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# Properties of Modified Cytochromes

## II. LIGAND BINDING TO REDUCED CARBOXYMETHYL CYTOCHROME *c**

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### SUMMARY

**Reduced ($Fe^{2+}$) carboxymethylated cytochrome *c* (Cm-cytochrome *c*) reversibly binds ligands of ferrous iron, *e.g.* CO, cyanide, ethyl isocyanide, and oxygen, at neutral pH.**

**Titrations of the reduced protein with CO show that over the pH range 6 to 9.5 the stoichiometry of binding is one, and that the spectrum of the Cm-cytochrome *c* complex with CO is practically pH-independent.**

**The kinetics of CO binding of Cm-cytochrome *c* have been studied as a function of pH. At low pH values the binding process as observed by stopped flow or photolysis techniques conforms to a simple bimolecular process ($l' = 1.6 \times 10^6\ M^{-1}\ s^{-1}$), whereas at high pH the process, although remaining simple, it is ~100-fold slower. At intermediate pH values, the binding of CO occurs in a biphasic reaction, the proportions of the fast and slow phases depending on the pH, on the monitoring wave length, and, in the case of photolytic experiments, on the CO concentration. The dissociation rate constants of CO from Cm-cytochrome *c* were measured by replacement with oxygen or ethyl isocyanide.**

**A model is proposed in which the acid and alkaline conformers of the ligand-free protein have different ligand-binding properties. It is suggested that there is a pH-dependent transition in the CO adduct of Cm-cytochrome *c* and the pK of this transition is reported. The model is consistent with simple thermodynamic considerations.**

**Binding between ferrous Cm-cytochrome *c* and $O_2$ has been examined. At pH 6, a stable $O_2$ complex, similar to myoglobin, is formed, whereas at pH 9 $O_2$ rapidly oxidized the heme iron.**

It has been reported that cytochrome *c* carboxymethylated at methionine 80, in contrast to the native molecule, is capable of binding ligands of the ferrous iron at neutral pH (2). However, to date, no kinetic studies on these binding processes have been undertaken. In the previous paper (3), it was shown that reduced carboxymethylated cytochrome *c* (Cm-cytochrome *c*) in the absence of any extrinsic heme ligand undergoes a proton linked conformational change; it was suggested that the ε-amino group of lysine 79 fills the vacant 6th coordination position of the iron at alkaline pH. In the light of this finding it might be expected that the binding of ligands to the reduced protein could well exhibit a marked pH dependence reflecting binding to the acid or alkaline conformers of the protein.

The present work investigates the ligand-binding properties of reduced Cm-cytochrome *c* over a wide pH range using rapid mixing and flash photolysis techniques. The bulk of the experimental work concerns binding of carbon monoxide; however, data on the reactions of Cm-cytochrome *c* with other ligands is also reported. Such an investigation appears of interest for the light it throws upon ligand-linked conformational processes occurring in the modified cytochrome *c* which may, in some respect, be considered as a model for similar processes occurring in more complex hemeproteins. In addition comparison with similar reactions taking place in other monomeric hemeproteins may yield useful information pertaining to regulation of the heme reactivity by the local protein environment.

### MATERIALS AND METHODS

Horse heart cytochrome *c* was purchased from Sigma Chemical Co. (type III) and used without further purification. Di-methionyl-carboxymethyl cytochrome *c* was prepared following the method of Schejter and George and has been reported in detail elsewhere (3, 4). Disc acrylamide gel electrophoresis revealed the presence of six components in the preparation. Gel filtration using a calibrated Sephadex G-100 column, however, clearly showed that something in excess of 95% of the modified protein was in the monomeric form, and thus the components seen on acrylamide gel electrophoresis differed in charge only. In spite of the chemical heterogeneity of the preparation, it appeared that the functional properties of the molecule, described below, were held in common by all the distinct species. This conclusion is supported by several lines of evidence, the strongest of which is that the components of the mixture, separated by isoelectric focusing, exhibited the same ligand-binding properties as did the mixture as a whole. In addition, if carboxymethylation was allowed to proceed in the absence of cyanide, the product of the reaction did not bind CO at neutral pH. Acrylamide gel electrophoresis of this material, however, showed that six bands were

* Part of this work has been presented in a preliminary form at the Metalloenzymes Conference, Oxford, September 1972 (1).

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again present, although not exactly in the same position as those observed when the reaction was carried out in the presence of cyanide. Assuming that in the absence of cyanide the heme crevice is unmodified, the spectral and ligand-binding properties being the same as for the native cytochrome, it would appear that the other products of carboxymethylation are species in which residues not directly involved in the heme site are modified.

Reduced Cm-cytochrome *c* was prepared by adding a slight excess of solid sodium dithionite to the oxidized material. Oxygenated Cm-cytochrome *c* was prepared by passing dithionite-reduced Cm-cytochrome *c* through a Sephadex G-25 column equilibrated with an aerated buffer at the required pH. All extinction coefficients were calculated with reference to the $\alpha$-band of ferrous cytochrome *c* which, at pH 10, was taken to have a value of $\epsilon_{mM} = 28.1$ (2).

Degassed distilled water was equilibrated at 20° with CO gas at 1 atm, the resulting solution being $10^{-3}$ M (5). Carbon monoxide solutions of the desired concentration were made from this stock solution by dilution.

Static spectra were obtained on a Cary 14 spectrophotometer. The pH values of all solutions were determined immediately after experimentation with a Radiometer model 4 pH meter fitted with a type E5021a electrode capable of measuring the pH of small volumes.

Stopped flow measurements were carried out using a Durrum instrument.

