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# Effect of Steady Illumination on the Binding of Carbon Monoxide by Carboxymethylated Cytochrome *c*

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The quantum yield, $\phi$, is reported for the photodissociation of CO from reduced carboxymethylated cytochrome *c*. The values of $\phi$ obtained are low relative to that for myoglobin and are pH-independent, being 0.23 at pH6.1 and 0.27 at pH9.7.

Reduced Cm-cyt-*c** has been found to exist in two interconvertible conformational states (Brunori *et al.*, 1972*a*). On the basis of spectral properties these conformations correspond to a high-spin form at acid pH and a low-spin form at alkaline pH (Schejter & Aviram, 1970). The apparent p*K* of the isomerization is about 7.15 at 20°C and the interconversion rates are in the millisecond time-range (Brunori *et al.*, 1972*a*). The ligand-binding properties of the acid and alkaline conformations are substantially different. The conformer predominant at acid pH is characterized by a higher ligand affinity and a larger combination velocity constant. Cm-cyt-*c* can bind CO and the resulting complex undergoes a pH-dependent transition with an apparent p*K* (approx. 8.3) that is higher than that with the ligand-free form (Brunori *et al.*, 1973). Unlike the ligand-free form, however, there is at present no evidence to indicate whether the pH-dependent transition in the ligand-bound form is conformational in nature or a simple ionization process.

The present communication reports an attempt to obtain information about the properties of the ligand-bound forms of Cm-cyt-*c*. As is the case for many haemoproteins, the binding of CO is photosensitive, and we have examined the effect of steady illumination on the binding of CO. We report here the quantum yield for Cm-cyt-*c* relative to that for myoglobin at two widely separate pH values.

## *Materials and methods*

Horse heart cytochrome *c* (type III) was purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A., and used without further purification.

The carboxymethylation reaction was performed by the method of Schejter & George (1965). The concentration of Cm-cyt-*c* was calculated from the extinction at 550nm by using $\epsilon = 28.1 \times 10^3$ litre·mol$^{-1}$·cm$^{-1}$ for the ferrous protein at pH10 (Schejter & Aviram, 1970). All experiments were performed in buffered solutions in the presence of $Na_2S_2O_4$ at a concentration of approx. 0.5mg/ml.

Stock solutions of CO (1mM) were prepared by equilibrating degassed distilled water with gaseous CO at 100kPa (1atm.) partial pressure and at 20°C. Solutions of the desired concentration were prepared from stock solutions by dilution with appropriate buffers.

The photochemical apparatus was a modification of the cross-illumination device described by Noble *et al.* (1967), in which the intensity of the actinic light could be varied with calibrated neutral filters.

## *Results and discussion*

At pH values far removed from the p*K* values of the transition of the ligand-free and ligand-bound forms, the system may be treated as a simple reversible ligand-binding reaction. Accordingly experiments were performed at pH6.1 and 9.7, where the reactions in the dark may be described by eqns. (1) and (2):

$$Fe^{2+} + CO \underset{l}{\overset{l'}{\rightleftharpoons}} Fe^{2+}\cdot CO \qquad (1)$$

$$L_D = \frac{l'}{l} \qquad (2)$$

$l'$ is the second-order combination constant, $l$ the first-order dissociation constant and $L_D$ the affinity constant of Cm-cyt-*c* for CO.

In the light photodissociation decreases the apparent equilibrium constant as shown in eqns. (3) and (4):

$$Fe^{2+} + CO \underset{l+w\cdot I}{\overset{l'}{\rightleftharpoons}} Fe^{2+}\cdot CO \qquad (3)$$

$$L_L = \frac{l'}{l + w\cdot I} \qquad (4)$$

The rate of photodissociation depends on the relative intensity, $I$, the extinction coefficient, $\epsilon$, and the quantum yield, $\phi$, with $w$ defined as $\epsilon\cdot\phi$ (Noble *et al.*, 1967).

At both pH6.1 and pH9.7 the effect of steady illumination on a solution of the protein in equilibrium

* Abbreviation: Cm-cyt-*c*, carboxymethylated cytochrome *c*.

