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ELECTRON-TRANSFER PROCESSES IN CARBOXY-CYTOCHROME *c* OXIDASE AFTER PHOTODISSOCIATION OF CYTOCHROME $a_3^{2+} \cdot CO$

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Summary

Under continuous illumination the CO binding curve of reduced carboxy-cytochrome *c* oxidase maintains the shape of the binding curve in the dark. The apparent dissociation constant calculated from the binding curves at various light intensities is a linear function of the light intensity.

Marked differences are observed between the light-induced difference spectra of the fully reduced carboxy-cytochrome *c* oxidase and the mixed-valence carboxy-cytochrome *c* oxidase. These differences are enhanced in the presence of ferricyanide as an electron acceptor and are explained by partial oxidation of cytochrome a_3 in the mixed-valence enzyme after photodissociation.

Upon addition of CO to partially reduced formate cytochrome *c* oxidase ($a^{2+}a_3^{3+} \cdot HCOOH$) the cytochrome $a_3^{2+} \cdot CO$ compound is formed completely with a concomitant oxidation of cytochrome *a* and the Cu associated with cytochrome *a*. During photodissociation of the CO compound the formate rebinds to cytochrome a_3 and cytochrome *a* and its associated Cu are simultaneously reduced. These electron transfer processes are fully reversible since in the dark the $a_3^{3+} \cdot HCOOH$ compound is dissociated slowly with a concomitant formation of the $a_3^{2+} \cdot CO$ compound and oxidation of cytochrome *a*.

When these experiments are carried out in the presence of cytochrome *c*, both cytochrome *c* and cytochrome *a* are reduced upon illumination of the mixed-valence carboxy-cytochrome *c* oxidase. In the dark both cytochrome *c* and cytochrome *a* are reoxidized when formate dissociates from cytochrome a_3 and the $a_3^{2+} \cdot CO$ compound is formed back. Thus, in this system we are able to reverse and to modulate the redox state of the different components of the final part of the respiratory chain by light.

Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) is an enzyme containing four redox centres, a non-ligand binding heme *a* group associated with cytochrome *a*, a ligand binding heme *a* group associated with cytochrome a_3 and two copper atoms [1]. During the reduction of oxygen to water by cytochrome *c* oxidase electrons are transferred through redox centres to oxygen (cf. Ref. 2). The mechanism, however, how the electrons donated by cytochrome *c* are transferred to oxygen is not fully understood, but the midpoint potentials of the different redox centres [3] being the electron affinity of a particular site may give an indication of the direction of electron flow.

A transfer of electrons between the redox centres of cytochrome *c* oxidase is observed by EPR after binding carbon monoxide to cytochrome a_3 in the partially reduced enzyme [4] and a reverse electron flow can be observed upon photodissociation of the mixed-valence CO-oxidase in both the absence and presence of azide [5]. These electron transfer reactions are explained by the change in the apparent midpoint potential of cytochrome a_3 when carbon monoxide binds to or photodissociates from the reduced cytochrome a_3.

The apparent midpoint potential of cytochrome a_3 in the presence of CO, assuming weak binding to oxidized cytochrome a_3 is given by [6]:

$$E_m^{(1)} = E_m + \frac{RT}{nF} \ln \frac{[CO] + K_r}{K_r} \tag{1}$$

where $E_m^{(1)}$ is the apparent midpoint potential of cytochrome a_3 in the presence of carbon monoxide, E_m the intrinsic midpoint potential and K_r the dissociation constant of CO with reduced cytochrome a_3. Under photodissociating conditions the apparent dissociation constants of hemoprotein-CO complexes increase [7]. For cytochrome *c* oxidase this will result in a lower apparent midpoint potential of cytochrome a_3 upon illumination.

The apparent midpoint potential of cytochrome a_3 in the presence of CO can be lowered to a greater extent by addition of ligands with a much higher affinity for oxidized cytochrome a_3 than for its reduced state, such as azide and formate [6]:

$$E_m^{(2)} = E_m^{(1)} - \frac{RT}{nF} \ln \frac{[L] + K_0}{K_0} \tag{2}$$

where $E_m^{(2)}$ and $E_m^{(1)}$ are the apparent midpoint potentials of cytochrome a_3 in the presence of CO with and without ligand L, respectively, and K_0 is the dissociation constant of the oxidized cytochrome a_3-ligand complex. Illumination of carboxy-cytochrome *c* oxidase in the presence of ligands can even result in an apparent midpoint potential of cytochrome a_3 as low as the midpoint potential of cytochrome *a* giving rise to a reverse electron flow from cytochrome a_3 to cytochrome *a*, while upon switching off the light an electron transfer occurs to cytochrome a_3.

A study of these electron redistributions may yield important information regarding the mechanism by which electrons are transferred from cytochrome *c* to the ligand binding heme *a* group of cytochrome a_3. In this paper the effects

of changes in the apparent midpoint potential of cytochrome a_3 upon combination and upon photodissociation with carbon monoxide were followed by optical spectroscopy and EPR. It will be shown that photodissociation of cytochrome $a_3^{2+} \cdot CO$ in the mixed-valence CO-oxidase causes an electron flow from cytochrome a_3 to cytochrome a. In the presence of formate and cytochrome c electrons could even be transferred to cytochrome c demonstrating that the final part of the electron transport chain can be reversed by a light-activated reaction.