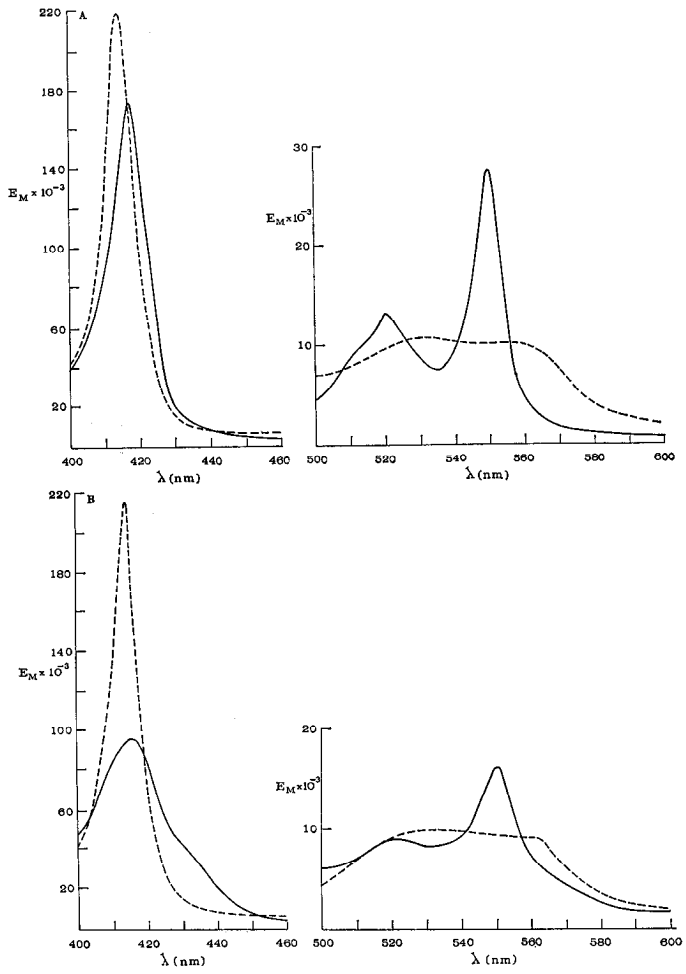



FIG. 1. Spectra of ferrous Cm-cytochrome *c* (——) and its CO complex (– – –). *A*, in 2% sodium borate buffer, pH 9.2; *B*, in 0.1 M potassium phosphate buffer, pH 6.5.

The procedure and the apparatus employed for the flash photolysis experiments have been described in detail elsewhere (6).

Isoelectric focusing of the products of carboxymethylation was carried out using the LKB apparatus. The ampholine carriers were chosen to give a pH gradient from 7 to 10.

CO titrations were performed by adding carbon monoxide solution of a known concentration to dithionite-reduced Cm-cytochrome *c*. The procedure has been described elsewhere (7).

Warburg manometric experiments were performed using a routine procedure (8). The manometer contained oxygenated Cm-cytochrome *c* in the main compartment and ferricyanide in the side arm. After temperature equilibration, the ferricyanide was poured into the main compartment and the resultant oxygen production was measured. After the reaction was complete, the Cm-cytochrome *c* was found to be entirely in the ferric form. Parallel experiments substituting oxyhemoglobin for oxygenated Cm-cytochrome *c* were conducted as controls.

## RESULTS

### *Reaction with Carbon Monoxide*

As previously reported, Cm-cytochrome *c* in the ferrous state binds CO at neutral pH (2). Fig. 1 reports spectra of the ligand-free and the CO complex of Cm-cytochrome *c* at two pH values. Fig. 2 shows the spectrophotometric titration of reduced Cm-cytochrome *c* with CO at pH 6.0 and it is evident that the titration displays good isosbestic points, which, in the Soret region, were located at 403.5 and 420 nm. Similar titration curves were obtained at pH 9.0 but here the isosbestic points were located at 399 nm and 417 nm.

Under the conditions of the experiments (*i.e.* protein concentration $\sim 5 \times 10^{-6}$ M), the titrations are practically stoichiometric up to high saturation (Fig. 2), the stoichiometry of binding corresponding to 1 CO eq per heme. Two conclusions may be drawn from these experiments: (*a*) only two spectroscopic species are dominant throughout the titration; and (*b*) the affinity

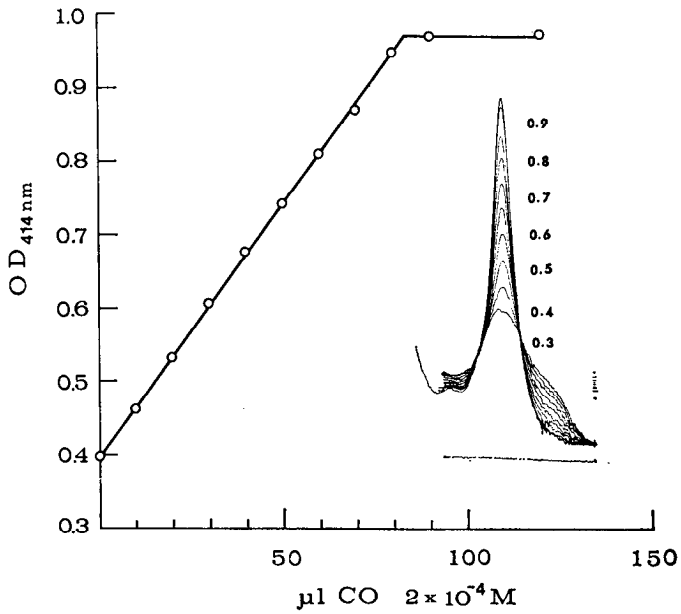



FIG. 2. Carbon monoxide titration of ferrous Cm-cytochrome *c* in 0.1 M potassium phosphate buffer, pH 6.0. The optical density at 414 nm is plotted against the number of microliters of CO solution added. The *inset* shows the spectral changes observed between 390 and 450 nm on making 10-μl additions of $2 \times 10^{-4}$ M CO solution. Protein concentration was 4.4 μM and the cuvette volume 3.65 ml.