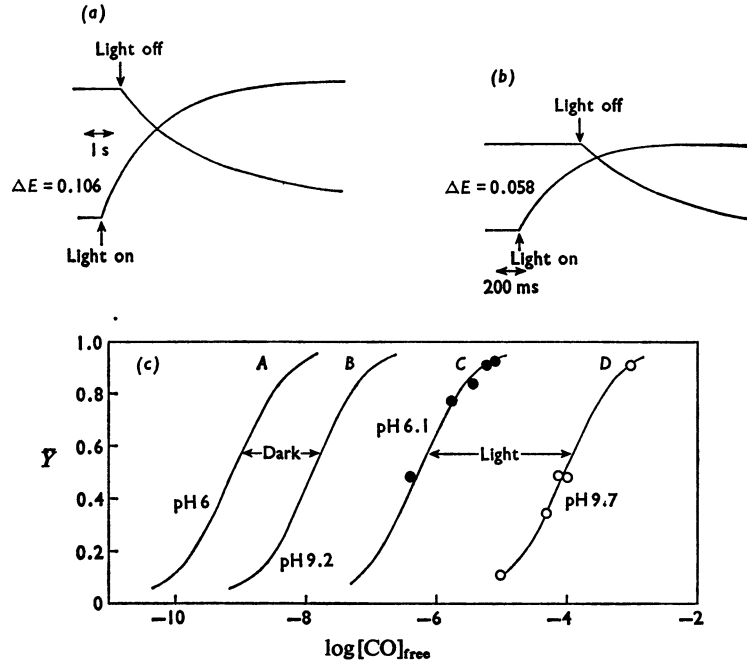


Fig. 1. *Effect of steady illumination on the CO complex of Cm-cyt-c*

(*a*) and (*b*) show the approach to a steady-state condition on illuminating Cm-cyt-*c*·CO complex (up) and the subsequent return to the dark equilibrium condition (down). The optical cell had a 2cm path length. (*a*) 3.3$\mu$M-Cm-cyt-*c* with 50$\mu$M-CO in 2% borate–NaOH buffer, pH9.7; $I_{rel.}$ was 1.0. $\bar{Y}$ changes from 100% to 33% on illumination. The wavelength was 413nm. (*b*) 3.3$\mu$M-Cm-cyt-*c* with 4$\mu$M-CO in 0.1M-potassium phosphate buffer, pH6.1; $I_{rel.}$ was 1.0. $\bar{Y}$ changes from 100% to 77% on illumination. The wavelength was 430nm. (*c*) shows a plot of $\bar{Y}$ versus log$[CO]_{free}$. The continuous lines are theoretical $n = 1$ curves for a simple binding process. Curves *A* and *B* are positioned from a knowledge of the affinity constant in the dark calculated from the known combination and dissociation rates at the two pH values (Brunori *et al.*, 1973). Curves *C* and *D* are positioned to give the best fit to the experimental points obtained under full illumination ($I_{rel.}$ 1.0) and in 0.1M-phosphate buffer, pH6.1 (●), or 2% borate–NaOH buffer, pH9.7 (○).

with CO induces a decrease in the saturation value, $\bar{Y}$, that can be monitored by virtue of the ensuing spectral changes. Fig. 1(*a*) illustrates the approach to a steady-state condition on illumination and the slower return to the equilibrium condition when light was removed. The difference between these two relaxation processes is due to the contribution of the photochemical dissociation constant, $w \cdot I$, to the overall relaxation time [see eqns. (1) and (3)]. In addition, the overall relaxation time of the reaction is shorter at low pH, owing to the higher value of the CO combination rate constant for the acid form (Brunori *et al.*, 1973).

From the shift in the saturation value induced by illumination it was possible to compute CO-binding curves in the light for the two forms of Cm-cyt-*c*. This can, of course, be performed under a variety of steady light-fluxes (in these experiments from maximum light-intensity, arbitrary value 1.0, to 0.2 of maximum intensity). Fig. 1(*c*) shows such binding curves, which, at both pH values, are compared with the corresponding curves in the absence of light. The binding curves conform to a simple formulation of the mass law with $n = 1$. It may be noticed that the difference in $C_{\frac{1}{2}}$ (defined as the CO concentration that gives half saturation) between acid and alkaline pH under photodissociating conditions is much larger than that observed in the dark.