Materials and Methods

Beef heart cytochrome c oxidase was prepared as described previously [8,9]. The absorption coefficient of cytochrome c oxidase (red. — ox.) was 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [10]. Horse heart cytochrome c was prepared by the method of Margoliash and Walasek [11]. The absorption coefficient (red. — ox.) was 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [12]. Chemicals were analar grade, mainly obtained from British Drug Houses. NADH, grade 2, was from Boehringer, the concentration was calculated with $\epsilon = 6.22\ mM^{-1} \cdot cm^{-1}$ at 340 nm [13]. Phenazine methosulphate was from Sigma. Carbon monoxide (Matheson gas products) was purified as described previously [14]. Low CO gas pressures were obtained using calibrated volumina to lower the gas pressure. The CO concentrations were calculated assuming Henry's Law and a solubility of 1 mM at a pressure of 100 kPa [15].

The experiments were carried out in Thunberg cuvettes having two or three side bulbs, one for NADH, one for phenazine methosulphate covered with black plastic to protect it from light and, if required, one for the addition of 10 mM potassium ferrocyanide and 10 mM potassium ferricyanide solution or for the addition of neutralized 10 M sodium formate solution. Mixed-valence CO-oxidase was prepared by anaerobic reduction ([NADH] : [aa_3] : [phenazine methosulphate] = 1 : 1 : 0.04) followed by CO addition as described previously [14] or by incubation of oxidized cytochrome c oxidase under CO at 20°C in the absence of O_2 [16].

Optical spectra were obtained with a Cary-14 or a Cary-17 recording spectrophotometer, modified to record spectra under continuous illumination, essentially as described by Noble et al. [17]. In the sample and the reference beam, blue filters (BG 12) were placed in front of the photomultiplier entrance. Illumination was carried out by rectangular irradiation with orange filtered light (OG 550 and infrared filter) from a 150 W xenon lamp or a 1000 W mercury lamp. Light intensity during illumination was measured with a Photometer/Radiometer (Model 450, E.G. and G.). Light intensities were varied by the use of a calibrated set of neutral-density filters (Oriel). Light minus dark difference spectra were obtained by measuring the change in absorbance upon illumination at several wavelength settings. EPR spectra were obtained with a Varian E-9 EPR spectrometer. At low field settings the magnetic field is corrected for remanent magnetism with a Varian Through-Zero-Field accessory. Temperature, magnetic field and microwave frequency were measured as described previously [14]. Illumination in the EPR experiments was performed as described [14]. The spin concentrations were calculated from the EPR spec-

tra following the methods of Aasa et al. [18,19]. If not specified otherwise, cytochrome *c* oxidase was dissolved in 100 mM potassium phosphate (pH 7.4) and 1% Tween 80.

Results

When carbon monoxide is added to fully reduced cytochrome *c* oxidase the γ-peak in the Soret region is shifted from 444 nm to 431 nm with a shoulder at 442 nm in accordance with earlier results [20]. These spectral changes are explained by binding of carbon monoxide to reduced heme of cytochrome a_3. From the absorbance change at 445 nm upon CO addition at low pressures it was possible to calculate a dissociation constant of 0.33 μM for the CO complex (Fig. 1) in agreement with other workers [16,21]. Upon illumination of the CO complex of cytochrome *c* oxidase, the CO affinity decreases. Fig. 1 shows the saturation curves of reduced cytochrome *c* oxidase with carbon monoxide at several light intensities. Under all conditions CO binds non-cooperatively to reduced cytochrome a_3. The calculated apparent dissociation constants for the CO complex of cytochrome *c* oxidase increase proportionally to the light intensity (Fig. 2), similarly as was found for other hemoprotein-CO complexes [7]. At the maximum light intensity available the apparent dissociation constant has increased to 130 μM. Within this light intensity it was possible to obtain more than 90% photodissociation at CO pressures below 1 kPa. From Eqn. 1 it is then possible to calculate the apparent midpoint potential of cyto-

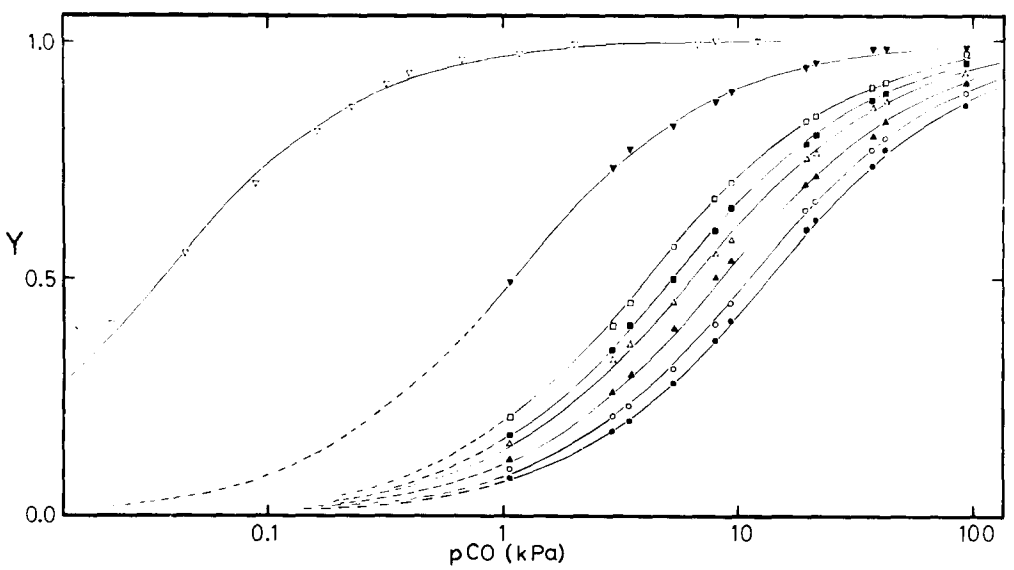
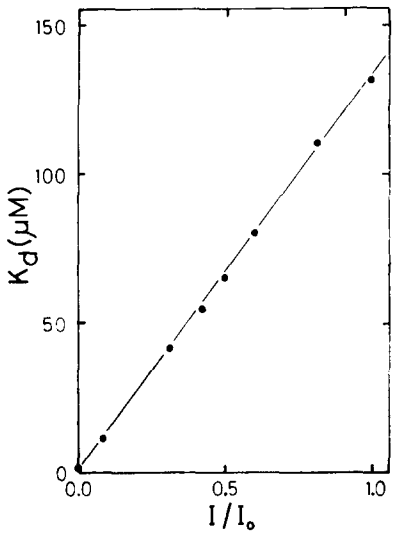