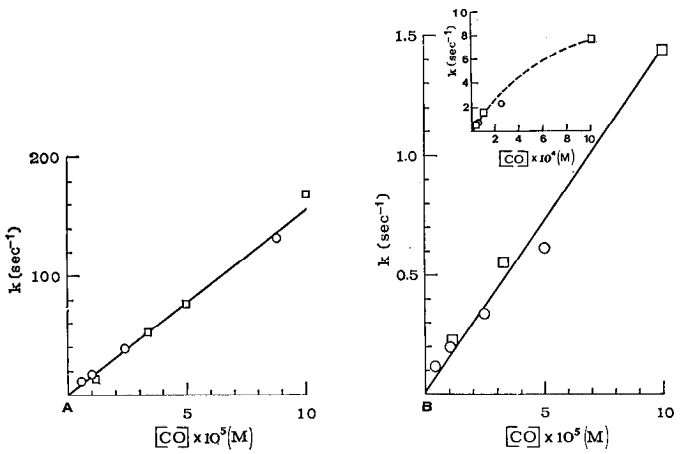



FIG. 3. Pseudo-first order rate constant ($k$) *versus* CO concentration for the combination of CO with ferrous Cm-cytochrome (2.5 $\mu$M). ○, stopped flow; □, flash photolysis. *A*, 0.1 M potassium phosphate buffer, pH 5.9, temperature = 20°; *B*, 0.1 M Tris-chloride buffer, pH 9.1. The *inset* shows the deviation from simple behavior at high CO concentrations, temperature = 20°.

TABLE I

*Kinetic and equilibrium constants for the reaction with CO of reduced Cm-cytochrome c at acid and alkaline pH values*

Conditions: temperature 20°; 0.1 M potassium phosphate buffer at pH 5.9; and 0.05 M sodium borate buffer at pH 9.1.

| | pH 5.9 | pH 9.1 |
|---|---|---|
| $l_{on}$ ($M^{-1}$ $s^{-1}$) | $1.6 \times 10^6$ | $1.4 \times 10^4$ |
| $l_{off}$ ($s^{-1}$) | $1.2 \times 10^{-3}$ | $1.7 \times 10^{-4}$ |
| $L_{eq}$ ($M^{-1}$)[a] | $1.3 \times 10^9$ | $8 \times 10^7$ |

[a] Calculated from the kinetic constants.

constant for the formation of the CO adduct is very high ($K > 10^6$ $M^{-1}$).

*Kinetics of CO Combination at Low pH*—At around pH 6, combination with CO is monophasic both by flow and by flash. The pseudo-first-order rate constant depends linearly on the CO concentration (see Fig. 3*A*), showing a simple bimolecular behavior. Both flow and flash yield the same value for the combination rate constant, which is given in Table I.

The dissociation velocity constant of CO from the complex was determined by replacement method in which $O_2$ was used as a competing ligand for CO. As shown later, the replacement process at low pH is fully reversible; thus a solution of the reduced Cm-cytochrome *c*-CO complex in the presence of a known concentration of both $O_2$ and CO was illuminated by a steady light, thereby shifting the equilibrium in favor of the oxygenated species. Spectroscopic observation after removal of light yields the rate of return to the dark equilibrium. In view of the conditions used (*i.e.* $l'(CO) > k'(O_2)$ the relevant equation is given by:

$$R = l + \frac{kl'[CO]}{k'[O_2]} \qquad (1)$$

where $l$ and $l'$ are the dissociation and combination rate constants for the reaction between Cm-cytochrome *c* and CO, $k$ and $k'$ are the corresponding constants for $O_2$, and $R$ is the observed first-order rate constant (9).

The replacement obeys first order kinetics, and, as shown in Fig. 4, $R$ is a linear function of the ratio [CO]:[$O_2$]. The intercept at [CO]:[$O_2$] = 0 yields the value of the dissociation velocity constant ($l$), which is reported in Table I.

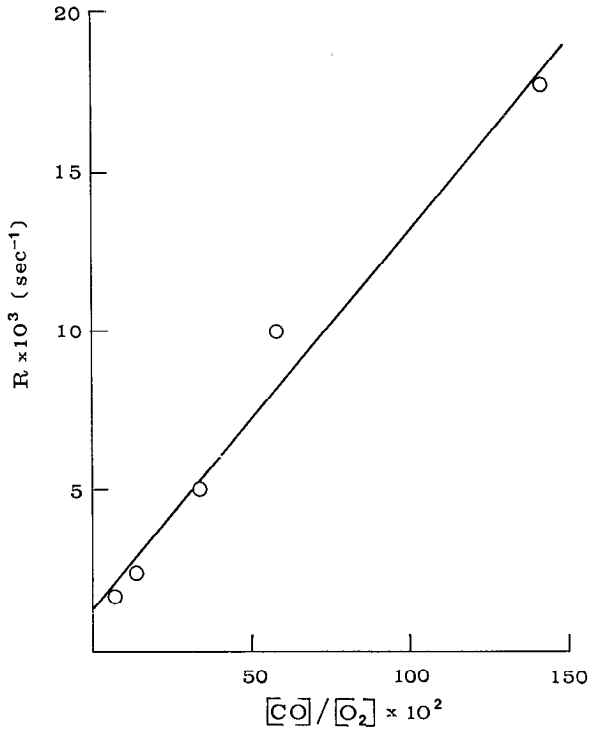



FIG. 4. Rate constant ($R$) for the replacement of $O_2$ by CO on ferrous Cm-cytochrome *c* plotted against [CO]:[$O_2$]; in 0.1 M potassium phosphate buffer, pH 6, 7 $\mu$M protein, temperature = 21°.