From eqns. (1) and (3) we may derive the following relationships:

$$(C_{\frac{1}{2}})_D \cdot l' = l \qquad (5)$$

and

$$(C_{\frac{1}{2}})_L \cdot l' = l + w \cdot I \qquad (6)$$

Thus a plot of $(C_{\frac{1}{2}})_L \cdot l'$ versus $I$ should give a straight line, the slope of which, $w$, is directly related to the

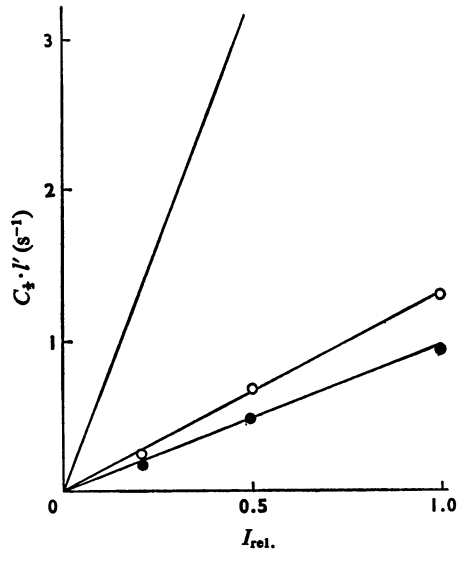


Fig. 2. *Plot of* $(C_{\frac{1}{2}})_L \cdot l'$ *versus relative light-intensity* ($I_{rel.}$)

The continuous line is for myoglobin in 0.2M-phosphate buffer, pH7, and has been calculated from the literature (Brunori *et al.*, 1972*b*; Antonini & Brunori, 1971). ○, 3.1 $\mu$M-Cm-cyt-*c* in 2% borate–NaOH buffer, pH9.7; ●, 2.6 $\mu$M-Cm-cyt-*c* in 0.1M-potassium phosphate buffer, pH6.1.

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quantum yield for CO photodissociation. Such a plot is reported in Fig. 2 for the experiments performed at the two pH values. The values of $l'$ are taken from previous work in which it was shown that the combination velocity constant at pH6 is about 115 times that at pH9.7 (Brunori *et al.*, 1973).

In principle the values of $l'$ and $w$ may be derived from the rate data exemplified in Fig. 1. The rate of approach, $1/\tau$, to the steady-state condition in the light may be expressed as a function of light-intensity as follows:

$$1/\tau = l' \cdot (\overline{Fe^{2+}} + \overline{CO}) + l + w \cdot I \qquad (7)$$

$\overline{Fe^{2+}}$ and $\overline{CO}$ are the equilibrium concentrations of the reactants.

Where the affinity for CO is high (i.e. $l' \gg l$) and where $w \cdot I$ makes the major contribution to the 'off' constant in the light, we may write:

$$1/\tau = l' \cdot (\overline{Fe^{2+}} + \overline{CO}) + w \cdot I \qquad (8)$$

Thus a plot of $(1/\tau)$ versus $I$ should yield a straight line of slope $w$ and intercept $l' \cdot (\overline{Fe^{2+}} + \overline{CO})$.

However, eqns. (7) and (8) imply that the perturbations from equilibrium are small. In most of the experiments reported here (for example see Fig. 1*c*) this is not so and thus the validity of these equations is called into question. We have therefore taken values of $l'$ from the literature (Brunori *et al.*, 1973).

Two conclusions may be drawn from the data in Fig. 2. The first is that the parameter $(C_{\frac{1}{2}}) \cdot l'$ is linearly dependent on (relative) light, as demanded by eqn. (6). The second is that the values of $\phi$ are very similar for the two pH values, being 0.23 at pH6.1 and 0.27 at pH9.7 relative to the value for sperm-whale myoglobin. The values of $\phi$ (relative to myoglobin) were calculated from the slopes of the lines in Fig. 2, which were corrected for protein concentration and relative absorption by using values of $\epsilon$ determined by integration of the area under the CO absorption spectra. This latter procedure was necessary as the actinic light was not monochromatic but irradiated the protein from approx. 350nm upwards.

Where $w \cdot I$ is large compared with $l$, and is the same at pH6.1 and pH9.7, it may be appreciated that the differences in $(C_{\frac{1}{2}})_L$ will be determined solely by the difference in $l'$, the combination velocity constants (see eqn. 4). Indeed the ratio of $(C_{\frac{1}{2}})_L$ at pH6.1 to that at pH9.7, calculated from Fig. 1, is close to the ratio (approx. 115) of the combination velocity constants at these two pH values (Brunori *et al.*, 1973).

The fact that the quantum yield for Cm-cyt-*c* is independent of pH implies either that the CO adducts of this protein have similar protein conformations at the two extremes of pH or that the quantum yield is insensitive to any proton-linked conformational changes that may occur in the ligand-bound form. If the latter is the case then these results may have an important bearing on the understanding of photochemical processes in more complex haemoproteins, e.g. haemoglobin.

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