Fig. 1. Carbon monoxide binding to reduced cytochrome *c* oxidase in the dark and at various light intensities. The curves are calculated assuming non-cooperative binding of CO to the enzyme. 8 μM cytochrome *c* oxidase reduced with $Na_2S_2O_4$ in 100 mM potassium phosphate (pH 7.4) and 1% Tween 80. For further conditions see Materials and Methods. Light intensities are expressed relatively (I/I_0). ▽———▽, 0; ▼———▼, 0.09; □———□, 0.31; ■———■, 0.42; △———△, 0.50; ▲———▲, 0.60; ○———○, 0.81; ●———●, 1. Y, fractional saturation with carbon monoxide as measured at 428 nm; pCO, carbon monoxide pressure.



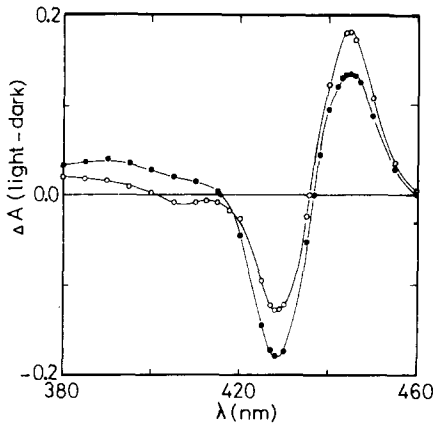


Fig. 2. Dependence of the apparent dissociation constants on the light intensity. The apparent dissociation constant (K_D) is calculated from the data of Fig. 1.

Fig. 3. Light minus dark difference spectra of fully and partially reduced cytochrome *c* oxidase in the presence of carbon monoxide. 8 μM cytochrome *c* oxidase in 100 mM potassium phosphate (pH 7.4) and 1% Tween 80; pCO, 30 kPa; temperature, 20°C; ○———○, $Na_2S_2O_4$-reduced enzyme; ●———●, enzyme partially reduced ($2\,e^-/aa_3$) with NADH.

chrome a_3 assuming that the midpoint potential of cytochrome a_3 in the absence of ligands is 375 mV under the conditions of our experiments [3]. The apparent midpoint potential of cytochrome a_3 at a CO pressure of 1 kPa in the dark will be 465 mV if cytochrome a_3 titrates as a one-electron acceptor [22] and 420 mV when two electrons are required to form the $a_3^{2+} \cdot$ CO compound [4,23]. Upon illumination of the cytochrome *c* oxidase-CO complex at a CO pressure of 1 kPa the apparent midpoint potential of cytochrome a_3 will decrease to its unliganded value of 375 mV.

In partially reduced cytochrome *c* oxidase a change in the apparent midpoint potential of one of the redox centres (cytochrome a_3) upon illumination may result in electron redistributions over the other redox sites of the enzyme. Therefore, the photodissociation of mixed-valence carboxy-cytochrome *c* oxidase was studied. Fig. 3 shows the light minus dark difference spectra of the dithionite-reduced carboxy-cytochrome *c* oxidase and the mixed-valence carboxy-enzyme. The trough at 428 nm can be assigned to photodissociation of the $a_3^{2+} \cdot$ CO complex and the peak at 445 nm to the formation of unliganded cytochrome a_3^{2+} [20]. There are, however, remarkable differences between the difference spectra of both compounds. The ratio $\Delta A_{445nm}/\Delta A_{428nm}$ changes from 1.4 in the fully reduced CO-enzyme to 0.8 in the mixed-valence CO-enzyme together with an increase in the absorbance at 412 nm and a change in the isosbestic point from 435.8 to 437.0 nm (Table I). In accordance with the results obtained by EPR [14] these changes suggest that a partial oxidation of cytochrome a_3^{2+} to a_3^{3+} takes place. This oxidation of cytochrome a_3 may account for the decrease in intensity of the peak at 445 nm and the slight

TABLE I

DIFFERENCES IN PHOTODISSOCIATION SPECTRA OF THE FULLY REDUCED AND MIXED-VALENCE CARBOXY-CYTOCHROME *c* OXIDASE

Conditions for the enzyme species are as in Fig. 3.

Enzyme species	$\Delta A_{445nm}/\Delta A_{428nm}$	$\Delta A_{412nm}/\Delta A_{428nm}$	Isobestic point (nm)
$a^{2+}a_3^{2+} \cdot CO$	1.40	—0.06	435.8
$a^{3+}a_3^{2+} \cdot CO$	0.80	+0.06	437.0

absorbance increase around 412 nm, which corresponds according to Vanneste [20] to the band of cytochrome a_3^{3+}.