*Kinetics of CO Combination at High pH*—At pH >9, the CO binding is again monophasic and second order; the dependence on CO of the observed rate constant is presented in Fig. 3*B*. However, as evident from these data, the bimolecular rate constant at pH 9 is about 100-fold smaller than that at pH ~6 (see Table I). As discussed below, this finding is probably related to the existence of an acid and an alkaline form of the unliganded reduced Cm-cytochrome *c* (2). Obviously the high "on" constant reflects the binding of CO to the acid form, while the lower one reflects a similar process involving the alkaline form. Flash photolysis experiments performed at high CO concentration ($10^{-3}$ M) showed a deviation from simple bimolecular behavior, the apparent second order rate constant being smaller at high CO concentration. The reasons for this may become apparent in the discussion.

In view of the instability of the oxygenated complex of Cm-cytochrome *c* at high pH (see below), the dissociation velocity constant for CO was determined under these conditions by using ethyl isocyanide as the competing ligand. In view of the extremely high affinity for CO, it was necessary to use high concentrations of ethyl isocyanide. The dissociation velocity constant was determined to be $1.7 \times 10^{-4}$ $s^{-1}$ (Table I).

*Kinetics at Intermediate pH Values*—As shown by the experiments reported in Fig. 5, the combination with CO measured by flow is clearly biphasic at $\lambda$ = 414 nm, the proportion of the fast component being much higher at lower pH values. The over-all kinetic difference spectrum at pH 7 corresponds closely to that obtained statically (see Fig. 6). The separation of the progress curve into two components allows one to calculate the relative contribution of the fast and slow species at every wave length and, therefore, to construct two kinetic difference spectra. This is also reported in Fig. 6. The spectral contribution of the two

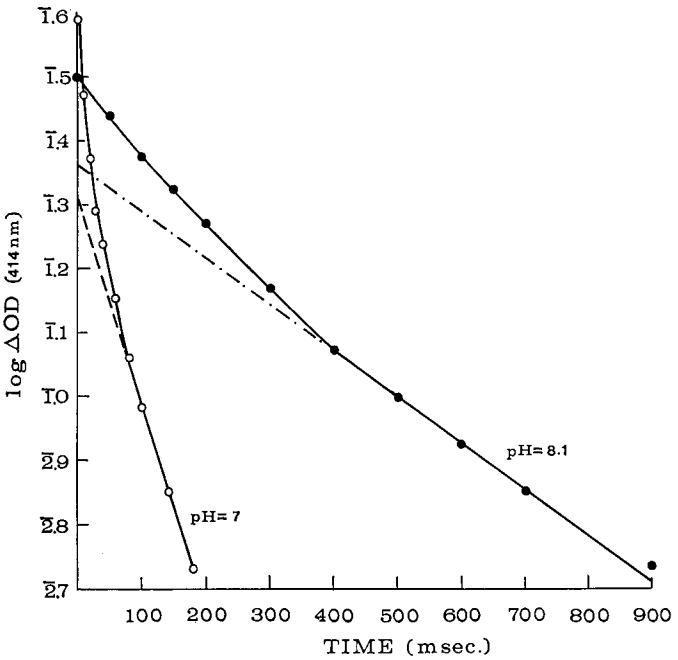



FIG. 5. Kinetics of CO combination with ferrous Cm-cytochrome *c* measured in a stopped flow apparatus. ○, 0.1 M potassium phosphate buffer, pH 7; ●, 0.1 M Tris-chloride buffer, pH 8.1. The *broken lines* represent the slow component's contribution extrapolated back to zero time. λ = 414 nm; 2.5 μM Cm-cytochrome *c*; $5 \times 10^{-5}$ M CO; temperature = 21°.

components at this pH is the same at some wave lengths (*e.g.* 414 nm), while in other regions the two contributions are different or even opposite.

Since, as shown above, the acid and alkaline forms of Cm-cytochrome *c* are characterised by very different values of the CO combination velocity constant, it seems natural to associate the biphasic nature of the progress curves at intermediate pH values with the presence of different amounts of the two species. In fact when monitored at λ = 414 nm, the per cent of slow component observed at any pH in flow experiments corresponds, to a first approximation, to the per cent of the alkaline form. However, since the two forms present in the absence of the ligand are in dynamic equilibrium (3), a strict correlation between per cent of slow component and per cent of alkaline form may only be expected under conditions in which the conformational re-equilibration is slow in comparison with the ligand-binding process, *i.e.* at very high CO concentrations. Similarly, the rates of the two components will correspond to those of the pure forms only when CO binding is much faster than the conformational relaxation.

As shown previously (3), the re-equilibration in the unliganded reduced Cm-cytochrome *c* is pH-dependent. It is therefore reasonable that, at pH 7 (where $1/\tau = 20\ s^{-1}$) (3), the CO dependence of the rate for the fast component (observed in flow experiments) is linear (Fig. 7) and the second order constant for CO binding ($l' = 1.5 \times 10^6\ M^{-1}\ s^{-1}$) is almost identical with that measured at pH 6 for the pure acid form. On the other hand at pH 8, where $1/\tau = 100\ s^{-1}$ (3), the kinetics of the process will be more complex due to the coupling between ligand-binding and conformational change. Of course similar and more serious reservations apply to any comparison of the rate of the slow process seen at intermediate pH values to that obtained for the pure alkaline form. The actual findings concerning the dependence of the rate constant for the slow component on CO concentration are given in Fig. 8.

