number of
ligands which usually react with unsaturated heme proteins.
The picture which emerges is of both hemes, together with their
ligands, constrained in a "cage" region of the protein. Thus,
photolysis would not remove the ligand CO immediately from
the vicinity of the heme, so that it could react preferentially and
produce results such as that shown in Fig. 10. A quantitative
treatment of the flash dissociation kinetics could be attempted
but seems premature in the present state of our research.

The "cage" hypothesis can be used to explain the relatively
sluggish oxidations by oxygen, compared with the rapid electron
transport noted when the proteins are in the presence of ferri-
cyanide as an oxidant, or dithionite as a reductant. (Despite
the bulkiness of the ferricyanide group, oxidation takes place
with an apparent second order constant of approximately 1 ×
109 M⁻¹ sec⁻¹.) The fact that reduction by dithionite is rapid,
even in the presence of oxygen, has been exploited in some recent
researches (14) on the possible role of the cytochromes cc' as
oxidases.

The phenomenon of rapid electron transport helps to explain
the variable behavior of cytochrome cc' with oxygen and, in par-
icular, the complex reactions exhibited in the presence of excess
of reductant. Thus, in R-cyto-c', the appearance of a rapid oxida-
tion by oxygen after prolonged incubation in excess ascorbate
may be the result of production of ascorbate degradation prod-
acts which mediate the protein reaction with oxygen, rather than
of changes in the protein.

In summary, the ligand-binding character of cytochromes cc'
determined by a balance between affinity of the heme group
for the ligand, as governed by the constants k1 and k2, and a
hindered diffusion of ligand to the reaction site, as determined by
the constants k8 and k9.

The difference in behavior between the two proteins can be ex-
plained by assigning suitable values to these kinetic constants.
The equilibria favor a relatively large amount of Intermediate
II for the R. rubrum protein and a relatively low amount of
Intermediate II for the Chromatium protein under most experi-
mental conditions.

REFERENCES
    Falk, and R. Lemberg (Editors), Haematin enzymes,
    (1963).
    (1958).
    (1938).
8. Gibson, Q. H., in G. Porter (Editor), Progress in reaction
    (Editors), Bacterial photosynthesis, Antioch Press, Yellow
13. Gibson, Q. H., Greenwood, C., Wharton, D. C., and Pal-
14. Chance, B., Bonio, T., Kamen, M. D., and Taniguchi, S.,
Kinetic Analysis of the Reaction of Cytochrome cc' with Carbon Monoxide
Quentin H. Gibson and Martin D. Kamen


Access the most updated version of this article at http://www.jbc.org/content/241/9/1969

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/241/9/1969.full.html#ref-list-1