Another explanation, as given by Greenwood et al. [16], who found similar difference spectra by extrapolating kinetic differences to $t = 0$ after flash photolysis of the mixed-valence and the fully reduced enzyme, is that the differences between these compounds are caused by site-site interactions and not by a change in redox state. Therefore, the light minus dark difference spectra of the mixed-valence CO compound were also recorded after addition of equal amounts (1 mM) of potassium ferrocyanide and potassium ferricyanide, that act as a redox buffer. The difference spectrum observed (Fig. 4) appears now to be dependent on the period of illumination. After 90 s illumination the peak at 445 nm of cytochrome a_3^{2+} in the difference spectrum has disappeared and a new absorption band is formed at 412 nm, which was assigned to cytochrome a_3^{3+} [20]. In the dark the mixed-valence CO-enzyme was formed back completely within 10 min, showing that the oxidation of cytochrome a_3 was reversible. These light-dark activated reactions could be repeated several times. The increae in absorbance difference at 428 nm upon prolonged illumination is probably due to incomplete photodissociation at short illumination times at a CO pressure of 13 kPa. At prolonged illumination,

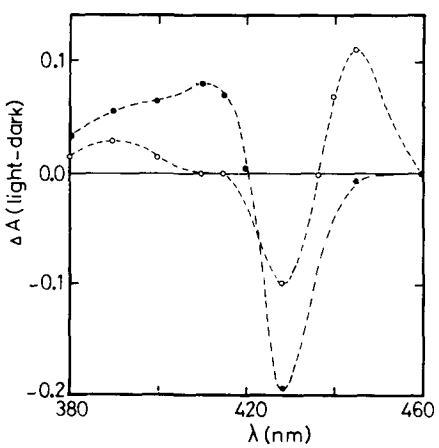


Fig. 4. Effect of illumination on the light minus dark difference spectrum of partially reduced cytochrome *c* oxidase in the presence of CO and equal amounts of ferro- and ferricyanide. 8 μM cytochrome *c* oxidase in 100 mM potassium phosphate (pH 7.4) and 1% Tween 80 was partially reduced by incubation with CO after which 1 mM ferri- and 1 mM ferrocyanide were added anaerobically. pCO, 30 kPa; temperature, 20°C; o———o, illumination time of 1 s, and •———•, 90 s.

however, the photodissociation effect is determined by the slowly attained redox equilibrium between cytochrome a_3^{2+} and ferricyanide and not by the fast photolysis and CO recombination reactions. At CO pressures lower than 1 kPa no such increase in ΔA_{428nm} is observed upon prolonged illumination, since the photodissociation is already complete at the short illumination time. However, at such low CO pressures the apparent midpoint potential of cytochrome a_3 is not high enough in the dark compared to the 420 mV of the ferro-ferricyanide couple, causing oxidation of cytochrome a_3 which results in a decreased photodissociation effect.

These results show that during illumination of the mixed-valence oxidase cytochrome a_3 is oxidized. The cause of this electron transfer between cytochrome a_3 and ferricyanide is the drop in potential of cytochrome a_3 below that of the ferro-ferricyanide couple. In the presence of ligands like azide or formate, which bind to oxidized cytochrome a_3, the apparent midpoint potential of cytochrome a_3 decreases by an amount depending on the ligand concentration (cf. Eqn. 2). Illumination under these conditions can result in an apparent midpoint potential of cytochrome a_3 lower than that of cytochrome *a* and, therefore, photodissociation experiments were carried out in the presence of formate.

When sodium formate is added to oxidized cytochrome *c* oxidase, the band at 598 nm (not shown) is slightly diminished (6%), a deeper trough is observed at 575 nm and the 650 nm band is increased by about 10%. In the Soret region the peak at 424 nm is shifted to 417 nm. From the absorbance changes at various formate concentrations, it is possible to calculate a dissociation constant of 1 mM (pH 7.4). These results are comparable with those reported previously [24]. When dithionite (0.3 mM) is added to oxidized formate oxidase the mixed-valence formate compound was formed with cytochrome *a* reduced and cytochrome a_3 oxidized (not shown). The stability of the mixed-valence formate compound can be explained by the low k_{off} of $13 \cdot 10^{-4}$ s^{-1} of the $a_3^{3+} \cdot$ HCOOH complex [24]. Upon addition of more dithionite (5 mM), the fully reduced enzyme is formed. When sodium formate is added to fully reduced cytochrome *c* oxidase, no spectral effects are observed, indicating that formate binds more strongly to oxidized heme of cytochrome a_3. From Eqn. 2 it is then possible to calculate that addition of 1 M sodium formate to oxidized cytochrome *c* oxidase results in a decrease of the apparent midpoint potential of cytochrome a_3 from 375 mV to 195 mV ($n = 1$). An apparent midpoint potential of cytochrome a_3 lower than that of cytochrome *a* results in electron redistributions within the enzyme upon illumination of the mixed-valence CO compound of cytochrome *c* oxidase.