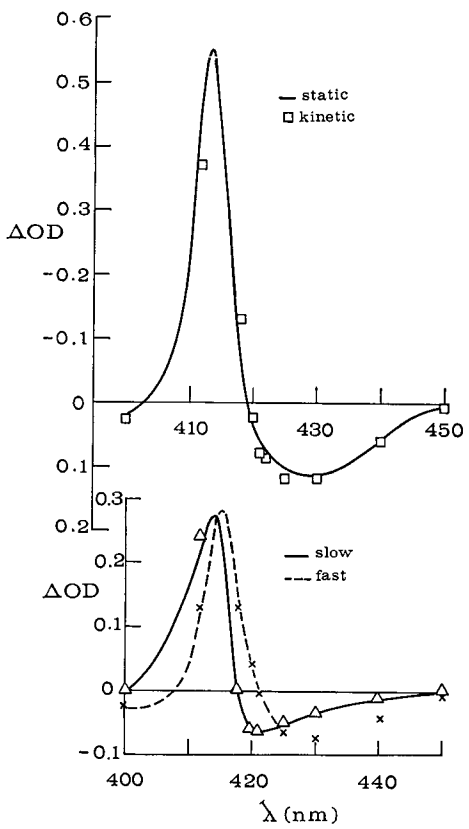



FIG. 6. Difference spectra of Cm-cytochrome *c*-CO minus ferrous Cm-cytochrome *c*. *Top*, over-all difference spectrum: ——, static; □, kinetic, obtained in stopped flow apparatus; *bottom*, separation of the over-all kinetic difference spectrum into two components. The relative Δ O.D. contributions for the fast and the slow components at each wave length have been obtained by analysis of the kinetic records as shown in Fig. 5. △——△, ΔO.D. *versus* λ for the slow kinetic process; ×- - -×, ΔO.D. *versus* λ for the fast kinetic process; 2.5 μM Cm-cytochrome *c*; $2.5 \times 10^{-5}$ M CO; 0.1 M potassium phosphate buffer, pH 7; 2-cm light path; temperature = 20°.

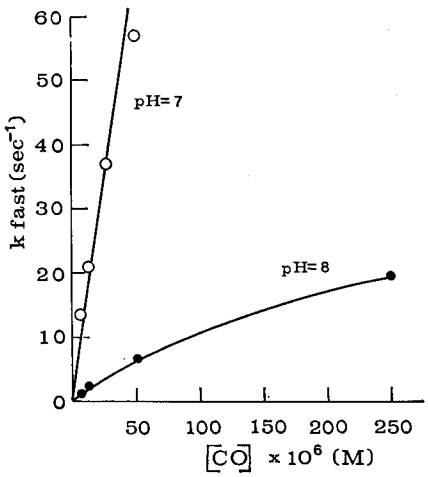



FIG. 7. Pseudo-first order rate constant for the fast CO combination process plotted against CO concentration; 2 μM Cm-cytochrome *c*; temperature = 20°; ○, 0.1 M potassium phosphate buffer, pH 7; ●, 0.1 M Tris-chloride buffer, pH 8.

The time course of CO recombination after flash photolysis also proved to be biphasic both at pH 7 and 8. Monitoring the reaction at different wave lengths, it was found that, at 421 nm, close to the isosbestic point, the optical density change occurs in oppo-

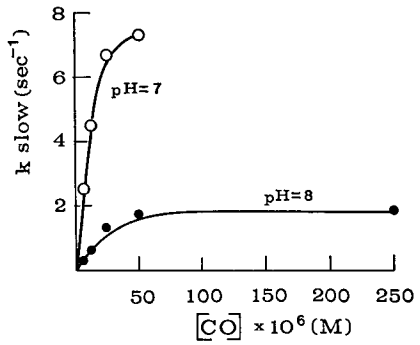



FIG. 8. Pseudo-first order rate constant for the slow CO combination process plotted against CO concentration; $2 \times 10^{-6}$ M Cm-cytochrome *c*; temperature = 20°; ○, 0.1 M potassium phosphate buffer, pH 7; ●, 0.1 Tris-chloride buffer, pH 8.

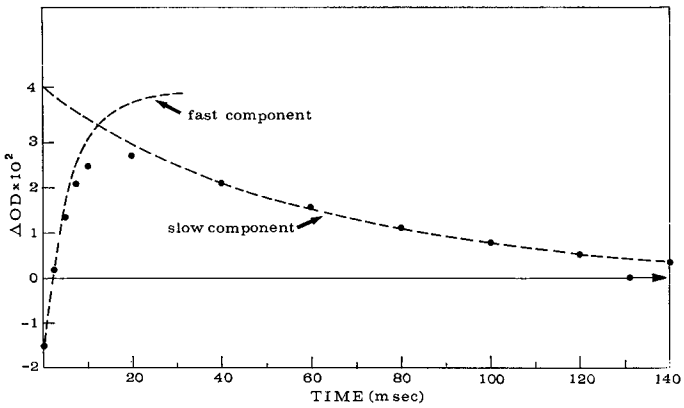



FIG. 9. Progress curves for the combination of CO with Cm-cytochrome *c* after intense photolytic flash. 7 μM Cm-cytochrome *c*; $4.5 \times 10^{-5}$ M CO; 0.1 M potassium phosphate buffer, pH 7.1; 2-cm path length; λ = 421 nm; temperature = 20°; ●, experimental data; – – –, exponential curves.

site directions for the two kinetic components. A typical flash-photolysis experiment at this wave length is depicted in Fig. 9. The progress curve at this, and all of the other wave lengths examined, could be analyzed in terms of two exponential decays. The analysis given in Fig. 9 shows the extent to which the two phases overlap under the conditions used. Similarly to what was observed in the flow experiments, the relative amplitudes of the fast and slow phases are pH-dependent at constant CO concentration. However, over and above this, it was found that the proportion of the fast component increases as the CO concentration is increased. It may be appreciated that this behavior is consistent with the presence of a monomolecular decay involving the ligand-free molecule, a decay which, from previous work, has been shown to involve two conformational states which relax at a pH-dependent rate. Therefore, at a given pH, as the CO concentration is increased, the bimolecular processes become faster and faster; when the CO concentration is high enough, recombination of the photoproduct with the ligand will occur prior to any re-equilibration in the ligand-free form. In the limit of infinite CO concentration, the proportion of the fast and slow components will reflect some distribution of species characteristic of the ligand-bound form. In fact it is shown in Fig. 10 that the ratio $\Delta$ O.D.$_{slow}$:$\Delta$ O.D.$_{fast}$ decreases, and tends to an asymptote as CO $\rightarrow \infty$. In conjunction with the known pH-dependent distribution of forms in the ligand-free Cm-cytochrome *c*, these results allow us to calculate the pH-dependent equilibrium constant for the acid and alkaline forms of the CO

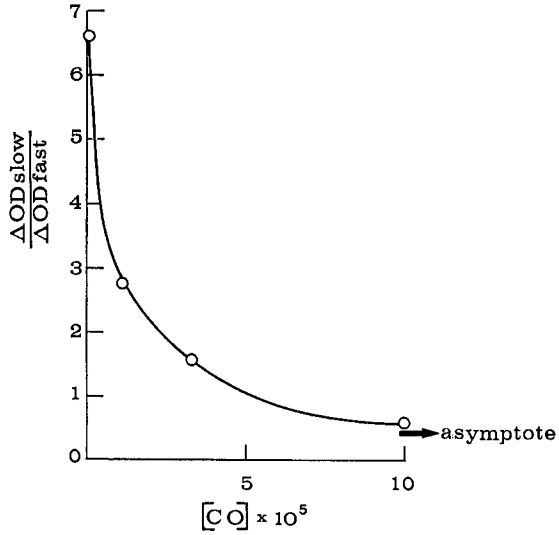



FIG. 10. Ratio of ΔO.D. in the slow kinetic phase to that in the fast kinetic phase seen after photolysis of the Cm-cytochrome *c*-CO complex, here plotted as a function of CO concentration. The data point for CO = 0, *i.e.* where full equilibration in the ligand-free form occurs prior to any combination with CO, was taken from flow data under identical conditions except that the [CO] was high. The proportions of the acid and alkaline forms of the ligand-bound Cm-cytochrome *c* at this pH may be calculated from the value of the ratio at infinite [CO] (asymptote); 0.1 M potassium phosphate buffer, pH 8.0; 2.5 μM Cm-cytochrome *c*; λ = 410 nm; temperature = 20°.

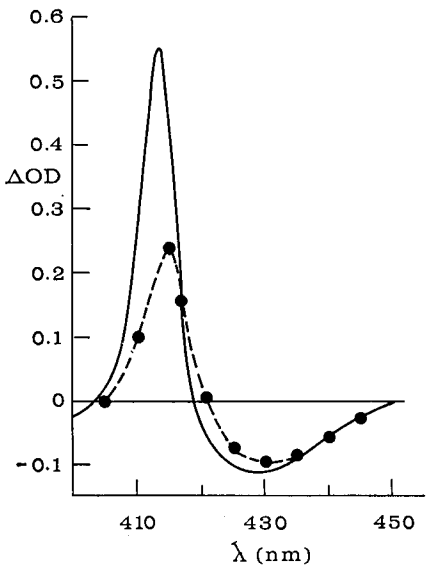



FIG. 11. Difference spectra of Cm-cytochrome *c*-CO minus ferrous Cm-cytochrome *c*; ——, obtained statically; ●– – –●, photochemical difference spectra; 2.5 μM Cm-cytochrome *c* in 0.1 M potassium phosphate buffer, pH 7, equilibrated with $2.5 \times 10^{-5}$ M CO; temperature = 20°; 2-cm path length.

complex. The apparent pK value for the CO form is about 8.3.

Contrary to the flow experiments, the kinetic difference spectrum by flash is obviously different from the static spectrum (Fig. 11). The flash data may also be analyzed into two parts, one of which (the fast component) has great similarities with the fast component observed in flow experiments.

### *Reaction with Oxygen*

Using the procedure given under "Materials and Methods," we have confirmed that in the reduced state Cm-cytochrome *c*

forms a fairly stable complex with $O_2$, which has a characteristic absorption band at 570 nm. Attempts to measure directly the stoichiometry of binding (at pH 7) using a Warburg manometric apparatus (see "Materials and Methods"), gave nonintegral values ranging from 0.4 to 0.6 moles of $O_2$ per mole of iron. It is possible that such low values for the $O_2$:heme ratio reflect the fact that, during the time from the preparation of the oxyenated complex on the column to introducing the complex into a Warburg flask (approximately 10 min), substantial amounts of the complex had decayed to the oxidised form.

Attempts to check the stoichiometry of binding by titrating the dithionite-reduced Cm-cytochrome *c* with air equilibrated buffer, however, always led to the formation of oxidized and not oxygenated Cm-cytochrome *c*. This was the case even at pH 6.0, where it was found that the oxygenated complex prepared by the column method decayed only ~10% on standing 30 min at room temperature.