When oxidized formate oxidase is partially reduced by NADH (2 e^-/oxidase) a mixed-valence compound, $a^{2+}a_3^{3+} \cdot$ HCOOH, is formed with peaks at 445 nm and 415 nm (Fig. 5) which are assigned to cytochrome a^{2+} and cytochrome $a_3^{3+} \cdot$ HCOOH, respectively. After addition of carbon monoxide to $a^{2+}a_3^{3+} \cdot$ HCOOH, the spectrum of the mixed-valence CO compound, $a^{3+}a_3^{2+} \cdot$ CO, is observed with a peak at 428 nm belonging to cytochrome $a_3^{2+} \cdot$ CO and a shoulder at 415 nm of cytochrome $a_3^{3+} \cdot$ HCOOH not affected by CO. The reduction of cytochrome a_3 at the expense of cytochrome *a* is explained by the increase in the apparent midpoint potential of cytochrome a_3 in the presence

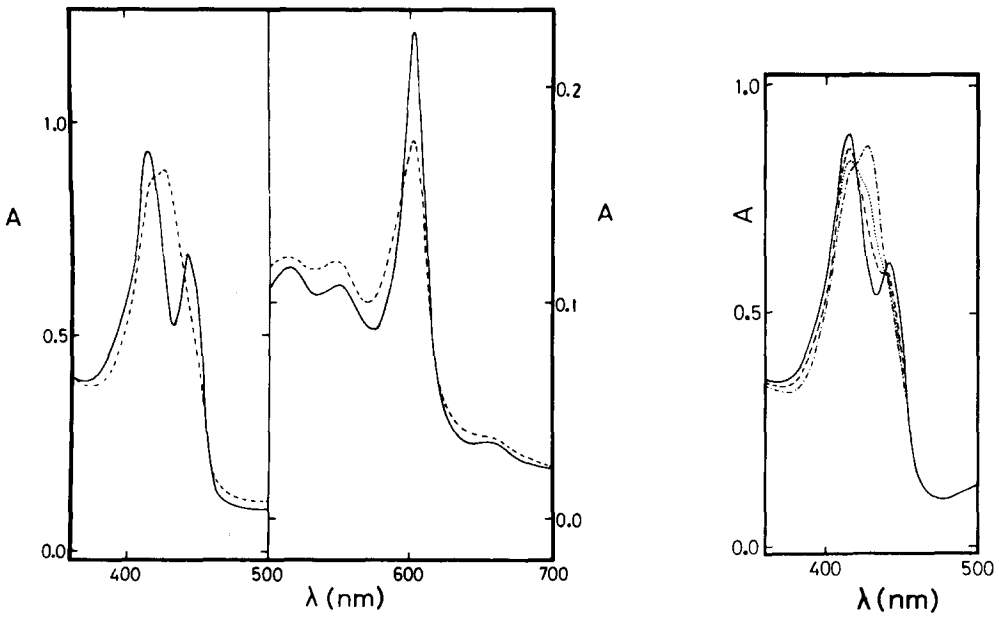


Fig. 5. Absorption spectra of partially reduced cytochrome *c* oxidase in the presence of formate and after addition of carbon monoxide. The mixed-valence complex of cytochrome *c* oxidase was obtained by partial reduction of the enzyme (2 e^-/aa_3) with NADH and phenazine methosulphate in the presence of 1 M sodium formate. 7.5 μM cytochrome *c* oxidase in 100 mM potassium phosphate (pH 7.4) and 1% Tween 80. Before (———), and after (- - - - -) addition of CO; pCO, 40 kPa.

Fig. 6. Time course of the changes in the absorption spectrum occurring after illumination of partially reduced carboxy-cytochrome *c* oxidase in the presence of formate. Conditions as in Fig. 5. ———, spectrum during illumination; - - - - - -, after 1 min in the dark; · · · · · ·, after 5 min in the dark; · — · — ·, after 30 min in the dark.

of CO (pCO = 40 kPa) to 285 mV (n = 2).

Fig. 6 shows that continuous illumination of the mixed-valence CO-enzyme, $a^{3+}a_3^{2+} \cdot CO$, reveals the mixed-valence formate enzyme $a^{2+}a_3^{3+} \cdot HCOOH$ as judged from the appearance of the bands at 445 and 415 nm. This indicates an electron transfer from cytochrome a_3 to cytochrome *a*. In the dark, the formate complex dissociates slowly (with $k = 17 \cdot 10^{-4}\ s^{-1}$) giving rise to the formation of the mixed-valence CO compound (Fig. 6). As this reaction in the dark is so slow, it is possible to measure complete spectra of the progress of this reaction after illumination.

In the presence of CO and formate the redox state of cytochrome *a* (with a midpoint potential of 230 mV) is affected by light. It is conceivable that in the presence of cytochrome *c* (E_m of 260 mV) reduction of this component takes place enabling us to study the redox reactions between cytochrome *a* and its natural electron donor.

When the mixed-valence CO-enzyme (2 e^-/oxidase) in the presence of 7 μM cytochrome *c* and 1 M sodium formate was illuminated both reduction of cytochrome *a* (peak at 605 nm) and reduction of cytochrome *c* (peaks at 550 nm and 520 nm) were observed (not shown). In the dark, cytochrome *a* and cytochrome *c* are slowly reoxidized, while cytochrome a_3 is reduced forming the

$a_3^{2+} \cdot CO$ complex (peak at 428 nm). After 20 min in the dark the same spectrum was obtained as before illumination. Because of the low cytochrome *c* oxidase concentration used, it was not possible to measure any effect at 830 nm. On the basis of the known red. — ox. absorption coefficients for cyto-