At neutral pH, or below, the complete reversibility of the $O_2$ complex of Cm-cytochrome *c* was tested, taking advantage of the the competition between $O_2$ and CO and of the photosensitivity of the CO complex. Thus, a solution of reduced Cm-cytochrome *c* in the presence of both ligands is in the CO form when the CO concentration is equal or somewhat higher than the $O_2$ concentration. Upon illumination of the solution, the equilibrium is shifted in favor of the oxygenated derivative in view of the photosensitivity of the Cm-cytochrome *c*-CO complex. The approach to the steady state situation in the light and the return to the dark equilibrium upon termination of illumination correspond to a first order process in both directions, as expected in view of the high concentration of the two competing ligands which maintains the system fully saturated at all times. The rate of approach to the steady state in the light is, of course, faster than the rate of return due to the contribution of the photochemical dissociation constant to the over-all relaxation time (10). The cycle could be repeated at will with complete reproducibility. Fig. 12 reports the difference spectrum between the CO and $O_2$ forms obtained by repeating photochemical experiments at various wave lengths. It is apparent that this is the same as the difference spectrum obtained statically, a finding which shows that the $O_2$ complex has a significance stability under these conditions.

In view of the stability of the $O_2$ adduct, it was possible to measure the dissociation velocity constant for $O_2$ using the dithionite method. The measured rate constant, which is independent of dithionite concentration, was found to be 1.7 $s^{-1}$ at pH 6 and 20°. Using this value and the data in Fig. 3, it is possible to obtain an estimate of the $O_2$ combination velocity constant applying Equation 1. Table II reports the relevant constants for $O_2$ at pH 6.

We attempted to perform similar experiments with $O_2$ at pH 9 to 10. However under these conditions, after photolysis of the CO complex, the Cm-cytochrome *c* undergoes, in the presence of $O_2$, an irreversible change, being presumably transformed into the oxidized species within the dead time of the flash apparatus (~100 μs). This point deserves further investigation.

### *Reaction with Cyanide and Isocyanides*

We have shown that reduced Cm-cytochrome *c* at neutral pH binds both cyanide and ethyl isocyanide. This emphasizes the broad similarities between this modified cytochrome *c* and other hemeproteins, such as myoglobin or peroxidase (11, 12). In addition it was shown that both complexes are photodissociable, the process being fully reversible.

Although a complete characterization of the reactivity towards these ligands remains to be done, it was shown that the process observed after flash photolysis reflects a bimolecular combination of the ligands to the protein. Table II gives the combination velocity constant for ethyl isocyanide at pH 6. In addition, it was seen that the kinetics of the reaction is pH-dependent, the combination rate constant being smaller at high pH (~$10^3$ $M^{-1}$ $s^{-1}$ at pH 9.2). This shows that the behavior observed for CO is not a unique feature of this ligand but is a general feature of the protein.

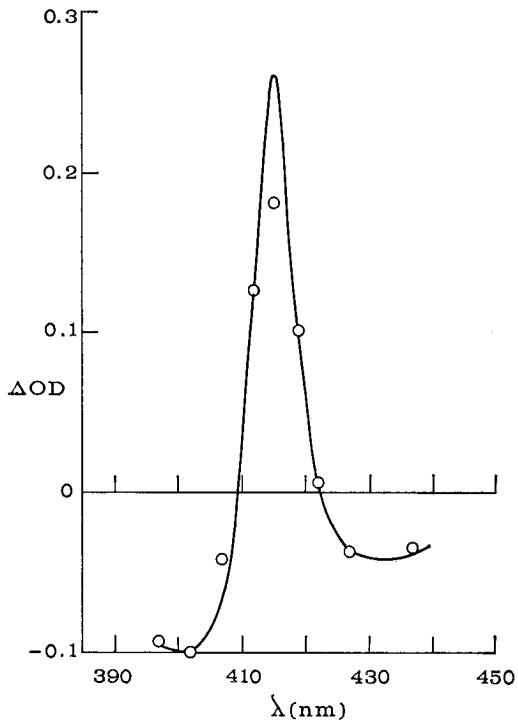



FIG. 12. Difference spectrum of Cm-cytochrome *c*-CO minus Cm-cytochrome *c*-$O_2$; ——, static difference spectrum; ○, photochemical difference spectrum obtained from replacement experiments under the following conditions: 0.1 M potassium phosphate buffer, pH 7; 2.7 × $10^{-4}$ M $O_2$; $10^{-3}$ M CO; path length, 1 cm; λ = 412 nm; temperature = 20°.

TABLE II

*Constants for the reaction of reduced Cm-cytochrome c with oxygen and ethyl isocyanide (EIC)*

Conditions: Temperature 20°; pH 6, 0.1 M potassium phosphate buffer.

| | $O_2$ | EIC |
|---|---|---|
| $k_{on}$ ($M^{-1}$ $s^{-1}$) | $10^8$ | 6.4 × $10^5$ |
| $k_{off}$ ($s^{-1}$) | 1.7 | |
| $K_{eq}$ ($M^{-1}$)[a] | 0.6 × $10^8$ | |

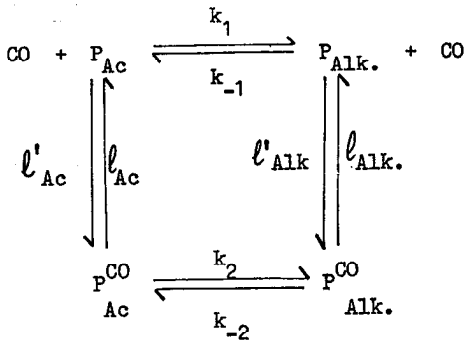
[a] Calculated from the kinetic constants.

## DISCUSSION

A certain body of data using different approaches indicates that the reduced form of Cm-cytochrome *c* exists in at least two different conformational states in pH-dependent equilibrium. The static and kinetic features of this pH linked conformational transition have been investigated previously (3). Thus it appears that in the reduced state Cm-cytochrome *c* exists essentially in an acid conformation at pH 6 and in an alkaline one at

pH >9, since the apparent pK of the transition is 7.16 at 20°. On the basis of spectral properties (see Fig. 1) (2), as well of NMR measurements (13), the acid conformation corresponds to a species in which the high spin iron is pentacoordinated and presumably out of the porphyrin plane, similarly to what is known for myoglobin or hemoglobin (9). On the other hand, the alkaline conformation appears to correspond to a state in which the iron is hexacoordinated and low spin.

In this paper we have shown that, correspondingly, the ligand-binding properties of reduced Cm-cytochrome *c* vary greatly with pH, and that the two states are characterized by different affinity and rate constants for CO as well as for other heme ligands. The simplest scheme which incorporates both the intramolecular conformational changes and the ligand-binding properties of both states is represented below:




The CO binding is indicated by the reactions along the vertical axis, while the horizontal axis indicates the pH-dependent conformational transitions. The ability of this scheme to describe quantitatively the experimental results may now be examined.

The scheme is obviously consistent with the experimental findings at extremes of pH. When the pH is low enough (pH ~6), the only form present in significant amounts is the acid conformation both in the ligand-free and ligand-bound derivatives; accordingly, the kinetics of the reaction with CO under these conditions conforms to a simple process. Therefore the determination of the on and off rates yields directly $l'_{Ac}$ and $l_{Ac}$, respectively (see Table I). Likewise, when the pH is high enough (pH ~9.5) the behavior characteristic of the alkaline form should be observable (see Table I).

On the basis of thermodynamic considerations, there must be a balance in free energy along any of the possible paths around the square depicted in the scheme. Thus the difference in free energy of binding of CO (or any other ligand) between the acid and alkaline forms should balance the difference in free energy for the pH-linked conformational transition in the ligand-free and ligand-bound forms. It follows that the apparent pK of the transition in the ligand-bound species should be about 1.2 pH unit higher than the value of 7.15 found for the ligand-free form since the difference in binding constant for CO is a factor of about 16 in favor of the acid conformation (Table I). As given under "Results," an accurate measure of the pH-dependent conformational transition in the CO form is not feasible by static methods in view of the very small (but still significant) spectral changes involved; it was, however, possible to measure this pK indirectly. Thus, the relative proportion of the acid and alkaline deoxy species after a brief, intense, flash will reflect the distribution characteristic of the CO form when the CO concentration is high enough to make the ligand-binding steps much faster than any conformational re-equilibration. The value obtained by this method (pK ~8.3) is indeed consistent, within the experimental errors, with the simple thermodynamic considerations reported above.

At intermediate pH values, the observed kinetic pattern and the associated spectral changes will of course depend on the relative speed of the processes occurring along the various reaction paths. A complete analysis may be accomplished by computer simulation of the scheme reported above, after inserting the proper values of rate and equilibrium constants. Experimental values are available for all equilibrium constants and for all rate constants except those involved in the conformational transition of the ligand-bound form (*i.e.* $k_2$ and $k_{-2}$). Attempts to determine these rates, as well as their pH-dependence, are in progress and computations will be reported in a subsequent publication.

In addition, it may be noticed that no direct information (of the type reported for the ligand-free form) is available about the difference in conformation between the acid and alkaline forms of the CO derivative. Experiments to elucidate this point are highly desirable.

It was shown in the previous paper (3) that the conformational transition in the deoxy form may be described with a minimum of two steps reflecting, respectively, the protonation of a group and a conformational change linked with it. Therefore, a complete treatment of all possible reaction coordinates should involve a more complex formulation, which might be represented by a "cube." Two of the faces would describe the pH-dependent processes in the ligand-bound and ligand-free forms, while the four edges connecting these two faces would represent the ligand binding processes. In spite of the relative simplicity of the system, a complete quantitative treatment of all the events required by a cube is presently out of reach. It is self evident, however, how elucidation of the details of the reaction mechanism in Cm-cytochrome *c* may be relevant to the understanding of the behavior of more complex molecules (such as hemoglobin) involving allosteric phenomena.

One interesting aspect of the work presented above is that Cm-cytochrome *c* in its reaction with ligands displays a variety of behavior depending on pH. At pH 6.0 the reduced unliganded Cm-cytochrome *c* exhibits an absorption spectrum which is similar to myoglobin for which the iron is out of the porphyrin plane (9). At this pH, the modified cytochrome *c* resembles myoglobin or the isolated chains of human hemoglobin (14) also in the kinetics of the reaction with CO, the value of the combination velocity constant being however somewhat higher in the case of Cm-cytochrome *c*. Even more significant may be the fact that under these conditions the Cm-cytochrome *c* is capable of forming a stable oxygen adduct when the protein is brought into contact with an excess concentration of oxygen. However, even at pH 6, reduced Cm-cytochrome *c* is rapidly oxidized if oxygen is present in considerably less than stoichiometric amounts. A possible explanation for this behavior may be that where the "oxygenated" complex is formed in the presence of reduced Cm-cytochrome *c*, intermolecular electron transfer can occur, thus supplying the bound oxygen with either 2 or 4 electron equivalents needed for its reduction to peroxide or water.

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