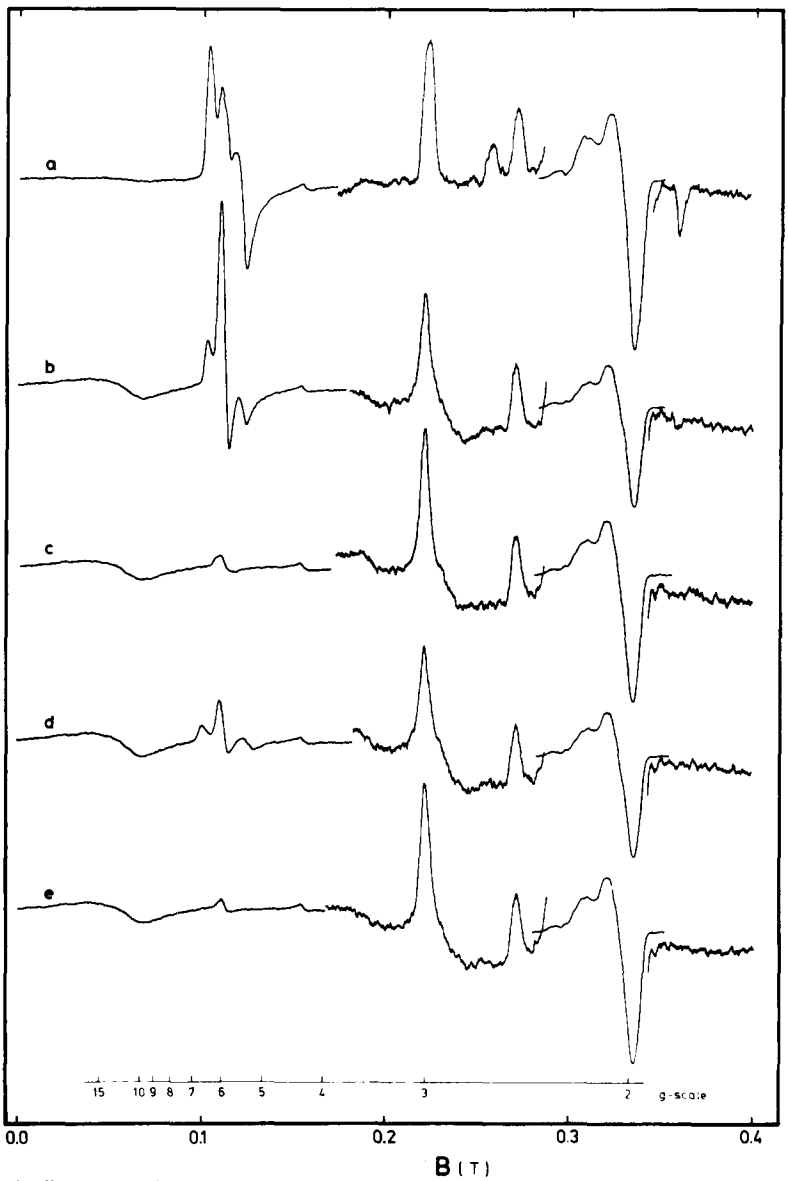


Fig. 7. Effect of formate and CO on the EPR spectrum of partially reduced cytochrome *c* oxidase. (a) 0.30 mM cytochrome *c* oxidase partially reduced (2 e^-/aa_3) with NADH and phenazine methosulphate in 50 mM potassium phosphate (pH 7.4) and 0.5% Tween 80. (b) After anaerobic addition of 1 M sodium formate to (a). (c) After anaerobic addition of carbon monoxide (pCO, 40 kPa) to (b). (d) After illumination of (c) for 3 min and subsequent freezing under illumination in liquid N_2. (e) After incubation of (d) for 10 min at room temperature in the dark. Conditions for EPR spectroscopy: frequency, 9.328 GHz; microwave power, 2 mW; modulation amplitude, 10^{-3} T; scanning rate, $5 \cdot 10^{-2}$ T $\cdot$ min^{-1}; microwave time constant, 1 s; temperature, 14.5 K. The signal at $g = 2$ was recorded at a 20-fold lower receiver gain, the signal at $g = 6$ at an 8-fold lower gain.

TABLE II

EFFECT OF FORMATE AND CO ON VARIOUS EPR SIGNALS OF PARTIALLY REDUCED CYTOCHROME *c* OXIDASE (FIG. 9)

Successive additions are as in Fig. 9.

Successive additions	EPR intensities from resonance at *g* value listed (% of one heme in aa_3)			Spectrum
	$g = 6$	$g = 3$	$g = 2$	
$2e^-/aa_3$	50	25	70	a
1 M NaHCOO	30	20	45	b
0.4 mM CO	—	25	60	c
Illuminated	10	15	45	d
Dark	—	25	60	e

chrome *c* at 550 nm [12], and for cytochrome *a* at 605 nm [10], it was calculated that in the dark after photodissociation cytochrome *a* and cytochrome *c* donated 0.19 and 0.05 e^-/aa_3, respectively, back to cytochrome a_3. Therefore, the value of the midpoint potential of cytochrome *a* must be below that of cytochrome *c*. From the estimated ratios a^{3+}/a^{2+} and c^{2+}/c^{3+} and a midpoint potential of 260 mV for cytochrome *c* a midpoint potential around 240 mV is calculated for cytochrome *a*. The number of electrons required to form cytochrome $a_3^{2+} \cdot CO$ is more difficult to calculate since the absorbance at 428 nm should be corrected for large contributions from cytochrome *c* and cytochrome *a*.

Similar ligand-induced redox changes were observed by EPR spectroscopy upon addition of sodium formate to partially reduced ($2\ e^-/aa_3$) cytochrome *c* oxidase (Fig. 7, spectrum a). A decrease is observed (spectrum b) in the $g = 2$ signal, assigned to low potential Cu^{2+}, and a decrease in the $g = 3$ signal, originating from the low-spin ferric heme *a* of cytochrome *a*. This shows that upon stabilization of the oxidized form of cytochrome a_3 by formate cytochrome *a* and its associated copper are reduced partly. Simultaneously an increase is observed of the axial $g = 6$ signal, assigned to the cytochrome $a_3^{3+} \cdot$ HCOOH complex and a broad trough is formed around $g = 12$. It is apparent from Table II, which shows the integrated intensities of the EPR signals, that the total amount of oxidized redox centres ($0.95/aa_3$) after the addition of formate is lower than that before the addition ($1.45/aa_3$). This indicates that not all cytochrome $a_3^{3+} \cdot$ HCOOH is EPR detectable, but that part of it is coupled to its associated Cu as in oxidized cytochrome *c* oxidase [25]. The addition of carbon monoxide to this mixed-valence formate enzyme (spectrum c) results in an increase of both the $g = 2$ and the $g = 3$ signals and a decrease of the axial $g = 6$ signal. After photodissociation of the formed mixed-valence CO-enzyme (spectrum d) the reverse effects were observed, showing oxidation of cytochrome a_3 and reduction of Cu^{2+} and cytochrome *a*. The broad trough at $g = 12$, however, is not effected by CO or illumination. The photodissociation reaction was completely reversible (spectrum e). These results support our findings from optical spectroscopy.

The $g = 12$ signal has been observed earlier [26] at lower temperatures but

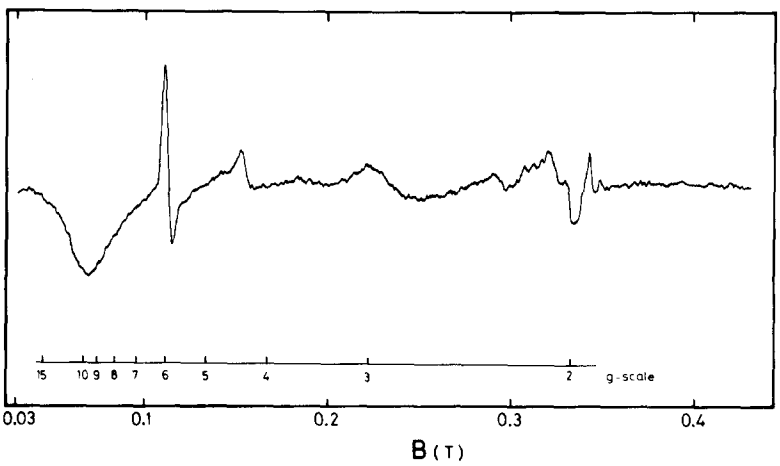


Fig. 8. The EPR spectra of cytochrome *c* oxidase reduced in the presence of formate. The enzyme (0.18 mM) was reduced by incubating with ascorbate (10 mM) and cytochrome *c* (14 μM) for 7 min. EPR conditions as in Fig. 7.

its origin is not clear. As shown in Fig. 8, an intense signal at $g = 12$ could be formed upon reduction of cytochrome *c* oxidase with ascorbate and cytochrome *c* in the presence of sodium formate, simultaneously with a broad resonance at $g = 2.9$. The latter resonance is not originating from cytochrome *c* since this component is completely reduced and its concentration (14 μM) is too low to allow detection by EPR under these conditions.

Discussion

From the binding studies of carbon monoxide to fully reduced cytochrome *c* oxidase under photodissociating conditions it was found that cytochrome a_3 binds carbon monoxide in a similar way as myoglobin and other hemoproteins [7].

In principle, the quantum yield of the photodissociation reaction, i.e. the ratio of the number of photodissociated molecules and the number of excited molecules, can be calculated from Fig. 2. To determine the number of excited molecules a knowledge of the rate constant for the formation of the cytochrome $a_3 \cdot CO$ complex and of the absorption coefficient of this complex is necessary, but since the only reported absorption coefficient [27] was calculated on the assumption of a quantum yield of 1 we were not able to calculate the quantum yield. It is interesting to note that with a quantum yield of 1 and a rate constant of $8 \cdot 10^4\ M^{-1} \cdot s^{-1}$ [28], the same absorption coefficient of $11.4\ mM^{-1} \cdot cm^{-1}$ at 590 nm from our data could be calculated as found by Chance [27].

The photodissociation experiments with partially reduced cytochrome *c* oxidase-CO complexes presented here show that electron redistribution can occur during illumination. We assign the differences in photodissociation behaviour of the mixed-valence CO compound and the fully reduced CO-cytochrome *c* oxidase partly to an oxidation of the heme of cytochrome a_3,

caused by the change in apparent midpoint potential of cytochrome a_3 in the presence of carbon monoxide upon illumination. This assignment is in contrast to other studies [16,29] where these differences in photoinduced spectra were explained by a heme-heme interaction, in which the optical spectrum of cytochrome a_3 is affected by the redox state of the other sites in the enzyme. Evidence for electron redistribution is given by experiments with mixed-valence CO-oxidase in the presence of ferricyanide where these differences are enhanced and could only be explained by complete oxidation of cytochrome a_3 by ferricyanide. Also by EPR it was shown previously [14,30] that photodissociation of the mixed-valence CO-enzyme could result in partial oxidation of cytochrome a_3.

One might speculate why this oxidation was not observed in the flash-photolysis experiments of Greenwood et al. [16]. Firstly, we observed greater differences in photodissociation behaviour between the fully reduced and the mixed-valence CO-enzyme, simplifying the assignment of these differences. Secondly, the oxidation of cytochrome a_3 by ferricyanide is slow and as a consequence can be observed only under continuous illumination of the CO-enzyme complex.

If, however, the observed differences in photodissociation behaviour between mixed-valence enzyme in the absence of ferricyanide and fully reduced CO-cytochrome *c* oxidase are ascribed to an oxidation of cytochrome a_3 an electron acceptor must be present in the enzyme. Thus, simultaneously with the photodissociation of the mixed-valence CO-cytochrome *c* oxidase and the partial oxidation of cytochrome a_3, a partial reduction of the other redox centres must be observed. From our optical experiments on the photodissociation of the mixed-valence CO-oxidase we were not able to assign considerable reduction of other redox centres concomitantly with the oxidation of cytochrome a_3, because of overlap of absorbance bands in the Soret region. However, the possibility exists that the deeper trough at 428 nm observed in the photodissociation difference spectrum of mixed-valence CO-cytochrome *c* oxidase compared to fully reduced CO-cytochrome *c* oxidase (see Fig. 3) is caused by the reduction of cytochrome a^{3+}, since this component absorbs at 425 nm [20]. Such a view is qualitatively supported by EPR experiments with mixed-valence CO-cytochrome *c* oxidase: after illumination of this compound a rhombic high-spin heme-iron signal at $g = 6$, assigned to cytochrome a_3^{3+} [14,30], is formed and the $g = 3$ and $g = 2$ signals, characteristic for cytochrome a^{3+} and Cu^{2+}, respectively, decrease a few procent, in agreement with previous results [14,31]. The less quantitative correlation between the optical and EPR experiments might be caused by a different temperature dependence of the midpoint potentials of the various redox centres, since the two types of experiments are carried out at very different temperatures. Such differences in temperature behaviour of the redox centres were also shown to exist in ceruloplasmin, a copper-containing oxidase [32] and in mixtures of ferri-ferrocyanide and cytochrome *c* [33].

When carbon monoxide recombines with mixed-valence cytochrome *c* oxidase, a mixture of $a^{3+}a_3^{2+}$ and $a^{2+}a_3^{3+}$, a biphasic type of recombination might be expected when the electron transfer between cytochrome *a* and cytochrome a_3 is slow compared to the recombination of CO with cytochrome a_3^{2+}. Since

Greenwood et al. [16] did find a single exponential process for the recombination with a second-order rate constant $k = 5 \cdot 10^4\ M^{-1} \cdot s^{-1}$ at CO concentrations to 1 mM we must conclude that the electron transfer between cytochrome *a* and a_3 is much faster than $50\ s^{-1}$. This is in agreement with the molecular activity of cytochrome *c* oxidase which requires that the electron transfer must be faster than $240\ s^{-1}$ [34]. From the data of Leigh et al. [35] it can be concluded that the oxidation of cytochrome a_3 during photodissociation at 5 K is shorter than 5 ms. These fast electron transfer processes at low temperature suggest that the electrons are tunnelled between the two cytochromes [36].

In the presence of sodium formate photodissociation of the mixed-valence CO-cytochrome *c* oxidase resulted also in oxidation of cytochrome a_3 but in addition a considerable reduction of cytochrome *a* is observed. This conclusion is reliable since the band of the cytochrome $a_3^{3+} \cdot HCOOH$ complex hardly overlaps the band of reduced cytochrome *a* in the Soret region. Furthermore, formate lowers the apparent midpoint potential of cytochrome a_3 to a value close to that of cytochrome *a*. This effect of formate results in greater electron redistributions in mixed-valence CO-cytochrome *c* oxidase during photodissociation. The redox reaction of mixed-valence CO-cytochrome *c* oxidase upon illumination in the presence of formate is described by the following equation:

$$a^{3+}a_3^{2+} \cdot CO + HCOOH \underset{\text{dark}}{\overset{h\nu}{\rightleftharpoons}} a^{2+}a_3^{3+} \cdot HCOOH + CO \qquad (3)$$

The light reaction shows that a decrease of the apparent midpoint potential of cytochrome a_3 reveals an electron transfer from cytochrome a_3 to cytochrome *a*, opposite to the normal direction of electron flow in this part of the electron transport chain. On the other hand, a forward electron transfer from cytochrome *a* to cytochrome a_3 is observed, when the apparent midpoint potential of cytochrome a_3 due to its combination with CO increases in the dark. A similar electron transfer might occur when oxygen binds to cytochrome a_3.

As we have shown, electrons can also be transferred from cytochrome *a* to cytochrome *c* when mixed-valence CO-cytochrome *c* oxidase is photodissociated in the presence of sodium formate and cytochrome *c*. Upon illumination the electron transport chain is partly reversed according to Eqn. 3 followed by:

$$a^{2+}a_3^{3+} \cdot HCOOH + c^{3+} \rightleftharpoons a^{3+}a_3^{3+} \cdot HCOOH + c^{2+} \qquad (4)$$

In principle it is possible to calculate from our observations how many electrons are involved in electron transfer on each redox centre during illumination, since most absorption coefficients (red. — ox.) of these centres are known. From such calculations one can establish the midpoint potentials for the different redox sites involved in electron transfer. For cytochrome *a* we find a midpoint potential of around 240 mV, which agrees well with the results from potentiometric titrations [3,37,38].

Another application of this light-induced reversal of the electron transport chain is that kinetic experiments can be carried out on the transfer of electrons from one redox centre to the other when cytochrome a_3 binds a ligand. However, the system with formate is not very apt for such measurements because the electron flow velocities are now governed by the slow on and off

velocities of formate in the cytochrome a_3^{3+}-HCOOH complex. It is conceivable that in the presence of other ligands (azide) for oxidized a_3 which have a faster on and off rate constant than formate, this method may be a valuable tool in studying electron transfer processes in the final part of the electron transport chain.

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